

# *In vitro* and *ex vivo* evaluation of a multi-epitope heparinase vaccine for various malignancies

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## Key words

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Advanced cancers are characterized by a high occurrence of invasion and metastasis, low success of surgical resection and a high recurrence rate, and to date have no known cure. The importance of T-cell-mediated antitumor immunity has been demonstrated in both animal models and human cancer immunotherapy. Animal models have shown that the generation of protective antitumor immunity depends on the presentation of tumor antigens by dendritic cells (DC).<sup>(1,2)</sup> These vaccines have been used to treat cancer<sup>(3)</sup> and hold promise for clinical applications.<sup>(4–6)</sup>

Heparanase (Hpa) is the only known glycosidic enzyme that can degrade the extracellular matrix (ECM) and heparan sulfate glycoprotein in the basement membrane.<sup>(7)</sup> Heparanase is extensively expressed in the majority of advanced cancers and is rarely expressed in normal mature tissues, except for low-level expression in lymphocytes and bone marrow. Heparanase plays a very important role in tumor progression, because cleavage of the ECM and basement membrane by activated heparanase releases a variety of cytokines, such as hepatocyte growth factor, epidermal growth factor and basic fibroblast growth factor. These cytokines can promote tumor angiogenesis and provide nutritional support for local tumor colonization.<sup>(8,9)</sup> Heparanase activation is also very important for tumor metastasis.<sup>(10,11)</sup> Many groups have reported a nega-

Previous studies have indicated that heparanase (Hpa) might represent a candidate universal tumor-associated antigen. However, vaccine therapy targeting only one cytotoxic T lymphocyte (CTL) epitope is suboptimal in preventing cancer. In the present study, we designed heparanase multi-epitope vaccines to increase the immune response to standard single heparanase epitopes. The results showed that multi-epitope vaccines Hpa525 + 277 + 405 + 16 and Hpa8 + 310 + 315 + 363 induced higher Hpa-specific lysis of various cancer cells from different tissues in a HLA-A2-restricted and heparanase-specific manner compared with the single epitope vaccines Hpa525, Hpa277, Hpa405, Hpa16, Hpa8, Hpa310, Hpa315 and Hpa363, both *in vitro* and *ex vivo*. Heparanase multi-epitope vaccines not only induced the heparanase-specific CTL to lyse tumor cells but also increased CTL secretion of interferon- $\gamma$ . However, these heparanase-specific CTL did not lyse heparanase-expressing autologous lymphocytes and dendritic cells, which confirms the safety of these multi-epitope vaccines. Therefore, the present study provides theoretical evidence for the use of heparanase multi-epitope vaccines for clinical application.

tive correlation between heparanase expression and tumor patient prognosis, and inhibiting heparanase activity can significantly reduce tumor metastasis.<sup>(12,13)</sup>

We previously showed that DC transfected with full-length heparanase cDNA can induce the generation of heparanase-specific cytotoxic T lymphocytes (CTL). These CTL have significant cytotoxicity to the heparanase-positive and MHC-I-matched gastric cancer cells and no cytotoxicity on normal lymphocytes and DC, which express low levels of heparanase.<sup>(14)</sup> This indicates that the use of heparanase as a tumor-associated antigen in cancer immunotherapy is safe and feasible. Furthermore, we predicted and identified three epitopes from human heparanase (Hpa(525-533)(PAFSYSFFV), Hpa(277-285)(KMLKSFLKA) and Hpa(405-413)(WLSLLFKKL))<sup>(15,16)</sup> and two epitopes from mouse heparanase (mHpa(398-405)(LSLLFKKL) and mHpa(519-526)(FSYGFFVI))<sup>(17)</sup> using bioinformatics technology and reverse immunological techniques. We found that epitopes from both human and mouse heparanase could induce an effective antitumor immune response. *In vivo* studies have shown that the above heparanase epitopes play a significant role in immune protection and immune therapy in a tumor-bearing mouse model. However, the major drawback to using peptide epitope vaccines is that these vaccines only represent one single CTL epitope, while expression of an entire transcript can produce several or

even dozens of epitopes, which can also combine with different MHC-I molecules. Therefore, the heparanase epitope peptide vaccine has weak immunogenicity and the CTL responses it induces are weaker than the CTL responses induced by a vaccine strain expressing the full-length heparanase cDNA.

Based on the above analysis and other previous studies, the goals of the present study were to use bioinformatics techniques to analyze and identify additional heparanase HLA-A2.1-restricted polypeptide epitopes, combine the effective epitope peptides, prepare heparanase multi-epitope vaccines and identify the vaccines that induced maximum killing activity by CTL, providing a theoretical basis for the potential anti-tumor activity of heparanase peptide vaccines.

## Materials and Methods

**Cell lines and animals.** All cell lines were maintained in our laboratory (Laboratory of Gastroenterology Institute, the Third Military Medical University, Chongqing, China), including osteogenic sarcoma cell line U2OS (Hpa<sup>+</sup>, HLA-A2<sup>+</sup>), colon cancer cell line SW480 (Hpa<sup>+</sup>, HLA-A2<sup>+</sup>), gastric cancer cell line KATO-III (Hpa<sup>+</sup>, HLA-A2<sup>+</sup>), liver cancer cell line HepG2 (Hpa<sup>+</sup>, HLA-A2<sup>-</sup>) and breast cancer cell line MCF-7 (Hpa<sup>-</sup>, HLA-A2<sup>+</sup>). The U2OS, MCF-7 and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone<sup>TM</sup>, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The KATO-III and SW480 cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin (200 U/mL) and streptomycin (100 µg/mL). The human TAP-deficient T2 cell line was a gift from Professor Ying WAN (Institute of Immunology, Third Military Medical University); this cell line was used for examination of HLA-A2-binding activity in a previous study.<sup>(15)</sup> All of these cell lines were maintained in a 37°C humidified atmosphere containing 5% CO<sub>2</sub>. C57BL/6/HLA-A2 transgenic mice were provided by Professor Bing NI (Institute of Immunology, Third Military Medical University). These transgenic mice express a chimeric MHC-I heavy chain (containing the transmembrane and intracytoplasmic domains HLA-A-2.1 a1, a2 and H-2K<sup>b</sup>). All animal study protocols were approved by the local ethics committee of the Third Military Medical University. All cell lines mentioned above have been used in previously studies.<sup>(15,18,19)</sup>

**Prediction of heparanase epitopes.** Human Hpa peptides with HLA-A2-binding motifs were predicted using a bioinformatics method described previously.<sup>(15,20,21)</sup> Twenty-eight nonapeptides derived from human heparanase and one nonapeptide derived from human immunodeficiency virus (HIV) [HIVpol (476-484) (ILLEPVHGV)],<sup>(1)</sup> which served as the positive control in the HLA-A2-binding assay and a negative control in the CTL-killing assay, were synthesized by the Chinese Peptide Company (Hangzhou, China) using Fmoc solid-phase peptide synthesis on the basis of the selected amino acid sequences.<sup>(22)</sup> The purity of these peptides was confirmed using analytic high-pressure liquid chromatography (HPLC) and their identity was confirmed using mass spectrographic analysis. All of the HPLC-purified peptides exhibited a purity of 93% or more.

**T2 *in vitro* HLA-A2-binding assay.** The binding activity of candidate peptides to the HLA-A2 molecule was determined semi-quantitatively by measuring peptide-induced expression of HLA-A2.1 molecules on T2 cells with flow cytometry according to our previously study.<sup>(15)</sup> We measured HLA-A2 expression by T2 cells in the absence or presence of individual predicted peptides using flow cytometry. The human HLA-

A2-restricted HIV epitope HIVpol(476-484)(ILLEPVHGV) was used as a positive control and the H-2K<sup>b</sup>-restricted mouse heparanase epitope mHpa(519-526)(FSYGFFVI) was used as a negative control in the HLA-A2-binding assay.

**Dendritic cell generation from human peripheral blood mononuclear cells (PBMC) and mouse bone marrow.** The DC were isolated from PBMC using the method described by Romani *et al.*<sup>(23)</sup> This *in vitro* study was approved by the ethics committees of Xinqiao Hospital, Third Military Medical University. All peripheral blood donors signed written informed consent in order to participate in the study. Briefly, 200 mL peripheral blood from healthy HLA-A2<sup>+</sup> donors was used to isolate PBMC. The PBMC were isolated using Ficoll-Hypaque density gradient centrifugation and then seeded into culture flasks in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). After 2 h of incubation, during which the monocytes adhered to the culture flasks, non-adherent cells were collected and frozen in freezing medium (30% FBS, 60% RPMI-1640 and 10% dimethyl sulfoxide) for later use in the CTL assays. The adherent cells were cultured in RPMI-1640 containing granulocyte-macrophage colony-stimulating factor (GM-CSF; 1000 U/mL, R&D Systems, Inc., Minneapolis, MN, USA) and interleukin-4 (IL-4; 1000 U/mL, R&D Systems, Inc.) for 7 days and then an additional day in the presence of tumor necrosis factor-α (TNF-α) (2000 U/mL, R&D Systems, Inc.), which induces final maturation. The mature DC were harvested on the eighth day. The phenotypic markers of these DC were analyzed using flow cytometry.

The DC from mouse bone marrow (mDC) were generated as described previously.<sup>(17,24)</sup> Briefly, the bone marrow was flushed from the femurs and tibias of C57BL/6-Tg mice. The cells were washed twice with serum-free RPMI-1640 medium and then cultured in a 100-mm plastic Petri dish at 1 × 10<sup>5</sup> cells/mL with RPMI-1640 medium containing recombinant murine GM-CSF (mGM-CSF; 200 U/mL, R&D System, Inc.) and recombinant murine IL-4 (mIL-4; 400 U/mL, R&D System, Inc.). Half of the media was refreshed without discarding any cells and fresh cytokine-containing (mGM-CSF and mIL-4) media was added on days 3, 5 and 7. Mouse TNF-α (R&D System, Inc.) was added on day 8 of culture. On day 9, any non-adherent cells obtained from these cultures were considered to be mature bone marrow-derived DC. The phenotypic markers of mDC were confirmed by flow cytometry using a BD FACSAria II.

**Induction of peptide-specific CTL with synthetic heparanase peptides.** These assays were conducted as described previously.<sup>(15-17)</sup> Briefly, we loaded the DC derived from PBMC with different heparanase peptides as well as a negative control peptide at a final concentration of 40 µmol/L for 4 h (the proportion of each epitope in multi-epitope vaccine is 1:1:1:1), then irradiated with 20 Gy to prevent outgrowth of the control cultures. Autologous T cells were restimulated every 7 days for three times to generate peptide-specific CTL with the peptide-pulsed DC, as mentioned previously. Recombinant IL-2 at a final concentration of 50 U/mL (R&D Systems, Inc.) was added to the culture medium on day 1. Five days after the final stimulation, CTL activity was assessed using a 4-h <sup>51</sup>Cr release assay.<sup>(16-18)</sup>

The DC generated from mouse bone marrow were cultured in 3 mL of RPMI-1640 supplemented with fetal calf serum (FCS; 10%), L-glutamine (2 mmol/L), 2-mercaptoethanol (50 mmol/L), penicillin (100 U/mL), streptomycin (100 Ag/mL) and 40 mmol/L of each heparanase polypeptide. Cells were then cultured at 37°C for 3 h with gentle shaking every 30 min and then washed twice with phosphate-buffered saline

(PBS). Next, the above-mentioned DC ( $2 \times 10^6$ ) loaded with the peptides were used to immunize C57BL/6-Tg(HLA-A2)1ENGE/J mice subcutaneously three times at 7-day intervals. Seven days after the last vaccination, the mice were killed and the spleen cells ( $4 \times 10^6$ /mL) were cultured with different heparanase peptides in six-well plates in complete medium containing 50 U/mL IL-2. After 5 days of co-culture, the *in vivo* restimulated splenocytes were analyzed using a 4-h  $^{51}\text{Cr}$  release assay.

**Cytotoxicity assay.** To evaluate the levels of CTL activity, a 4-h  $^{51}\text{Cr}$  release assay was used, as previously described.<sup>(15–17)</sup> Briefly, the target cells were incubated with  $^{51}\text{Cr}$  (100 mCi per  $1 \times 10^6$  cells) for 2 h in a 37°C water bath. After incubation with  $^{51}\text{Cr}$ , the target cells were washed three times with PBS, resuspended in RPMI-1640 medium and mixed with effector cells at a 10:1, 20:1, 40:1 or 80:1 effector to target (E/T) ratio. The assays were conducted in triplicate for each sample at each ratio in a 96-well round-bottomed plate. After 4 h of incubation, 100  $\mu\text{L}$  of supernatant was harvested from each well and the amount of released  $^{51}\text{Cr}$  was measured with a gamma counter. The percentage of specific lysis was calculated using the following formula:

$$\text{Specific lysis (\%)} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100\%$$

**Determining interferon (IFN)- $\gamma$  production using an enzyme-linked immunospot assay.** The heparanase-specific CTL were assayed for IFN- $\gamma$  production using a 24-h enzyme-linked immunospot (ELISPOT).<sup>(15–17)</sup> Briefly, CD8+ T cells were sorted using flow cytometry and plated in triplicate at a final concentration of  $1 \times 10^5$  or  $4 \times 10^5$  cells per well in 96-well coated microtiter plates (DAKEWE, China). The effector cells were stimulated with candidate peptides at a final concentration of 30 mmol/L. The plates were incubated at 37°C with 5%  $\text{CO}_2$  for 24 h. The plates were processed using a biotin-labeled anti-mouse IFN- $\gamma$  antibody, an enzyme-labeling marker and an anti-marker. Freshly prepared developer was then added and the plates were incubated in the dark at 37°C for 8 min (Quick Spot Mouse IFN- $\gamma$  Precoated ELISPOT kit; DAKWE, Shenzhen, China). Spot density was quantified using the ELISPOT reader (BioReader 4000 Pro-X, BIOSYS, Karben, Frankfurt, Germany).

**Statistics.** All experiments were performed in triplicate and the results are given as mean  $\pm$  SD. The statistical analysis was conducted using a Student's *t* test. Differences were considered statistically significant when the *P*-value was  $<0.05$ . All statistical analyses were conducted using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

## Results

**Epitope prediction, polypeptide synthesis, purification and identification.** HLA-A2-binding scores of epitopes predicted from heparanase using the quantitative motif method are shown in Table 1. We synthesized 28 peptides using solid-phase peptide synthesis. The purity of the peptides was confirmed using HPLC. The purity of the peptides was 93% or more, which meets the international reference standard for polypeptide experiments (data not shown). The identity of the peptides was confirmed using mass spectrographic analysis. There was no significant discrepancy between the observed

**Table 1.** HLA-A2-binding scores of epitopes predicted from heparanase using the quantitative motif method

| Position      | Amino acid sequence | HLA-A2-binding score |
|---------------|---------------------|----------------------|
| Hpa (363-371) | FMWLDKLG            | 41.31                |
| Hpa (315-323) | VLDIFISSV           | 25.37                |
| Hpa (456-464) | NLHNVTKYL           | 21.72                |
| Hpa (16-24)   | LLLGLGPL            | 13.93                |
| Hpa (412-420) | KLVGTVKLM           | 13.84                |
| Hpa (64-72)   | NLATDPRFL           | 9.98                 |
| Hpa (303-311) | RTATREDFL           | 7.02                 |
| Hpa (393-401) | LVDENFDPL           | 6.68                 |
| Hpa (8-16)    | ALPPPLMLL           | 45.41                |
| Hpa (174-182) | YTFANCSGL           | 24.63                |
| Hpa (310-318) | FLNPDVLDI           | 21.11                |
| Hpa (241-249) | QLGEDFIQL           | 13.85                |
| Hpa (82-90)   | TLARGLSPA           | 12.28                |
| Hpa (380-388) | VMRQVFFGA           | 8.05                 |
| Hpa (72-80)   | LILLGSPKL           | 6.91                 |
| Hpa (322-330) | SVQKVFQVV           | 5.59                 |
| Hpa (1-9)     | LLRSKPAL            | 31.72                |
| Hpa (494-502) | QLNGLTLKM           | 22.93                |
| Hpa (51-59)   | LVSPSFLSV           | 19.07                |
| Hpa (487-495) | GLLSKSVQL           | 13.64                |
| Hpa (365-373) | WLDKLGLSA           | 11.76                |
| Hpa (449-457) | DLTYAINL            | 7.63                 |
| Hpa (66-74)   | ATDPRFLIL           | 6.86                 |
| Hpa (13-21)   | LMLLLGLPL           | 5.15                 |
| Hpa (183-191) | DLIFGLNAL           | 41.12                |

value and the theoretical value of the molecular weight of the human heparanase epitopes, confirming the identity of the peptides.

**Peptide-binding assay with flow cytometry.** As shown in Figure 1, we found that all epitopes from human heparanase upregulated the expression of HLA-A2 on T2 cells except Hpa (184-192)(LIFGLNALL). Expression of the peptides increased the expression of HLA-A2 molecules on T2 cells in a dose-dependent manner. When the concentration of peptides reached 40  $\mu\text{mol/L}$ , the relative binding affinity exceeded 1.5 (Fig. 1). These results indicate that the human heparanase epitopes could bind to HLA-A2 molecules on T2 cells.

**Identification of DC.** The DC phenotypes were assessed using flow cytometry. The results showed that the expression of CD86, CD83, CD1a and HLA-A2 in the DC derived from PBMC was 82.6%, 91.3%, 87.8% and 99.8%, respectively, whereas the expression of CD86, CD11c, MHC-II and HLA-A2 in DC isolated from mice was 92.1%, 73.6%, 86.5% and 89.5%, respectively. These data indicate that these DC are mature and have antigen-presenting capacity.

**Heparanase multi-epitope vaccines are more potent than single epitope vaccines in inducing CTL killing activity.** Next, we assessed the killing effect of lymphocytes induced by all of the predicted epitopes. Our results showed that Hpa16, Hpa8, Hpa310, Hpa315 and Hpa363 were able to elicit an effective antitumor immune response (Fig. 2). To detect whether the heparanase multi-epitope vaccines could elicit stronger heparanase-specific CTL than the corresponding single heparanase peptides vaccines, DC were loaded with heparanase multi-epitope vaccines and their corresponding single peptides to induce heparanase-specific CTL. The cytotoxicity of heparanase-specific

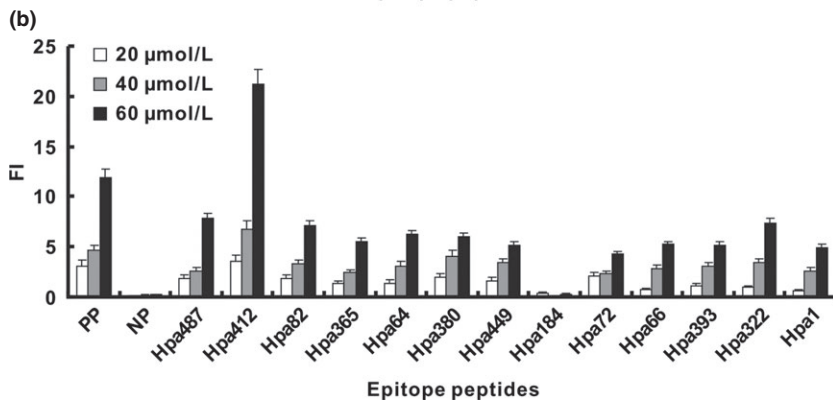
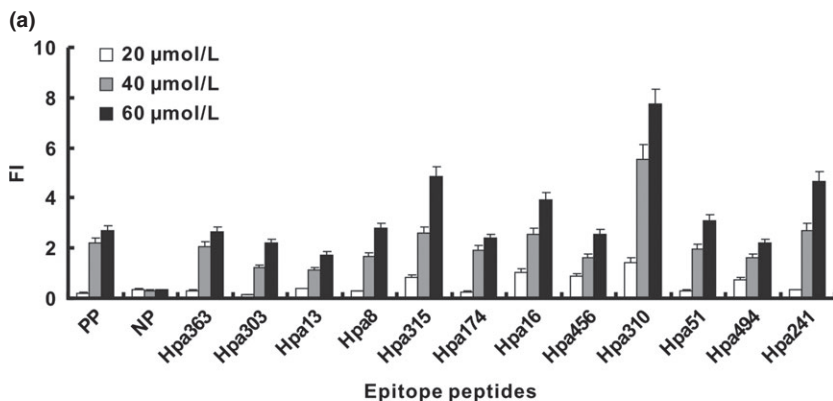


Fig. 1. Analysis of the HLA-A2-binding affinity of epitopes derived from human heparanase. (a, b) Fluorescence index (FI) of human heparanase-derived peptides binding to HLA-A2. NP, negative peptide control; PP, positive peptide control.

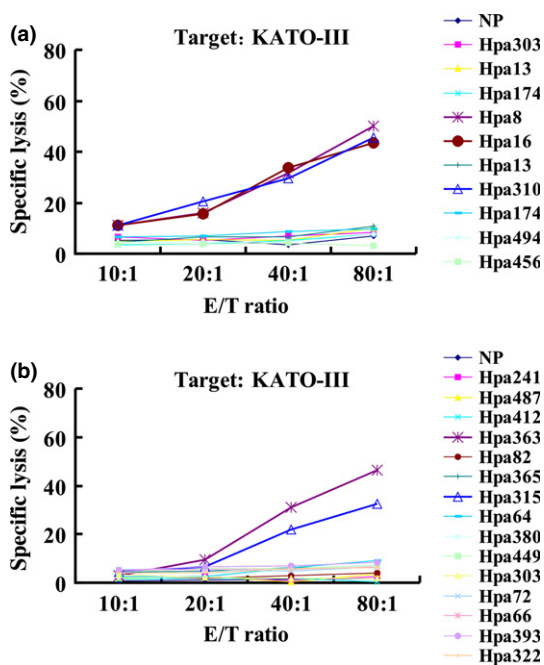
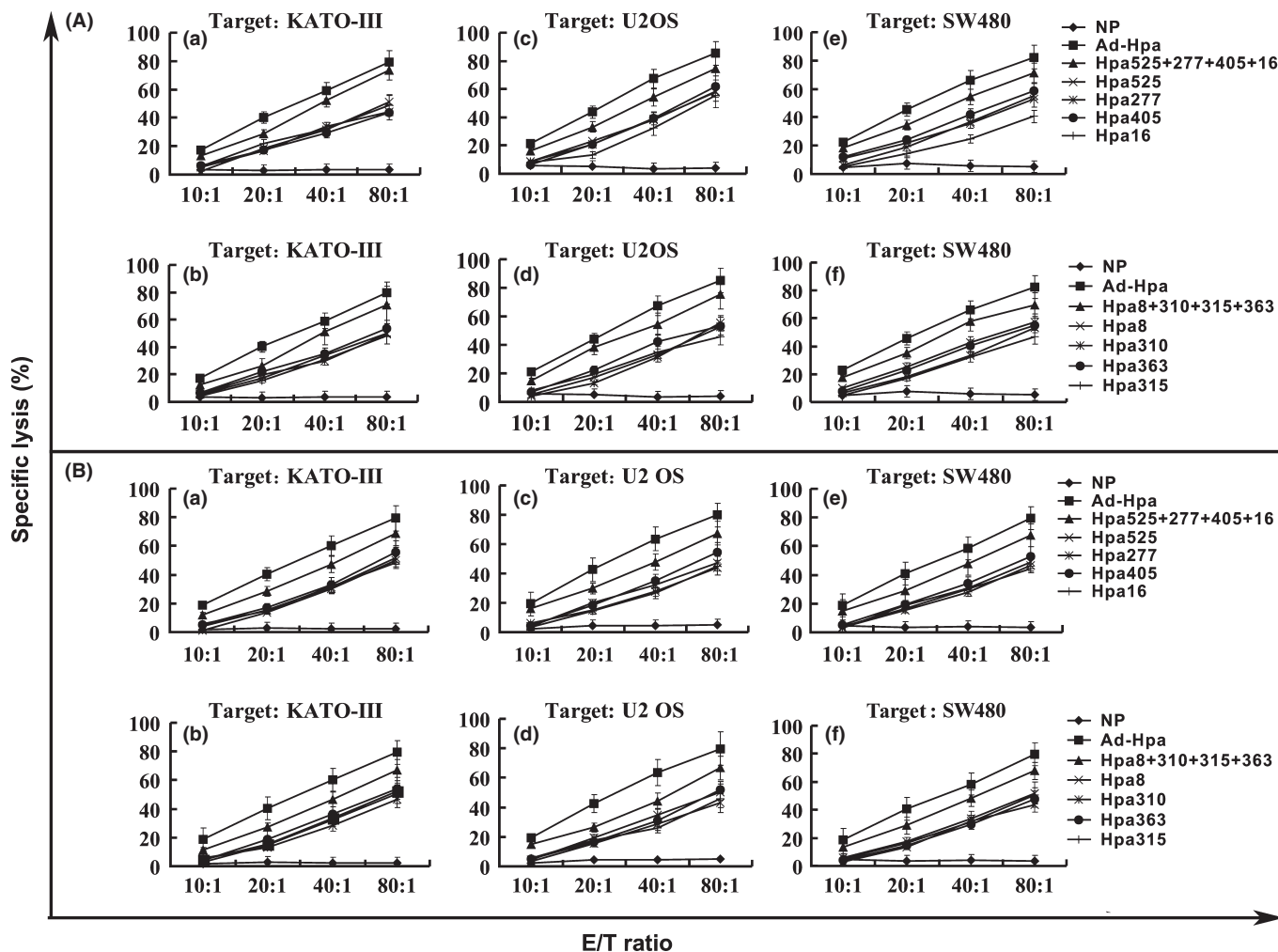


Fig. 2. Identification of efficient human heparanase epitopes. (a, b) Specific cytotoxic T lymphocyte (CTL)-mediated lysis of KATO-III gastric carcinoma cells induced by epitope peptides derived from heparanase was detected using a  $^{51}\text{Cr}$  release assay. The CTL generated from the HIV<sub>pol</sub>(476-484)(ILLEPVHGV) peptide derived from HIV were used as the negative peptide control (NP). E/T, effector/target.

CTLs to various tumor cell lines was measured using a 4-h  $^{51}\text{Cr}$  release assay. The results showed that the multi-epitope vaccines from human heparanase Hpa525 + 277 + 405 + 16

and Hpa8 + 310 + 315 + 363 and their corresponding single peptides Hpa525, Hpa277, Hpa405, Hpa16, Hpa8, Hpa310, Hpa315 and Hpa363 could induce heparanase-specific killing of KATO-III gastric cancer cells, U2OS osteogenic sarcoma cells and SW480 colonic cancer cells, which express both heparanase and HLA-A2 (Fig. 3). At the highest E/T ratio (80:1), the lysis rates of CTL generated from Ad-Hpa were 85.3% *in vitro* and 79.6% *ex vivo*. The lysis rates of CTL generated from the multi-epitope vaccines were 73.9%, 71.1% and 74.4%, respectively, *in vitro* and 68.5%, 67.6%, and 67.8%, respectively, *ex vivo*. The highest lysis rates of CTL generated from Hpa525, Hpa277, Hpa405, Hpa16, Hpa8, Hpa310, Hpa315 and Hpa363 were 58.2%, *in vitro* and 54.3%, *ex vivo*. The induced effector cells generated from the negative peptide could not lyse the target cells. These results indicate that multi-epitope vaccines generated from human heparanase are capable of eliciting much more potent killing of tumor cells than their corresponding single peptides *in vitro* and *ex vivo* (Fig. 3).

**Multi-epitope vaccines of heparanase are cytotoxic to several tumor cell lines in a heparanase-specific and MHC-restricted manner.** To further confirm the heparanase specificity of the CTL *in vitro* and *ex vivo*, we used the HLA-A2-positive, heparanase-negative breast cancer cell line MCF-7 (Fig. 4). MCF-7 cells were transfected with recombinant, replication-defective adenovirus (Ad-Hpa) encoding a full-length cDNA of human heparanase at a multiplicity of infection of 200 and cultured for 2 days in fresh DMEM medium containing 10% FCS. In our previous study, western blot analysis showed that heparanase protein could be detected in MCF-7/Hpa cells, whereas in MCF-7 cells the expression of heparanase is very weak.<sup>(19)</sup> Heparanase-specific CTL were generated using peptide-pulsed DC from HLA-A2-positive PBMC and DC from the bone marrow of C57BL/6-Tg transgenic mice. After being stimulated



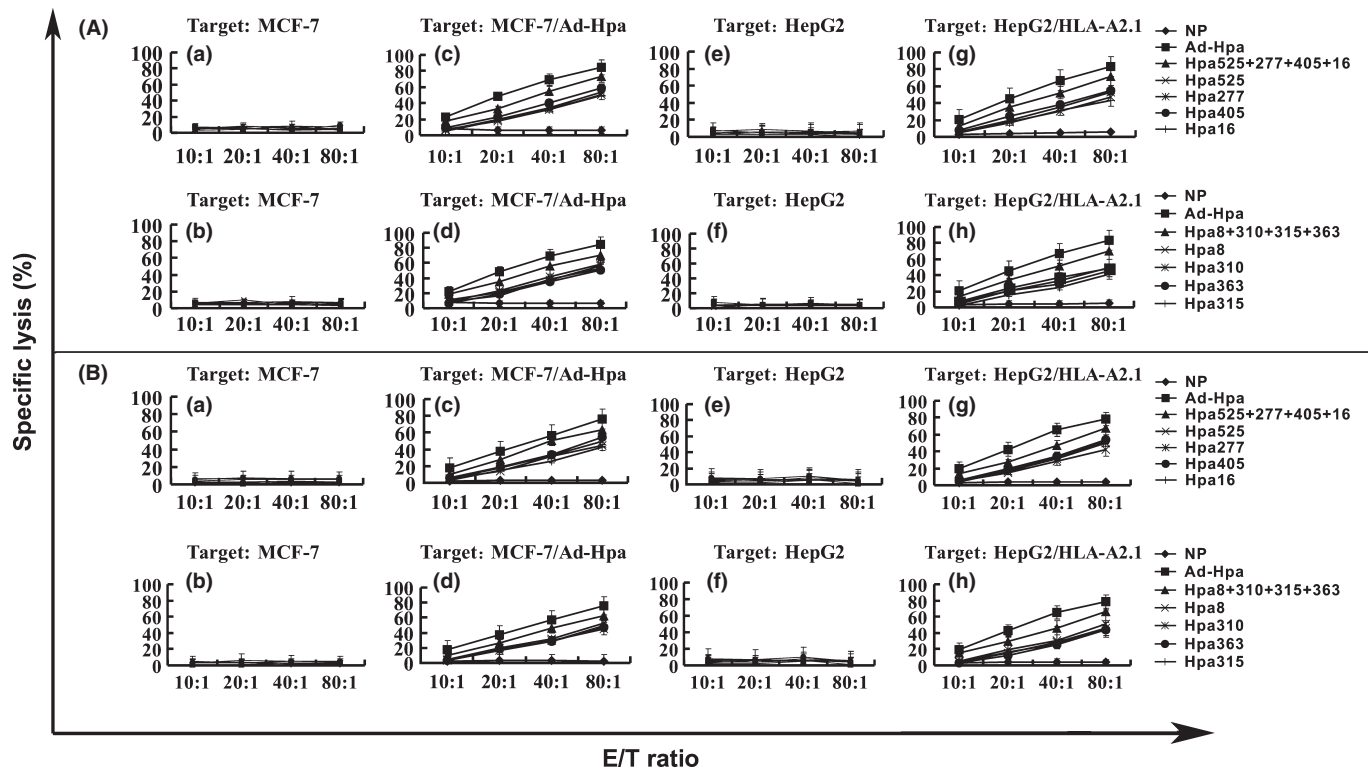
**Fig. 3.** Specific cytotoxic T lymphocyte (CTL)-mediated lysis of various target cells *in vitro* and *ex vivo* by CTL generated from different heparanase-derived peptides. (A) Specific lysis of CTL induced by multi-epitope vaccines or the single epitope-pulsed dendritic cells derived from PBMC against KATO-III (a, b), U2OS (c, d) and SW480 (e, f) cells. (B) Specific lysis of CTL induced by multi-epitope vaccines or the single epitope-pulsed dendritic cells derived from HLA-A2 transgenic mice bone marrow against KATO-III (a, b), U2OS (c, d) and SW480 (e, f) cells. The CTL generated from the HIV<sub>po1</sub>(476-484)(ILLEPVHGV) peptide derived from HIV served as the negative peptide control (NP). E/T, effector/target.

three times, the killing effect of CTL induced by Hpa525 + 277 + 405 + 16 and Hpa8 + 310 + 315 + 363 and their corresponding single peptides Hpa525, Hpa277, Hpa405, Hpa16, Hpa8, Hpa310, Hpa315 and Hpa363 were determined at various E/T ratios using a 4-h <sup>51</sup>Cr release assay. The results showed that these heparanase-specific CTL could lyse MCF-7/Hpa cells, whereas no obvious lysis of MCF-7 cells was detected even at the highest E/T ratio. Furthermore, compared with the corresponding single heparanase peptides, heparanase multi-epitope vaccines elicited more robust specific lysis of MCF-7/Hpa cells (Fig. 4). These results clearly indicate that the CTL specifically targeted heparanase peptides that were presented in the context of HLA-A2 and the multi-epitope vaccines of human heparanase could elicit much more potent killing effects compared with their corresponding single peptides.

To further confirm that these CTL epitopes were restricted by HLA-A2, we took advantage of the heparanase-positive HLA-A2-negative liver cancer cell line HepG2 (Fig. 4). HepG2 cells were transfected with a eukaryotic vector containing the full-length HLA-A2 cDNA using the DOTAP (Roche Ltd. CO., Mannheim, Germany) lipofection method according to the man-

ufacturer's protocol. After 24 h of transduction, 400 mg/mL G418 was added to the RPMI-1640 medium. After selecting with G418 for 4 weeks, a drug-resistant clone was randomly selected from the transduced cultures. The selected clone was named HepG2/HLA-A2. HLA-A2 was expressed in 68.5% of HepG2/HLA-A2 cells, but only 1.3% of control HepG2 cells. The <sup>51</sup>Cr release assay showed that CTL generated from Hpa525 + 277 + 405 + 16 and Hpa8 + 310 + 315 + 363 and their corresponding single peptides Hpa525, Hpa277, Hpa405, Hpa16, Hpa8, Hpa310, Hpa315 and Hpa363 peptide-pulsed DC could lyse HepG2/HLA-A2 cells *in vitro* and *ex vivo*. However, the polypeptide-induced effector cells could not lyse HepG2 cells, even at the highest E/T ratio. Moreover, compared with their corresponding heparanase single peptides, heparanase multi-epitope vaccines could elicit much more potent lysis of HepG2/HLA-A2 cells (Fig. 4). These results clearly show that the polypeptide-induced CTL are restricted by MHC-I molecules.

**Heparanase-specific CTL do not lyse autologous lymphocytes or DC.** Heparanase is expressed in immunologically competent cells, natural killer cells and inflammatory cells such as neutrophils, granulocytes and activated T and B cells.<sup>(25)</sup>



**Fig. 4.** Heparanase specificity and HLA-A2 restriction of cytotoxic T lymphocytes (CTL) generated from different heparanase vaccines *in vitro* (A) and *ex vivo* (B). The CTL generated from the HLA-A2-restricted nonapeptide HIV<sub>po1</sub>(476-484)(ILLEPVHGV) derived from HIV served as the negative peptide control (NP). (A) The cytotoxic activity of CTL induced by heparanase multi-epitope vaccines and single epitope peptides against MCF-7 (A[a, b]), MCF-7/Hpa (A[c, d]), HepG2 (A[e, f]) and HepG2/HLA-A2.1 (A[g, h]) *in vitro*. (B) The cytotoxic activity of CTL induced by heparanase multi-epitope vaccines and single epitope peptides against MCF-7 (B[a, b]), MCF-7/Hpa (B[c, d]), HepG2 (B[e, f]), and HepG2/HLA-A2 cells (B[g, h]) *ex vivo*. E/T, effector/target.

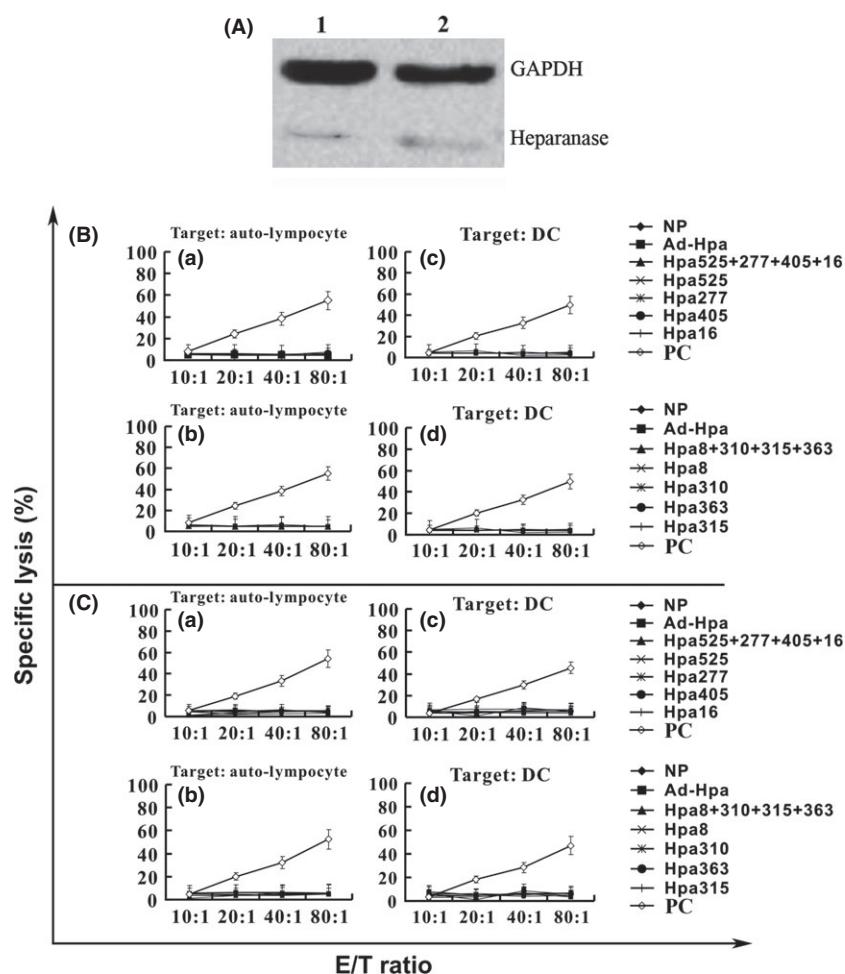
Theoretically, immunotherapy targeting heparanase might have side-effects on the immune system. To investigate the effect of heparanase-specific CTL on immunologically activated lymphocytes, CTL induced by heparanase-specific peptides were also used to lyse autologous lymphocytes and DC. The results indicate that multi-epitope vaccines and their corresponding single peptides from heparanase did not lyse autologous lymphocytes or DC *in vitro* or *ex vivo* (Fig. 5).

**ELISPOT assay for IFN- $\gamma$ .** Because CTL are known to produce the Th1 cytokine IFN- $\gamma$ , we quantified peptide-specific T cells by measuring IFN- $\gamma$ -producing cells with an ELISPOT assay. As shown in Figure 6, Hpa525 + 277 + 405 + 16 and Hpa8 + 310 + 315 + 363 and their corresponding single peptides generated strong peptide-specific T-cell responses by inducing a higher frequency of IFN- $\gamma$ -producing T cells compared with a control peptide ( $P < 0.05$ ). We also found that mice immunized with Hpa525 + 277 + 405 + 16 and Hpa8 + 310 + 315 + 363 produced many more IFN- $\gamma$  spots (indicating peptide-specific T cells) than mice immunized with vaccines expressing the corresponding single peptides ( $P < 0.05$ ) (Fig. 6).

## Discussion

Immunotherapy for cancer patients is expected to become a fourth option for the treatment of cancer, in addition to the currently available therapeutic arsenal of surgical excision, chemotherapy and radiotherapy.<sup>(26–28)</sup> Cancer immunotherapy is a field of intense research, particularly studies focusing on

the induction of active immunity. Recent advances in immunology and the identification of promising tumor-associated antigens introduced the possibility of treating cancer patients using tumor-associated antigen-specific immunotherapy.<sup>(29,30)</sup> The immune response to tumor-associated antigens (TAA) is well established.<sup>(31)</sup> Cancer immunotherapy is based on the capacity of CD8<sup>+</sup> T lymphocytes to eliminate malignant cells.<sup>(32,33)</sup> The activated CTL recognize tumor associated antigenic peptides on the surface of tumor cells in association with MHC class I molecules and lyse the cancer cells expressing this tumor-associated antigen.<sup>(34)</sup> Because many tumor-associated antigens are normal ‘self-proteins’, epitope peptides derived from these tumor-associated antigens are often affected by T-cell tolerance. Previous studies have indicated that spontaneous antitumor T-cell responses in cancer patients are controlled by regulatory T cells (Tregs). Bonertz *et al.*<sup>(35)</sup> showed that colorectal cancer patients develop multivalent and individual T-cell responses against a broad variety of different colorectal cancer-associated TAA; however, Tregs that were responsive to heparanase were not found in patients with colorectal cancer. Heparanase is the only endogenous endoglycosidase that can degrade the heparan sulfate glycoprotein in ECM and basement membrane. Heparanase can be expressed in almost all of the metastatic malignant tumor cells. It is not expressed in normal mature non-immune tissue, such as heart, lung and liver, skeletal muscle, pancreas and so on. The occurrence of metastasis depends mostly on activation of heparanase, which makes tumor cells break through the basement membrane and ECM, release many kinds of cytokines,



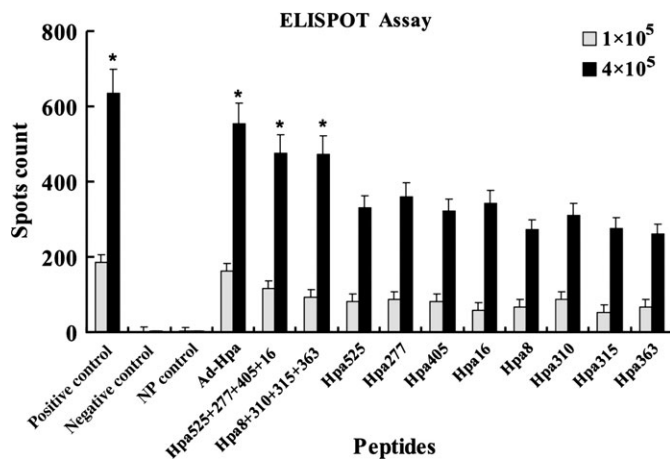
**Fig. 5.** Western blot analysis of dendritic cells (DC) and lymphocyte for heparanase expression and heparanase-specific cytotoxic T lymphocyte (CTL) killing of autologous lymphocytes and DC *in vitro* and *ex vivo*. (A) Western blot analysis of DC and lymphocyte for heparanase expression. (1) Heparanase expression in lymphocytes. (2) Heparanase expression in dendritic cells. (B, C) The CTL generated from the HLA-A2-restricted nonapeptide HIV<sub>pol</sub>(476-484)(ILLEPVHGV) derived from HIV serves as the negative peptide control (NP). The KATO-III cells serve as a target for the positive control (PC). Specific lysis of CTL generated from heparanase multi-epitope vaccines and single epitope peptides against autologous lymphocytes and DC *in vitro* (B) and *ex vivo* (C).

provoke the formation of new vessels and cause the local permanent planting of tumor cells. Inhibition of heparanase can obviously inhibit the proliferation and metastasis of tumor cells. Furthermore, if some tumors develop immune escape variants against heparanase-specific CTL through downregulating Hpa expression, such variants might have inhibited the proliferation and metastasis of tumor cells by reducing their antigenicity. Heparinase is a potential universal TAA for immunotherapy of advanced-stage tumors. Therefore, epitopes derived from heparanase could be good candidates for inducing an antitumor immune response because they are not affected by tolerance and/or ignorance due to low recognition efficiency.

Previous studies indicate that cancer-specific CTL were induced by HLA class I-binding short peptides of 8–9 amino acids.<sup>(36)</sup> Cancer immunotherapy with short peptide vaccines could induce increased cancer-specific CTL, but vaccine therapy using only one CTL epitope appears to be suboptimal in treating cancer.<sup>(37,38)</sup> Therefore, the development of immunization strategies that induce strong multi-valent T-cell responses is crucial for tumor immunotherapy. In the present study, we demonstrated the multi-epitope vaccines from heparanase have the capacity to elicit a much more potent immune response against different cancer cells than single epitope vaccines. The multi-epitope vaccines Hpa525 + 277 + 405 + 16 and Hpa8 + 310 + 315 + 363 induced increased Hpa-specific lysis of KATO-III gastric carcinoma cells, SW480 colon cancer cells and U2OS osteogenic sarcoma cells in a HLA-

A2-restricted and heparanase-specific manner compared with Hpa525, Hpa277, Hpa405, Hpa16, Hpa8, Hpa310, Hpa315 and Hpa363, both *in vitro* and *ex vivo*. These results suggest that a heparanase epitopes multi-epitope vaccine is superior to single epitope vaccines. Moreover, our results suggest that the protective immune response observed in HLA-A2-transgenic mice immunized with multi-epitope vaccines could be greater than that observed when a single epitope peptide vaccination is used.

We further showed that CD8<sup>+</sup> T epitope peptide-pulsed DC stimulated a large number of IFN- $\gamma$ -producing cells. The immune-modulatory cytokine IFN- $\gamma$  is a pivotal player in the immune system; this cytokine is known to enhance the expression and function of various antigen-processing components. Interferon- $\gamma$  has been considered to provide antitumor benefits through its ability to enhance antigen processing for both MHC-I and MHC-II pathways.<sup>(39)</sup> A previous study showed that IFN- $\gamma$  induces the expression of the immunoproteasome, which is associated with more efficient MHC class I antigen processing.<sup>(40)</sup> The secretion of IFN- $\gamma$  might sensitize tumor cells to CTL-mediated lysis by upregulation of MHC-I molecules, stimulate the innate arm of the immune system at the tumor site and prevent local angiogenesis.<sup>(41)</sup> Our results suggest that heparanase multi-epitope vaccines could induce a heparanase-specific immune response, leading to tumor cell killing. These results also suggest that heparanase is an ideal broad-spectrum TAA for clinical use in the treatment of patients with advanced-stage tumors.



**Fig. 6.** Interferon (IFN)- $\gamma$ -producing cell quantification using an enzyme-linked immunospot (ELISPOT) assay. The negative peptide (NP), HLA-A2-restricted nonapeptide HIV<sub>pol</sub>(476-484)(ILLEPVHGV) derived from HIV, serves as the NP control. Phytohemagglutinin (PHA) was used as a positive control. The column indicates the mean; bars, SE. \*Statistically significant values ( $P < 0.01$  using a paired Student's *t* test) were assessed compared with the NP group.

Several previous studies have indicated that heparanase is also expressed in some immune cell populations, such as T and B lymphocytes, DC, macrophages, mast cells and neutrophils, mediating extravasation and traffic to inflammatory sites.<sup>(25,42-44)</sup> Using multi-epitope vaccines for cancer treatment poses a risk of autoimmune adverse effects. We previously showed that CTL induced by heparanase epitopes could not lyse autologous lymphocytes and DC, which express heparanase at low levels.<sup>(15-18)</sup> However, it is unclear if the heparanase-specific CTL induced by multi-epitope vaccines, due to their enhanced immunogenicity, could lyse autologous lymphocytes and DC. To investigate this, we used autologous lymphocytes and DC as target cells for lysis by CTL induced by multi-epitope vaccines. The results indicate that the CTLs

induced by heparanase multi-epitope vaccines could not lyse these lymphocytes. Taken together, our results indicate that the lysis of lymphocytes or DC does not occur *in vitro* or *ex vivo*. This result is similar to the studies by Vonderheide *et al.*<sup>(45)</sup> Vonderheide and colleagues used human telomerase reverse transcriptase (hTERT) as a universal TAA. The authors found that hTERT-specific CTL induced by hTERT-transfected DC could not lyse hTERT-positive lymphocytes. It was explained in the study that expression of hTERT in lymphocytes was below the threshold that hTERT-specific CTL could recognize. In the present study, we also deduced that the level of heparanase expressed in normal cells was below the threshold recognized by the heparanase peptide-specific CTL. Our results suggest that the heparanase multi-epitope vaccines are safe and effective.

In summary, the present study suggests that multi-epitope vaccines based on HLA-A2-restricted CTL epitopes of human heparanase are capable of inducing HLA-A2-restricted and heparanase-specific CTL *in vitro* and *ex vivo*. Furthermore, compared with their corresponding single peptides, heparanase multi-epitope vaccines elicit a much more robust lysis of target cells by activating CD8<sup>+</sup> T lymphocytes. However, these heparanase-specific CTL do not lyse heparanase-expressing autologous lymphocytes and DC, confirming the safety of these multi-epitope vaccines. Therefore, the present study provides theoretical evidence of the suitability of heparanase multi-epitope vaccines for clinical application.

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#### Disclosure Statement

The authors have no conflict of interest.

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