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Prediction and identification of novel HLA-A*0201-restricted cytotoxic T lymphocyte epitopes from endocan

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Abstract

Background: Prediction and identification of cytotoxic T lymphocyte (CTL) epitopes from tumor associated antigens is a crucial step for the development of tumor immunotherapy strategy. Endocan has been identified as antigen overexpressed in various tumors.

Methods: In this experiment, we predicted and identified HLA-A2-restricted CTL epitopes from endocan by using the following procedures. Firstly, we predicted the epitopes from the amino acid sequence of endocan by computer-based methods; Secondly, we determined the affinity of the predicted peptide with HLA-A2.1 molecule by peptide-binding assay; Thirdly, we elicited the primary T cell response against the predicted peptides in vitro; Lastly, we tested the specific CTLs toward endocan and HLA-A2.1 positive target cells.

Results: These data demonstrated that peptides of endocan containing residues 4–12 and 9–17 could elicit specific CTLs producing interferon-γ and cytotoxicity.

Conclusions: Therefore, our findings suggested that the predicted peptides were novel HLA-A2.1-restricted CTL epitopes, and might provide promising target for tumor immunotherapy.

Keywords: HLA-A*0201, Cytotoxic T lymphocyte, Epitopes, Endocan

Introduction

Gliomas are the most common primary central nervous system tumors [1–3]. Glioblastomas represent 50% of all gliomas in adults with an extremely poor prognosis. Despite innovative therapeutic methods such as surgical resection, chemotherapy, and radiation therapy, the survival ratio is very low [4–6]. Therefore, the novel therapeutic approaches are in great needed.

Endocan is a soluble proteoglycan of 50 kDa, consisting of a mature polypeptide of 165 amino acids and a single dermatan sulfate chain [7]. Endocan was originally described as being secreted by human umbilical vein endothelial cells (HUVECs) [8]. Recently, endocan has been identified to be upregulated in various tumors [9–11]. In addition, endocan has also been found in common brain tumors and acted as a marker for malignancy [12–14]. In recent years, endocan has been reported as an important biomarker for inflammation [15]. The related results confirm the hypothesis that human endocan may have a protective effect against acute lung inflammation [16].

Cellular adoptive immunotherapy has been widely utilized in the tumor therapy [17]. Tumor-associated antigens (TAA) represent promising targets for anticancer immunotherapies [18]. Peptides derived from TAAs are presented on the surface of tumor cells in association with MHC class I (MHC-I) complexes [19]. These peptide-MHC-I (pMHC-I) complexes can generate CTL mediated antitumor responses [20].

To date, endocan related CTL epitopes have not been identified. In this experiment, HLA-A*0201-restricted cytotoxic T lymphocyte epitopes from endocan were predicted, and the potential of generating CTL responses were analyzed.

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Materials and methods

Cell lines and animals

The human TAP-deficient T2 cell line, BB7.2 cell line producing mAb against HLA-A2, human glioma cell line U251 (HLA-A2+), microglia cell line BV2 (HLA-A2-) were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 medium containing 10% FBS (Gibco, with endotoxin level < 10 IU/ml), penicillin (200 U/ml), and streptomycin (100 μg/ml). All cell lines mentioned previously were kept at 37 °C in a humidified atmosphere containing 5% CO_2. HLA-A*0201/Kb transgenic (Tg) mice, 8–12 weeks-old, were purchased from The Jackson Laboratory (USA). Mice were bred and maintained in specific pathogen-free (SPF) facilities. Animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of Chongqing Medical University.

Epitope prediction and synthesizing

A panel of endocan specific peptides (9 mer) containing HLA-A* 0201-binding motifs were predicted by software BIMAS (Section of BioInformatics and Molecular Analysis, National Institutes of Health, Bethesda, MD, USA) and SYFPEITHI (Institute for Immunology, University of Tübingen, Tübingen, Germany). The two 9-mer-peptides that demonstrated the highest scores with both SYFPEITHI and BIMAS were utilized for further analysis. Peptides were synthesized by F-moc chemistry. Before use, HPLC-purified peptides (> 95% pure) were dissolved at 40 mg/ml in DMSO and diluted to 1 mg/ml with Iscove's modified Dulbecco's medium (IMDM) (Life Technologies, Grand Island, NY).

Peptide-binding assay

HLA-A*0201 Ag-processing defective T2 cell line was cultured in RPMI 1640 containing 10% FBS. Before use, the cells were incubated for 6 h at 37 °C in serum-free IMDM. Then, cells were washed once, suspended in serum-free IMDM containing 20 μM of 2-ME and 15 μg/ml of human β2-microglobulin (β2m) (Calbiochem, La Jolla, CA), and pulsed with 50 μM peptide. HLA-A2.1-restricted MAGE-2 CTL epitope KMVELVHFL (amino acid position in MAGE-2; 112–120) and Kb-restricted Hpa CTL epitope FSYGFFVI (amino acid position in Hpa; 519–526) served as positive and negative controls. After a 24 h incubation at 37 °C, T2 cells were washed once with cold PBS containing 0.5% BSA and 0.02% NaN_3. They were then stained directly with primary anti-HLA-A2 Ab derived from BB7.2 and FITC-labeled goat-antimouse IgG (BD Biosciences Pharmingen, USA) secondary antibody. The percentage of FITC-positive cells as well as their staining intensity (mean fluorescence intensity (MFI)) was determined on an Epics Profile II (Coulter, Hialeah, FL). The Δ MFI for a particular mAb was calculated by subtracting the MFI of either the isotype-matched control mAb or the second-step Ab from each MFI value. The fluorescence ratio (FR) was calculated using the following formula: FR = (Δ MFI of peptide-treated T2 cells)/(Δ MFI of nontreated T2 cells).

RT-PCR analysis of endocan expression

The tumor cells were homogenized using RNAiso Plus (Takara) and ceramic beads for 1 min in a speedmill plus according to the instructions of the manufacturer (Alytik Jena). RNA was isolated according to the instructions of the manufacturer and reverse transcripted to obtain cDNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara). Real-time PCR was performed using cDNA samples with SYBR@Premix ExTaq™II (Takara, Tli RNaseH Plus) by the One-step Plus analyzer (ABI). We normalized the results for each individual gene using the housekeeping gene beta-actin. RT-PCR products were then run on a gel and visualized with ethidium bromide.

Western blot analysis of endocan expression

Proteins from cultured tumor cells were resolved using SDS-PAGE and transferred onto polyvinylidene fluoride membranes using electroblotting. The membranes were incubated with primary antibodies, all diluted to 1:1000 (Cell Signaling Technology), at 4 °C overnight. β-actin (1:200; Santa Cruz Biotechnology, Dallas, TX) was used as the loading control. The membranes were incubated with HRP-conjugated goat anti-rabbit secondary Abs (1:2500; Sigma-Aldrich, St. Louis, MO) at 25 °C for 1 h. Bound Abs were visualized using a chemiluminescence detection system. Protein levels were calculated as the ratio of the target protein value to the β-actin value.

Dendritic cell generation from human peripheral blood precursors

In brief, PBMC were isolated from HLA-A0201+ donors by ficoll-hypaque density gradient centrifugation. The cells were allowed to adhere in culture flasks for 2 h at 37 °C in RPMI1640 with 10% FBS. Then, non-adherent cells were collected and frozen in freezing media (60% RPMI-1640, 30% FBS and 10% DMSO) for later use in CTL assays. Adherent cells were cultured in 6 mL of RPMI-1640 with 10% FBS containing 800 U/mL recombinant human granulocyte-macrophage colony- stimulating factor (rhGM-CSF, R&D Systems, McKinley Place NE, MN, USA) and 1200 U/mL recombinant human interleukin-4 (rhIL-4, R&D Systems). On days 3, 5 and 7, half of the media was refreshed without discarding any cells and fresh cytokine-containing (rhGM-CSF and rhIL-4) media was added. On day 8 of culture, 1000 U/mL of tumor necrosis factor-a (TNF-a, R&D Systems)

was added to the media. On day 9, non-adherent cells obtained from these cultures were considered mature human PBMC-derived DC.

Induction of peptide-specific CTL with synthetic peptides

Mature human PBMC-derived DCs were pulsed with 100 lg/ml HLA-A0201- restricted CTL epitopes of endocan for 4 h. The DCs were then irradiated with 20 Gy to prevent outgrowth. Subsequently, the non-adherent autologous peripheral blood lymphocytes were co-cultured with the DC. After stimulation, 800 U/mL recombinant interleukin-2 (IL-2) was added. On day 7, and weekly thereafter, the autologous peripheral blood lymphocytes were restimulated with peptide-pulsed pulsed irradiated dendritic cells.

ELISPOT assay

ELISPOT assay were utilized to analyze the inferferon-gamma (IFN-γ) production of CTLs. Briefly, effectors were plated in triplicate at a final concentration of 1×10^5 cells/well in 96-well nitrocellulose plates. Effector cells were stimulated with candidate peptides at a final concentration of 30 μM. The plate was incubated at 37 °C, 5% CO_2 for 24 h. The plate was processed using a biotin labeled anti-mouse IFN-γ antibody, an enzyme labeling marker and an antimarker. Then, a freshly prepared developer was added and incubated in the dark at 37 °C for 8 min (Quick Spot Mouse IFN-γ Precoated ELISPOT kit, Dakewe). Spots were quantified using the ELISPOT reader (BioReader 4000 Pro-X, BIOSYS, Germany).

Cytotoxicity assay

To evaluate levels of CTL activity, a standard 4-h ^{51} Cr-release assay was used. In the autologous lymphocytes lysis assay, the autologous lymphocytes activated by PHA were used as target cells. Briefly, target cells were incubated with ^{51} Cr (100 μCi per 1×10^6 cells; Amersham Biosciences Corp) for 2 h in a 37 °C water bath. After incubation with ^{51}Cr, target cells were washed three times with PBS, resuspended in RPMI 1640, and mixed with effector cells at effector-to-target (E/T) ratios of 25:1, 50:1, or 100:1. Assays were performed in triplicate wells for each experiment at each ratio in a 96-well round-bottomed plate. After a 4-h incubation, the supernatants were harvested, and the amount of ^{51}Cr released was measured with a γ-counter. The percentage of specific lysate was calculated as 100 × (experimental release - spontaneous release) / (maximum release - spontaneous release). Maximum release was determined from supernatants of cells that were lysed by the addition of 2% Triton X-100.

Analysis of in vivo immunogenicity

12 weeks old HLA-A*0201/Kb mice were immunized three times once a week, by subcutaneous injection in the back with 1×10^6 syngeneic mature DC pulsed with the peptides. After 7 days, the spleens of the mice were removed and the splenocytes were harvested as effectors.

Statistical analysis

All the experiments were done in triplicate, and the results are given as Means ± S.E.M. of triplicate determinations. Statistical analyses were performed using Student's t-test. The difference was considered statistically significant when the P value was < 0.05.

Results

Prediction of HLA-A*0201 restricted CTL epitopes of endocan.

To predict the HLA-A*0201-restricted CTL epitopes of endocan, we utilized two programs (BIMAS and SYFPEITHI) to analyze the total amino acid sequence of the protein. Top four 9-amino-acid peptides with highest scores were used as candidates for further analysis (Table 1). These peptides were chemically synthesized, purified, and identified. The molecular weight and the purity (> 95%) of each peptide were determined by mass spectrometry assay and HPLC.

MHC peptide-binding assay

The high affinity peptides binding to HLA-A2.1 could enhance HLA-A2.1 expression of antigen processing-deficient T2 cells. Therefore, the binding affinity of the predicted peptides to HLA-A2.1 was determined by using antigen processing- deficient T2 cells. As shown in Table 2, the four peptides were bound to HLA-A2.1 molecules with various affinities. Of four peptides selected, endocan$_{\mathbf{4-12}}$ and endocan$_{\mathbf{9-17}}$ enhanced the HLA-A2.1 molecular expression and demonstrated high affinity to HLA-A2.1 molecule. However, endocan$_{\mathbf{6-14}}$ and endocan$_{\mathbf{3-11}}$ demonstrated low affinity to HLA-A2.1 molecule.

Endocan mRNA and protein analysis

To explore endocan levels of target cells, we analyzed endocan mRNA and protein in several cell lines by RT-PCR and Western blot assays. As shown in Fig. 1, endocan mRNA and protein could be detected in U251 cell

Table 1 Predicted endocan epitopes binding to HLA-A2.1

Position	Length	Sequence	BIMAS score	SYFPEITHI score
4–12	9	VLLLTTLLV	437.482	26
9–17	9	TLLVPAHLV	257.342	24
6–14	9	LLTTLLVPA	19.425	22
3–11	9	SVLLLTTLL	6.916	21

Table 2 HLA-A2-binding affinity of peptides

Name	Position	Length	Sequence	FI
endocan$_{4-12}$	4–12	9	VLLLTTLLV	1.81
endocan$_{9-17}$	9–17	9	TLLVPAHLV	1.76
endocan$_{6-14}$	6–14	9	LLTTLLVPA	0.49
endocan$_{3-11}$	3–11	9	SVLLLTTLL	0.43
MAGE-2	112–120	9	KMVELVHFL	1.76
Hpa $_{519-526}$	519–526	8	FSYGFFVI	0.34

lines. However, endocan mRNA and protein could not be detected in BV2 cell line and autologous lymphocytes.

IFN-γ production analysis

To detect whether predicted peptides could elicit CTL to produce IFN-γ, we analyzed IFN-γ-producing cells by ELISPOT assay. As shown in Fig. 2, endocan$_{4-12}$ and endocan$_{9-17}$ could elicit IFN-γ-producing cells. However, IFN-γ-producing cells could not be detected in endocan$_{6-14}$ and endocan$_{3-11}$ groups. These results suggested that.

endocan$_{4-12}$ and endocan$_{9-17}$ could effectively promote CTL response.

Cytotoxicity analysis

To detect whether predicted peptides could elicit CTL to lyze target cells, we utilized the peptides to elicit PBMCs from HLA-A2.1+ donors. As shown in Fig. 3, endocan$_{4-12}$ and endocan$_{9-17}$ could elicit specific CTL to lyse target cells expressing endocan and HLA-A2.1.

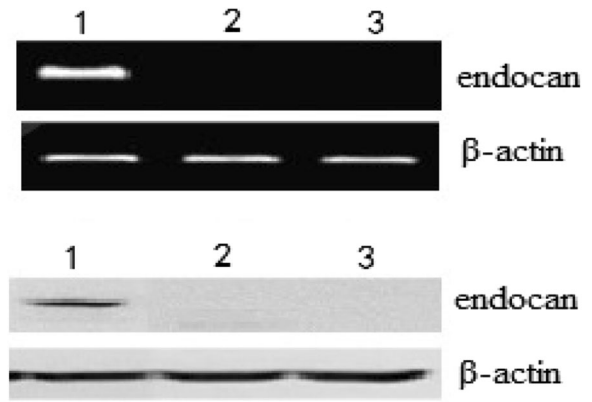

Fig. 1 Endocan expression in different target cells. The tumor cells were homogenized, and total RNA was isolated using Tripure Isolation Regent Kit. Two microliters RT product was amplified with PCR by using TaqDNA polymerase (using standard procedures). RT-PCR products were then run on a gel and visualized with ethidium bromide. For Western blot analysis, proteins in the cell extracts were separated by SDS-PAGE and were then analyzed with anti- endocan MAb. 1: U251 cells; 2: BV2 cells; 3: autologous lymphocytes

However, the lysate could not be detected in endocan$_{6-14}$ and endocan$_{3-11}$ groups. These results suggested that endocan$_{4-12}$ and endocan$_{9-17}$ could effectively elicit CTL mediated cytotoxicity.

Antibody inhibition assay

To further identify whether peptide induced effectors recognized target cells in an HLA-A2-restricted manner and whether the effectors were CD8+ T lymphocytes, HLA-A2 mAbs and CD8 mAbs were utilized to block recognition of target cells and effectors. In addition, HLA-B0702 mAbs and CD4 mAbs were served as negative controls. As Fig. 4 demonstrated, cytotoxicity of endocan$_{4-12}$ and endocan$_{9-17}$ specific CTLs was significantly attenuated after blocking by HLA-A2 mAbs and CD8 mAbs. However, cytotoxicity of endocan-specific CTLs could not been attenuated by negative controls. These results indicated that peptide induced effectors were HLA-A2.1- restricted and were mainly from CD8+ T lymphocytes.

Induction of epitope-specific CTLs in vivo

Furthermore, we investigated whether peptide could induce immune response in vivo. HLA-A*0201/Kb mice were immunized by subcutaneous injection with syngeneic mature DC pulsed with the peptides. After 7 days, the splenocytes were harvested as effectors to analyze the cytotoxicity. As Fig. 5 demonstrated, endocan$_{4-12}$ and endocan$_{9-17}$ could induce CTL response to lyse endocan and HLA-A2.1 positive cells with high efficiency. These results suggested that the peptides also had immunogenicity in vivo.

Discussion

Glioma is a fatal disease with median survival of 1.5 years [21]. Despite decades of therapeutial strategies in developing surgical and radiation techniques and chemotherapy agents, the survival of patients with glioma remains limited [22–24].

The challenge in treating glioma is the rapid proliferation of the tumor cells, the diffuse infiltrative nature of the disease, and resistant clones during treatment [25–27].

Recently, the immune system has been proved to recognize and eliminate the spontaneous development of glioma cells [28–30]. Because of its potential to manipulate and attack tumor cells, immunotherapy represents promising strategy in the clinic [31–33]. The related strategy to regulate immune system includes checkpoint inhibitors, gene vector, oncolytic virus and vaccine therapy [34–36]. Of the treatment strategies, tumor-associated antigens (TAAs) of inducing a specific host immune response to tumors are particularly appealing

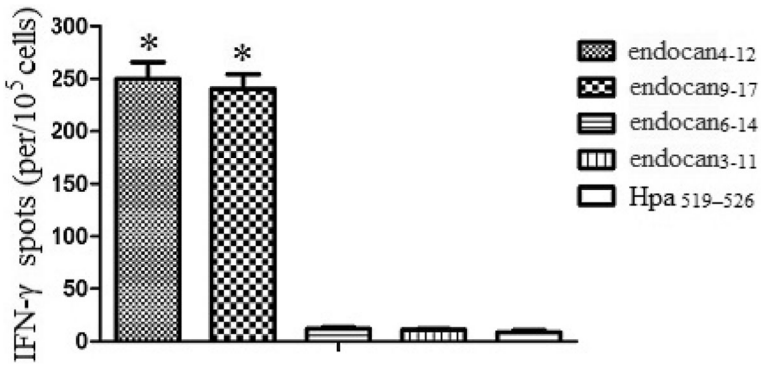

Fig. 2 ELISPOT assayThe PBMCs of human HLA-A2+ donors were obtained and then cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. DCs were generated, and loaded with different peptides at a final concentration of 100 μg/ml for 4 h and were then irradiated with 20 Gy, which prevented all outgrowths in the control cultures. Autologous T cells were restimulated every 7 days with the peptide-pulsed DCs to generate peptide-specific CTLs. The IFN-γ secretion was then assessed on day 23. Experiments performed in triplicate showed consistent results. Compared with controls, $P < 0.05$.

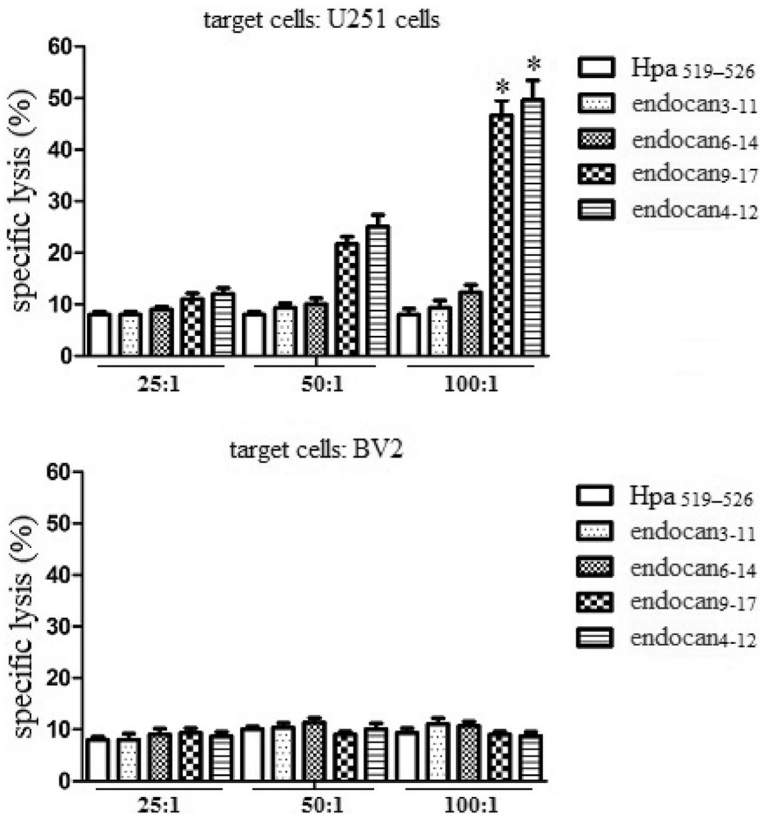

Fig. 3 Specific lysis of endocan-derived peptide elicited CTLs. The PBMCs of human HLA-A2+ donors were obtained and then cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. DCs were generated, and loaded with different peptides at a final concentration of 100 μg/ml for 4 h and were then irradiated with 20 Gy, which prevented all outgrowths in the control cultures. Autologous T cells were restimulated every 7 days with the peptide-pulsed DCs to generate peptide-specific CTLs. Target cells were incubated with ^{51} Cr (100 μCi per 1×10^6 cells; Amersham Biosciences Corp) for 2 h in a 37 °C water bath. After incubation with ^{51}Cr, target cells were washed three times with PBS, resuspended in RPMI 1640, and mixed with effector cells at effector-to-target (E/T) ratios of 25:1, 50:1, or 100:1. The percentage of cytotoxicity was calculated as follows: percentage of lysis = (sample cpm- spontaneous cpm)/(maximum cpm- spontaneous cpm) × 100%. Experiments performed in triplicate showed consistent results. Compared with control, $^*P < 0.05$

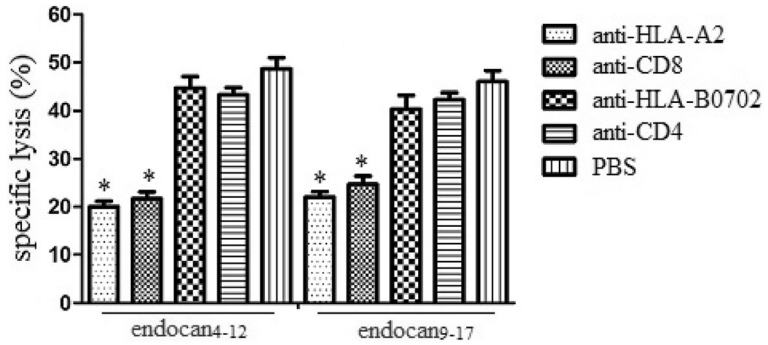

Fig. 4 Antibody inhibition assay by anti-HLA-A2 or anti-CD8 antibody. Peptide-coated T2 target cells were incubated with or without anti-HLA-A2 antibody from BB7.2 cells for 1 h at 4 °C. Moreover, effectors induced by different endocan -derived peptides were also incubated with or without anti-CD8 antibody for 1 h at 4 °C. The cytotoxic activities of CTLs against T2 cells were analyzed at various E/T ratios by 51 Cr release assay. Experiments performed in triplicate showed consistent results. Compared with control, *P < 0.05

because of high specificity for tumor cells and low toxicity to normal tissue [37–39].

DCs are regarded as the professional antigen-presenting cell that specializes in the initiating of antigen-specific CD8+ T cells [40]. DCs carry exogenous tumor antigens into endosomes and/or phagolysosomes and translocate these into the cytosol [41]. Furthermore, tumor antigens are delivered into the class I MHC processing pathway for presentation to tumor-specific CD8+ T cells [42]. Therefore, DCs loaded with TAAs represent ideal strategy for tumor antigen vaccination.

Endocan is a secreted proteoglycan that is upregulated by growth factors and chemokines in vitro and on tumor vasculature in various cancers [43, 44]. Endocan can be upregulated by angiogenic factors and inflammatory cytokines, such as tumor necrosis factor-α and interleukin-

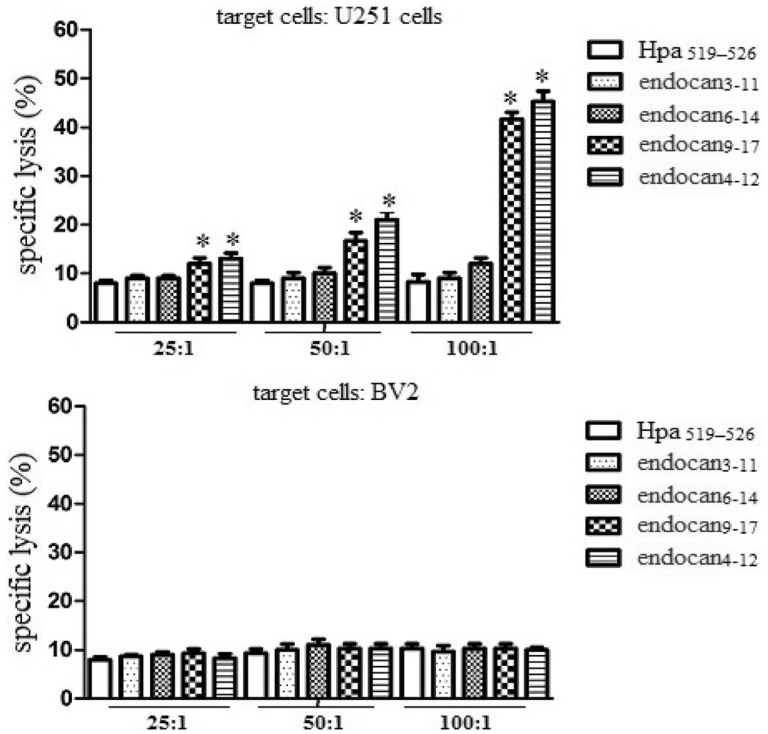

Fig. 5 In vivo induction of epitope-specific CTLs in vivo. 12 weeks old HLA-A*0201/Kb mice were immunized three times once a week, by subcutaneous injection in the back with 1×10^6 syngeneic mature DC pulsed with the peptides. After 7 days, the spleens of the mice were removed and the splenocytes were harvested as effectors. The cytotoxic activities of CTLs were determined against target cells at various E/T ratios using 51 Cr release assay. Experiments performed in triplicate showed consistent results. Compared with controls, *P < 0.05

1β [45]. In tumor tissue sections, a stromal inflammatory reaction was observed only in tumors overexpressing endocan polypeptide, and depletion of CD122+ cells was able to delete partially the anti-tumor effect of endocan polypeptide. These results reveal a novel pathway for endocan in the control of tumor growth, which involves inflammatory cells of the innate immunity [44]. In addition, an increase in tissue expression or serum level of endocan reflects endothelial activation and neovascularization which are prominent pathophysiological changes associated with inflammation and tumor progression. Consequently, enedocan has been used as a blood-based and tissue-based biomarker for various cancers and inflammation and has shown promising results [46].

In previous studies, endocan was reported to induce tumor formation and progression [11]. Recently, endocan has been identified to be overexpressed at the messenger RNA and/or protein levels in several tumor types, including glioblastoma, nonsmall cell lung cancer, gastric cancer, and hepatocellular carcinoma [13, 47, 48]. Most evidence suggested that endocan overexpression contributed to aggressive tumor progression and poor outcomes [9, 49, 50].

In current experiment, four candidate epitopes with highest scores from endocan were predicted according to HLA-A2.1-restricted epitope prediction algorithms. Secondly, the affinity of each epitope with HLA-A2.1 was analyzed by peptide binding assay. The data demonstrated that endocan$_{4-12}$ and endocan$_{9-17}$ showed high affinity to HLA-A2.1 molecule. Thirdly, the activity of epitope specific CTLs was measured by ELISPOT and ^{51}Cr release assay. The results demonstrated that endocan$_{4-12}$ and endocan$_{9-17}$ could elicit IFN-γ-producing cells and lyse target cells in an HLA-A2.1-restricted manner. Lastly, HLA-A*0201/Kb mice were immunized with predicted peptides, and the specific CTLs elicited in vivo were analyzed. The results demonstrated that endocan$_{4-12}$ and endocan$_{9-17}$ could also generate CTLs to lyse endocan and HLA-A2.1 positive target cells. These results suggested that the peptides also had immunogenicity in vivo.

Conclusions

Taken together, our results suggest that endocan$_{4-12}$ and endocan$_{9-17}$ could elicit specific HLA-A2.1-restricted CTLs and exert antitumor potential. In addition, the novel epitopes can be acted as a promising tumor vaccine, and may represent a new strategy for tumor immunotherapy in the clinic.

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None.

Authors' contributions
Conceptualization: GHS; Methodology: HS; Manuscript Preparation: ZY. The author(s) read and approved the final manuscript.

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Availability of data and materials
Please contact author for data requests.

Ethics approval and consent to participate
All animals received care in compliance with the Principles of Laboratory Animal Care and National standards.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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