Original Articles

Enhanced anti-colon cancer immune responses with modified eEF2-derived peptides

Weihong Sun [a,*], Xiaofang Wei [a], Airong Niu [b], Xuezhen Ma [c], Jian Jian Li [d,**], Daiqing Gao [a]

[a] Biotherapy Center, The Second Affiliated Hospital, Qingdao University Medical College, Qingdao 266042, China
[b] Department of Clinical Laboratory, The Second Affiliated Hospital, Qingdao University Medical College, Qingdao 266042, China
[c] Department of Oncology, The Second Affiliated Hospital, Qingdao University Medical College, Qingdao 266042, China
[d] Department of Radiation Oncology, NCI-designated Compressive Cancer Center, University of California Davis, Sacramento, CA 95817, USA

ABSTRACT

Eukaryotic elongation factor-2 (eEF2) is overexpressed in many human cancers and is an attractive target for cancer immunotherapy. The eEF2 derived polypeptides have been shown to be able to induce cytotoxic T lymphocytes from healthy donor. Here, we demonstrate the evidence indicating that modification of a segment of peptides from wild type eEF2-derived immunogenic peptides is able to further enhance its capacity of inducing antigen-specific cytotoxic T lymphocytes (CTLs) against colon cancer cells. Using peptide-MHC binding algorithms, potential HLA-A2.1-restricted epitopes capable of inducing specific CD8+ CTLs were identified. By analyzing HLA-A2.1 affinity and immunogenicity, we further identified one novel immunogenic peptide, P739–747 (RLMEPIYLV), that elicited specific CTL responses in HLA-A2.1/Kb transgenic mice and culture with peripheral blood lymphocytes from colon cancer patients. Furthermore, replacing certain amino acids (at positions 1, 3, 7) within the P739–747 sequence improved the immunogenicity against eEF2. Several analogs containing the auxiliary HLA-A*0201 anchor residues were able to stably bind to HLA-A*0201 and enhance CTL responses compared with the native sequence; two of them showed increased anti-tumor effects during the adoptive immunotherapy in vivo. Thus, these results support that modified immunogenic analogs are promising candidates for peptide-based cancer vaccination and immunotherapy.

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Introduction

Cytotoxic T cell (CTL)-based specific immunotherapy is considered one of the most promising strategies for tumor therapy. The CD8+ CTLs are capable of lysing tumor cells by recognizing peptides derived from tumor-associated antigens (TAAs) presented on MHC class I molecules [1,2]. Indeed, encouraging clinical responses have been observed in some cancer patients receiving peptide-based tumor immunotherapy [3,4]. As most TAAs are self-antigens, antigen-specific CTL repertoires may be significantly reduced in the host during the negative selection process, leaving

behind a T-cell repertoire that is poorly effective at mounting productive antitumor responses [5]. As a potential approach to enhance the immunogenicity of epitopes recognized by CTLs, modifications to the peptide sequences are applied to improve binding to MHC class I molecules [6–9]. However, replacing residues that are directed toward the TCR (T cell receptor) can also improve epitope immunogenicity [10–12]. Some of these peptides with enhanced immunogenicity have been successfully used to immunize cancer patients, improve detection of antitumor immune responses, and reverse non-responsiveness to tumor antigens [10].

Eukaryotic elongation factor-2 (eEF2) is previously identified as a novel tumor-associated antigen [13,14]. Clinical specimens of cancer tissues show that eEF2 protein is highly expressed in human breast, prostate, lung, gastric and colorectal carcinoma tissues, but not in normal tissues, as examined by immunohistochemical analysis [13,14], indicating eEF2 being an effective TAA target for immunotherapy. Oji et al. [14] have shown that eEF2-derived 9-mer peptides, EF786 (eEF2 786–794 aa) and EF292 (eEF2 292–300 aa), elicited cytotoxic T lymphocyte (CTL) responses in peripheral blood mononuclear cells (PBMCs) from an HLA-A*24:02- and an HLA-A*02:01-positive healthy donor, respectively, in an HLA-A-restricted manner. Following this line of study, we investigated whether additional eEF2-derived peptides could elicit CTL response, and whether

the CTL response by such eEF2-derived peptides could be further enhanced by peptide modifications. Here, we identified a nonamer peptide derived from the eEF2 protein, designated as P739–747 (RLMEPIYLV), which possessed the ability to provoke a peptide-specific, HLA-A2.1-restricted CTL response in HLA-A2.1/K^b transgenic (Tg) mice *in vivo* as well as in peripheral blood lymphocytes (PBLs) from HLA-matched colon cancer patients using dendritic cells (DCs) pre-pulsed with the peptides *in vitro*. In addition, amino acid substitution at HLA-A*0201-binding anchor positions in the native peptide enhanced the HLA-A*0201-binding affinity and immunogenicity of these modified peptides. These results provide the first evidence indicating the potential clinical application of this eEF2-derived analog in peptide-mediated immunotherapy for eEF2-expressing tumors.

Materials and methods

Synthetic peptides

All peptides utilized in this study were synthesized by GL Biochem (Shanghai, China) using fluorenylmethoxycarbonyl chemistry and purified to more than 95% by reversed phase high-performance liquid chromatography (HPLC), as confirmed by mass spectrometry. The lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO), diluted with phosphate-buffered saline (PBS; pH 7.4) at a concentration of 10 mM, and stored in aliquots at –80 °C. The amino-acid sequences and the predicted score for binding to HLA-A*0201 were generated by four databases available online (*ProPred-I*, http://www.imtech.res.in/raghava/propred1/; *SYFPEITHI*, http://www.syfpeithi.de/0-Home.htm).

Animals and cell lines

HLA-A2.1/K^b Tg mice, 5–6 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were bred and maintained under specific pathogen-free conditions. Mice were fed with an autoclaved laboratory rodent diet. The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Animal Subject of Qingdao University. Cell surface HLA-A*0201 expression was assessed by flow cytometry using fluorescein isothiocyanate (FITC)-labeled HLA-A2-specific monoclonal antibody (mAb) BB7.2 (Serotec, Oxford, UK). The human TAP-deficient T2 cell line (human B and T-cell hybrid expressing HLA-A2.1 molecules), the human breast adenocarcinoma MCF-7 (eEF2$^-$/HLA-A2.1$^+$), and the human colorectal adenocarcinoma SW480 (eEF2$^+$/HLA-A2.1$^-$) and Caco-2 cell (eEF2$^+$/HLA-A2.1$^+$) lines were obtained from American Type Culture Collection (ATCC, Manassas, VA).

Peptide-binding assay

A peptide-induced stabilization assay of the HLA-A*0201 molecule expressed by the TAP-deficient T × B-cell hybrid T2 cells [15] was performed according to a previously described protocol [16,17]. The HLA-A2.1-restricted carcinoembryonic antigen peptide-1 (CAP-1, YLSGANLNL) served as a positive control peptide and the ovalbumin (OVA)-derived H-2b-restricted peptide OVA$_{257-264}$ (SIINFKEL) was used as a negative control peptide. The fluorescence index (FI) was calculated as follows: FI = (mean FITC fluorescence with the given peptide – mean FITC fluorescence without peptide)/(mean FITC fluorescence without peptide). Peptides with an FI > 1 were regarded as high-affinity epitopes.

Measurement of the peptide-HLA-A*0201 complex stability

T2 cells were incubated overnight at 37 °C with 100 µM of each peptide in serum-free RPMI 1640 medium supplemented with 100 ng/mL β2-microglobulin as described [18]. The cells were then washed four times to remove free peptides, incubated with 10 µg/mL brefeldin A (Sigma-Aldrich, USA) for 1 hour to block cell-surface expression of newly synthesized HLA-A*0201 molecules, washed, and further incubated at 37 °C for 0, 2, 4, and 6 hours. Subsequently, the cells were stained with mAb BB7.2 to evaluate HLA-A*0201 molecule expression. Mean fluorescence intensity (MFI) measured at 0 hour was considered at 100%. MFI at all other time points are expressed relative to the MFI at 0 hour and calculated as follows: [MFI (0 hour) – MFI (2, 4, or 6 hour)/MFI (0 hour)] × 100%.

eEF2 RNA-interference assay

Transient knockdown of eEF2 using an shRNA assay with a chemically synthesized shRNA duplex and empty shRNA vector control was performed as previously described [13].

Generation of CTL in HLA-A2.1/K^b Tg mice

Bone marrow-derived DCs were generated from transgenic mice as previously described [19]. Vaccination of HLA-A2.1/K^b Tg mice was performed as previously described [20] with minor modifications. Briefly, on day 7, DCs were pulsed with 20 µM peptides in the presence of 3 µg/mL β2-microglobulin at 37 °C for 4 h and then washed. HLA-A2.1/Kb transgenic mice (5 mice per group for each peptide) were immunized intraperitoneally thrice at 1-week interval with 1 × 10^6 DCs per mouse. A control group was set simultaneously to receive PBS-treated DCs. According to a previously described protocol [21–23], on day 7 after the last immunization with peptide-pulsed DCs, total immune splenocytes from the pooled samples from the same group were cultured at a density of 1 × 10^7 cells per well in 6-well plates and stimulated with peptides (20 µM) for 7 days *in vitro*. Then, induced cells were harvested and functionally tested for IFN-γ and granzyme B by enzyme-linked immunospot (ELISPOT), ELISA, and intracellular staining assays as well as for cytotoxicity by ^{51}Cr-release cytotoxicity assays.

CTL induction in PBLs of colon cancer patients *in vitro*

We obtained 1.5 × 10^7 peripheral blood lymphocytes (PBLs) from 20 mL of heparinized peripheral blood collected from each HLA-A2.1$^+$ patient with colon carcinoma (who gave written informed consent and the protocol was reviewed and approved by the Ethic Committee for Application of Human Samples, Qingdao University Medical College) and human peripheral blood monocyte-derived DCs were generated as previously described [24–26]. Fifteen HLA-A2.1$^+$ patients with colon carcinoma were recruited in this study. Peptide-specific CTLs were generated as previously described [20] with minor modifications. After 7 days of co-culturing with peptide-pulsed autologous DCs, lymphocytes were re-stimulated with peptide-pulsed autologous DCs in medium containing 10 ng/mL IL-7, which was then supplemented with 20 IU/mL rhIL-2 (Sigma-Aldrich) 72 hours later. Lymphocytes were restimulated each week in the same manner. Half of the media volume was changed every 3 days in the presence of recombinant human rhIL-2 (20 IU/mL) and expanded as necessary. On day 7 after the third stimulation, 7 × 10^8 CD8$^+$ T lymphocytes were enriched by positive selection using magnetic immunobeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the procedure recommended by the manufacturer. IFN-γ secretion of these CD8$^+$ T cells was then examined by ELISPOT and ELISA, and the cells were evaluated for cytotoxic ability using both the granzyme B ELISPOT and ^{51}Cr-release assays.

ELISPOT assay

ELISPOT assays were performed using a commercially available kit (R&D Systems, Minneapolis, MN, USA) as described [20]. Splenocytes (1 × 10^5) from the immunized HLA-A2.1/K^b mice described above and purified CD8$^+$ T cells (more than 95% pure) were used as effector cells. Peptide-pulsed or unpulsed T2 cells or tumor cells (1 × 10^4) were used as stimulator cells. The data in the figures represent the mean of triplicate assays. Standard deviation (SD) was generally within 10% of the mean.

Intracellular detection of IFN-γ

Intracellular IFN-γ secretion was detected as described [27]. Briefly, effector T cells from immunized mice or colon cancer patients were re-stimulated in the presence of corresponding peptides or tumor cell lines (Caco-2, MCF-7, and SW480) for 48 hours. Brefeldin A (20 µg/mL) (Sigma-Aldrich) was added during the last 8 hours of culture to block protein secretion. Cells were harvested, washed, and stained with a FITC-conjugated anti-CD8 monoclonal antibody (BD Pharmingen, San Diego, CA). PE-conjugated anti-IFN-γ monoclonal antibody (BD Pharmingen, San Diego, CA) was added after cells were fixed and permeabilized using saponin (Sigma-Aldrich). After washing with PBS, stained cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (FASCcan or FACSVantage SE; BD Biosciences). Data analysis was performed using CellQuest software (BD Biosciences).

IFN-γ ELISA

ELISA was performed using a commercially available kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Peptide-pulsed or unpulsed T2 cells or tumor cells were used as stimulator cells. Splenocytes from immunized HLA-A2.1/K^b mice or purified CD8$^+$ T cells were used as effector cells. The amount of cytokine presented in the effector-cell culture supernatant was calculated based on the IFN-γ standard curve. The data in the figures represent the mean of triplicate assays. SD was generally within 10% of the mean.

Cytotoxicity assay

Cytotoxicity assays were performed using a standard 4-hour ^{51}Cr-release assay as previously described [20]. Percent specific lysis was determined according to the following formula: percent specific lysis = [(mean experimental cpm – mean spontaneous cpm)/(mean maximum cpm – mean spontaneous cpm)] × 100%. SD of triplicate wells was less than 15%.

Splenocytes from each group of immunized HLA-A2.1/K^b mice were stimulated with 20 μM P739–747 for 7 days as described in the Cytotoxicity assay section. Caco-2 tumor cells (5×10^6) were injected s.c. into the flanks of C57BL/6$^{nu/nu}$ mice, which formed homogeneous tumors in 100% of the mice. Three days later, mice were i.v. injected with splenocytes (1×10^8 cells/mouse) derived from peptide-immunized HLA-A2.1/K^b Tg mice. This adoptive transfer was performed twice at 1-week intervals followed by i.p. injection of 2000 IU of IL-2 every 2 days. Control mice received splenocytes from HLA-A2.1/K^b mice immunized with unpulsed DCs or received only IL-2.

Statistical analysis

Tumor size among the respective groups was compared with the Mann–Whitney *U*-test. Kaplan–Meier curves were plotted to evaluate survival. All other statistical analyses were based on the Student's *t*-test.

Results

Selection of potential eEF2-derived peptides binding to HLA-A*0201 molecules

Many types of human tumors overexpress the eEF2 protein compared to the related normal tissues, making it a potential TAA for immunotherapy. To begin more fully assessing this potential in terms of eliciting an immunogenic response in the host, we attempted to screen the eEF2 amino acid sequence for the presence of HLA-A*0201-binding motifs using computer-assisted analysis. We selected and synthesized 6 candidate nonameric peptides with the highest estimated half-life of dissociation from HLA-A*0201. Next, we analyzed the HLA-A*0201-binding affinity of these peptides using the HLA-A*0201 up-regulation assay on TAP-deficient T2 cells (Table S1). Only peptide No. 1 (referred to as P292–300), No. 2 (P739–747), and No. 5 (P661–669) were high-affinity epitopes (FI = 1.21, 1.18, and 1.03, respectively); all others were low-affinity epitopes (FI < 1; Table S1). The positive control CAP-1 peptide was strongly bound to HLA-A*0201 (FI = 1.15), whereas the negative control peptide exhibited no binding (FI = 0.04).

The eEF2-derived P739–747 peptide is immunogenic in HLA-A2.1/K^b Tg mice

Although eEF2-derived peptide is indicated to be able to induce CTL cytotoxicity [14], it is unknown whether the eEF2-derived peptides could induce immune response *in vivo*. To determine the *in vivo* immunogenic potential of the candidate peptides, we immunized HLA-A2.1/K^b Tg mice with syngeneic DCs pulsed with these high-affinity peptides. After 3 rounds of immunization *in vivo*, splenocytes were isolated, restimulated *in vitro* for an additional 7 days with the corresponding peptides, and then assayed for IFN-γ production and CTL cytotoxicity. Although bulk CTLs from Tg mice injected with the P292–300 and P661–669 peptides did not produce any significant IFN-γ to any stimuli (data not shown), bulk CTLs from Tg mice injected with the P739–747 peptide exhibited strong IFN-γ production (Fig. 1A and B); only low IFN-γ levels were secreted to any stimuli in the control groups.

In the cytotoxicity assays, T2 cells pulsed with the relevant eEF2-derived peptides were used to measure peptide-specific lysis by bulk CTLs, and T2 cells pulsed with irrelevant peptide CAP-1 measured nonspecific lysis. As shown in Fig. 1C, only the P739–747 peptide induced a specific bulk CTL response compared to the irrelevant- or no-peptide control. Similar to the IFN-γ production assay, cytotoxicity by P292–300 and P661–669-specific CTLs to their corresponding target cells were either less effective than the P739–747 peptide or near background levels (Fig. S1). Thus, the P292–300 and P661–669 peptides were excluded from further study. Taken together, the above results indicated that IFN-γ production

and cytotoxicity of the induced CTLs were specific for the P739–747 peptide within the eEF2 protein.

P739–747 is a naturally processed peptide from eEF2

To address whether the immunogenic peptide P739–747 was a naturally processed and presented peptide from eEF2 by tumor cells, which would support that eEF2 has the potential to serve as a tumor-rejection antigen, we tested the capability of peptide-specific CTLs to recognize HLA-A2.1$^+$ tumor cells endogenously expressing the eEF2 protein. Western blot analysis showed that Caco-2 and SW480, but not MCF-7, were tumor cell lines that expressed eEF2 (data not shown). Determining HLA-A2.1 expression on these cell lines by flow cytometry revealed that Caco-2 and MCF-7 cells were HLA-A2.1$^+$, while SW480 were HLA-A2.1$^-$. As shown in Fig. 1D, only the CTLs directed against the P739–747 peptide were able to specifically lyse the eEF2$^+$ and HLA-A2.1$^+$ Caco-2 cells, but not SW480 (eEF2$^+$/HLA-A2.1$^-$) or MCF-7 (eEF2$^-$/HLA-A2.1$^+$) cells. These data indicate that the P739–747 peptide is not only an immunogenic and naturally processed epitope from eEF2 but also an HLA-A2.1-restricted CTL epitope in HLA-A2.1/K^b Tg mice.

HLA-A2.1-restricted, P739–747-specific CD8$^+$ CTL responses can be induced in vitro *from PBLs harvested from colon cancer patients*

To investigate the ability of the P739–747 peptide to induce tumor-specific CD8$^+$ T-cell responses in T cells from HLA-A2.1$^+$ colon cancer patients *in vitro*, specific CTLs were generated by sensitizing PBLs from eEF2$^+$/HLA-A2.1$^+$ colon cancer patients with autologous DCs pre-pulsed with P739–747 *in vitro*. In more than 60% (7/11) of the eEF2$^+$/HLA-A2.1$^+$ colon cancer patients tested, P739–747-specific CD8$^+$ T cells were generated, eliciting significant IFN-γ production upon activation by P739–747-pulsed T2 (T2+P739–747) or Caco-2 cells (eEF2$^+$/HLA-A2.1$^+$) (Fig. 2A and B). Significantly lower amounts of IFN-γ near background levels were detected upon stimulation with irrelevant peptide CAP-1-pulsed T2 cells (T2+CAP-1) or with MCF-7 (eEF2$^-$/HLA-A2.1$^+$) or SW480 (eEF2$^+$/HLA-A2.1$^-$) tumor cells.

The cytotoxic ability of these activated bulk CTLs was then tested against T2 cells loaded with either the P739–747 (T2+P739–747) or irrelevant CAP-1 peptide (T2+CAP-1). As shown in Fig. 2C, DCs loaded with P739–747 could induce peptide-specific cytotoxicity from bulk CTLs. In addition, to investigate whether the P739–747 peptide was naturally processed and presented, peptide-specific CTLs were tested for their ability to lyse target cells endogenously expressing eEF2. As observed in HLA-A2.1/K^b Tg mice, bulk CTLs against the P739–747 peptide could specifically lyse Caco-2 (eEF2$^+$/HLA-A2.1$^+$), but not SW480 (eEF2$^+$/ HLA-A2.1$^-$) or MCF-7 (eEF2$^-$/HLA-A2.1$^+$) cells (Fig. 2D).

To further address whether the immunogenic P739–747 peptide is naturally processed and presented by tumor cells, bulk CTLs against P739–747 were tested for their ability to recognize eEF2$^+$/ HLA-A2.1$^+$ Caco-2 after genetic modification to silence express eEF2. For silencing eEF2 expression, Caco-2 cells were transiently transfected with an eEF2-specific shRNA duplex, as previously described [13]. As shown in Fig. 3, CTLs were incapable of killing eEF2-silenced Caco-2 cells. Thus, these results further confirmed that P739–747 was naturally processed and presented by tumor cells.

Designed analog peptides exhibit high affinity to HLA-A2.1

Enhancing immunogenicity of a CTL epitope may be achieved by replacing epitope residues that contact the MHC class I molecule and/or those that contact the TCR. Previous studies indicated that residues at positions 1, 2, 3, 6, 7, and 9 may be replaced [28–30], where residues at positions 2 and 9 are primary anchor motifs, and
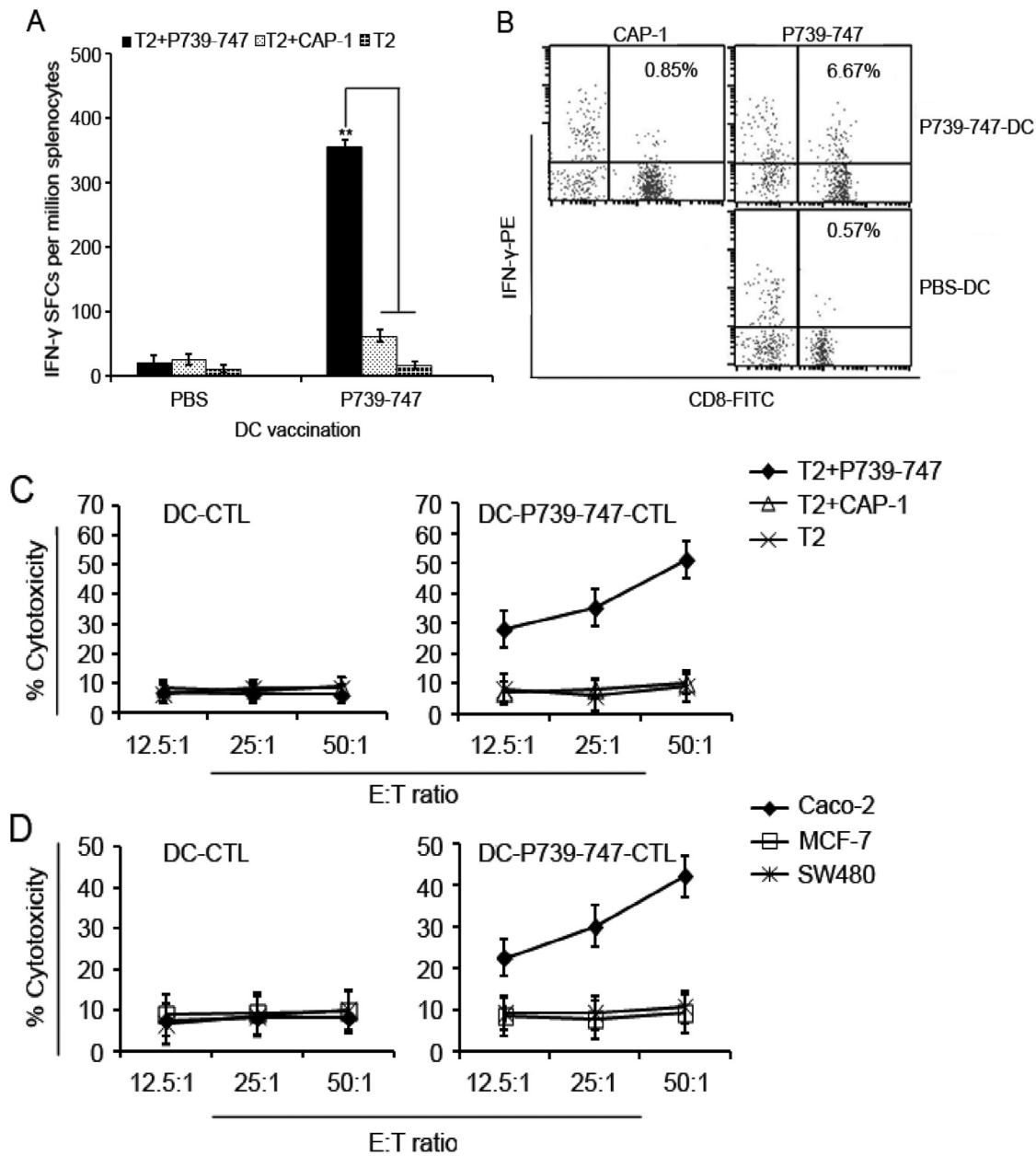
Fig. 1. Immunization with peptide-loaded DCs results in peptide-specific CD8+ T-cell responses in HLA-A2.1/Kb Tg mice. Splenocytes (DC-CTL or DC-P737–747-CTL) from HLA-A2.1/Kb Tg mice (5 mice per group) immunized with PBS or P739–747-pulsed DCs were stimulated *in vitro* with P739–747 for 7 days. (A) IFN-γ-positive SFCs/10^6 splenocytes detected by ELISPOT. Mean ± SD; n = 3; **P < 0.01 in comparison of IFN-γ production in P739–747-immunized CTLs stimulated with P739–747-pulsed T2 (T2+P739–747) and CAP-1-pulsed T2 (T2+CAP-1) or T2 alone cells (T2). (B) CD8+ T cells detected by FACS with IFN-γ staining (representative data from one of three independent experiments). (C and D) Cytotoxicity measured by 51Cr-release assay at the indicated E:T (Effector:Target cells) ratio. (C) P739–747-pulsed T2 and (D) Caco-2 (eEF2+/HLA-A2.1+) cells were used as peptide-specific targets, while (C) CAP-1-pulsed T2 or T2 alone cells and (D) MCF-7 (eEF2−/HLA-A2.1+) or SW480 (eEF2+/HLA-A2.1−) cells were used as control targets. n = 3; p < 0.05. All results.

those at positions 1, 3, 6, and 7 are secondary anchor motifs. The native P739–747 peptide eEF2 already contains the preferred residues (leucine [L], isoleucine [I], valine [V]) at positions 2, 6, and 9. We therefore performed *in silico* analyses to further improve the ability of the peptide to bind to the HLA-A*0201 molecule and found that modifying the following secondary anchor motifs may increase the binding and affinity of the native P739–747 peptide to the HLA-A*0201 molecule: at position 1 with L, V, I, tyrosine (Y) or phenylalanine (F); at position 3 with Y, F, L, I, or tryptophan (W); and at position 7 with W, F, I proline (P), or histidine (H). After these alterations, the computer algorithm predicted that binding affinity would increase from 2 ~ to 77 times that of the native peptide

sequence for HLA-A*0201, and we chose to select and synthesize the 9 candidate nonameric peptides with the higher estimated binding affinity to HLA-A*0201 than the native peptide (Table S2).

Analog peptides stably bind to HLA-A2.1

The immunogenicity of MHC class I-restricted peptides requires the capability to bind and stabilize MHC class I molecules on the live cell surface. We therefore sought to directly measure the interaction potential between the peptides and HLA-A2.1 using a conventional binding and stabilization assay with T2 cells. Of the 9 analog peptides, 8 (except 3W) showed a stronger affinity for
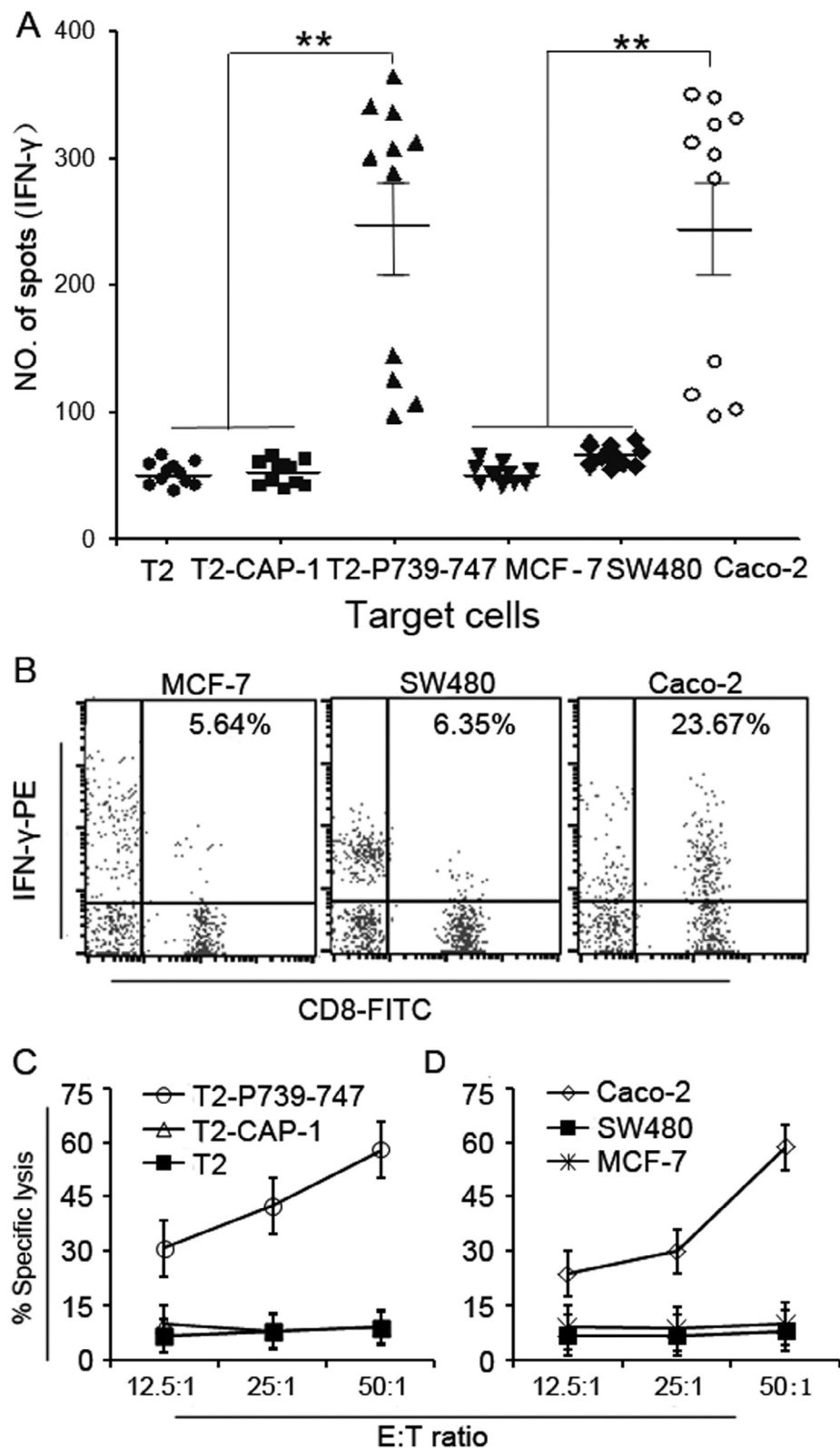
Fig. 2. CTL induction from PBLs of HLA-A*0201-positive colon carcinoma patients. CTLs were induced from PBLs of eEF2+/HLA-A2.1+ colon carcinoma patients through 3 sequential rounds of stimulation with autologous P739–747-pulsed DCs. On day 7 after the third stimulation, CD8+ T lymphocytes were enriched by positive selection using magnetic immunobeads for test. (A) IFN-γ-positive SFCs/10^6 CTLs detected by ELISPOT. Mean ± SEM; n = 3; **P < 0.01 for the number of IFN-γ-positive SFCs/10^6 CD8+ T cells between P739–747-pulsed and unpulsed or CAP-1-pulsed T2 stimulator cells; and between Caco-2 and MCF-7 or SW480 cells. Each data point represents one individual sample. (B) CD8+ T cells detected by FACS with IFN-γ staining (presentative data from one of three independent experiments). (C and D) Cytotoxicity measured by ^{51}Cr-release assay at the indicated E:T (Effector:Target cells) ratio. (C) P739–747-pulsed T2(T2+P739–747) and (D) Caco-2 (eEF2+/HLA-A2.1+) cells were used as peptide-specific targets, while (C) CAP-1-pulsed T2 (T2+CAP-1) or T2 alone cells (T2) and (D) MCF-7 (eEF2−/HLA-A2.1+) or SW480 (eEF2+/HLA-A2.1−) cells were used as control targets. n = 3; p < 0.05.
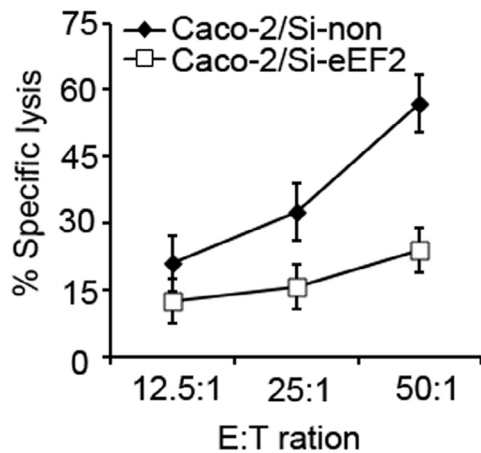
Fig. 3. Knockdown of eEF2 decreases in cytotoxicity of P739–747-specific CTL to cancer cells. eEF2-specific shRNA duplex (shEF2) was transfected into Caco-2 cells (Caco-2/Si-eEF2) or shMock vector was transfected into Caco-2 cells (Caco-2/Si-non). CD8^{+} CTLs from colon carcinoma patients generated as described in Fig. 2 were tested for P739–747-specific lysis using ^{51}Cr-release assay. CTL-mediated lysis to eEF2-silenced Caco-2 cells. n = 3; p < 0.05.

HLA-A2.1 compared with the affinity of native P739–747 peptide (Table S3). Recent studies [25,31,32] suggest that a stable peptide-MHC (pMHC) complex can facilitate synapse formation between T cells and APCs, and that the stability of the pMHC complex is a key factor for CTL activation. We investigated the stability of the pMHC complex when in contact with the native or analog peptides over a 6-hour observation period. Data in Table S4 showed that the stability of most analog peptides (except 1Y, 1F, and 3W) was enhanced compared with the native peptide when in complex with the HLA-A2.1 molecule.

Analog peptides can induce peptide-specific CD8^{+} CTLs in HLA-A2.1/K[b] Tg mice in vivo

Since 6 of the synthesized analog peptides exhibited a higher affinity and a relative stability for the HLA-A2.1 molecule, we were wondering if they would be more functionally effective than the native P739–747 peptide in inducing immunity *in vivo*. We first induced peptide-specific CD8^{+} CTLs in HLA-A2.1/K[b] Tg mice and then assayed for IFN-γ production and CTL cytotoxicity *ex vivo*. Once *ex vivo* restimulated with the native peptide, only splenocytes harvested from the 1Y3W7W-, 1Y7W-, and 7W-immunized Tg mice exhibited an elevated IFN-γ production compared with the splenocytes from the native-peptide–immunized mice (Fig. 4A and B).

To address whether IFN-γ-producing CTL lines could lyse T2 target cells pulsed with native peptide or eEF2-expressing tumor cells, granzyme B ELISPOT and ^{51}Cr-releasecytotoxicity assays were performed. As shown in Fig. 4C and D, 1Y3W7W-, 1Y7W-, and 7W-induced CTLs showed increased capacity to release granzyme B and to lyse P739–747-pulsed T2 cells compared to the native-peptide–induced CTLs, but only 1Y3W7W-and 1Y7W-induced CTLs exhibited stronger cytotoxicity to Caco-2. Cytotoxicity by the other analog-specific CTLs were either not significantly different from the native peptide or exhibited background cytotoxicity. Therefore, these analogs were excluded from further studies. Importantly, these data showed that splenocytes were able to recognize the native peptide sequence even in the presence of the synthetic analogs. Overall, these results indicated that the 1Y3W7W and 1Y7W could induce eEF2-specific CTLs more effectively than the native peptide.

Analog peptides more effectively induce peptide-specific CD8^{+} CTLs from colon cancer patients in vitro

We show above that analog peptides can induce more effective CTLs from Tg mice. To determine whether the analog peptides could also induce more effective CTLs from human colon cancer patients than the native peptide, we stimulated PBLs from 15 HLA-A2.1^{+} colon cancer patients with either analog or native-peptide-loaded autologous DCs. Seven days after the third *in vitro* stimulation, CD8^{+} T cells from each individual were tested for IFN-γ production and CTL cytotoxicity. For the IFN-γ-production assays, CTLs raised by the 1Y3W7W, 1Y7W, and 7W, and P739–747 peptides were challenged with P739–747-pulsed T2 cells or Caco-2 cells as stimulators. We observed that while 30% (5/15 patients) of CTLs raised by 1Y3W7W and 60% (9/15 patients) of CTLs raised by 1Y7W were able to generate better responses than P739–747, 7W were unable to elicit CTL responses to various stimulators (Fig. 5A and B).

We further tested CTL cytotoxicity using granzyme B ELISPOT and ^{51}Cr-release cytotoxicity assays. CTLs generated *in vitro* in the presence of 1Y3W7W or 1Y7W were able to specifically kill significantly more P739–747-pulsed T2 cells, and Caco-2 cells (both eEF2[+]/HLA-A2.1[+]) than those generated with P739–747, but not T2 cells without peptide, T2 cells pulsed with irrelevant peptide, MCF-7 (eEF2[−]/HLA-A2.1[+]), or SW480 (eEF2[+]/HLA-A2.1^{−}) cells. Therefore, the two synthetic peptides 1Y3W7W and 1Y7W were able to generate cytolytic responses from CTLs, but CTLs induced by 7W peptides did not generate any significant immune response against the various targets (Fig. 5C and D). More importantly, T cells stimulated with 1Y3W7W or 1Y7W were able to recognize the native sequence, confirming the necessary heteroclitic response.

To further confirm and characterize the increased immunogenicity of 1Y3W7W and 1Y7W analogs above that of the native peptide, specific CTLs were tested against T2 cells pre-pulsed with various concentrations (100, 10, or 1 μM) of the P739-747 peptide at a 25:1 effector:target (E:T) ratio and against Caco-2 cells at various E:T ratios (12.5:1, 25:1, and 50:1). Remarkably, when recognizing various concentrations, especially 10 or 1 μM P739–747-loaded T2 cells, 1Y3W7W- or 1Y7W-induced CTLs showed stronger cytotoxicity than P739–747-induced CTLs. Interestingly, CTLs induced by 1Y3W7W and 1Y7W analogs showed a similar capacity to lyse both the 1 and 10 μM P739–747-loaded T2 cells (Fig. 6A). As for the E:T ratio, the ^{51}Cr-release assay demonstrated that tumor cell lysis for 1Y3W7W- or 1Y7W-induced CTLs obviously increased over that of P739–747-induced CTLs (Fig. 6B).

We next determined the capacity of the 1Y3W7W and 1Y7W analogs to induce peptide-specific CTLs by stimulating PBLs from HLA-A2.1-positive colon cancer patients with autologous DCs loaded with various concentrations (100, 10, or 1 μM) of 1Y3W7W, 1Y7W, or control P739–747 peptides. Seven days after the third stimulation, the CD8^{+} T cells from each individual were tested in a ^{51}Cr-release assay against P739–747-pulsed T2 cells at a 25:1 E:T ratio. While no specific CTL response was detected when CTL cultures were stimulated with a low concentration of the P739–747 peptide (1 μM), both 1Y3W7W and 1Y7W were able to more efficiently elicit CTL responses to P739–747 peptide when cultures were stimulated with this low 1 μM peptide concentration (Fig. 6C). Together, these results suggest that 1Y3W7W- or 1Y7W-induced CTLs were able to recognize native-peptide–loaded T2 cells and eEF2-expressing tumor cells, and produce higher IFN-γ levels and better cytotoxicity than P739–747 or other analogs.

*Adoptive immunotherapy of C57BL/6[nu/nu] mice bearing human colon carcinoma*

We next tested the *in vivo* antitumor reactivity of 1Y3W7W- and 1Y7W-induced lymphocytes in a mouse model of human
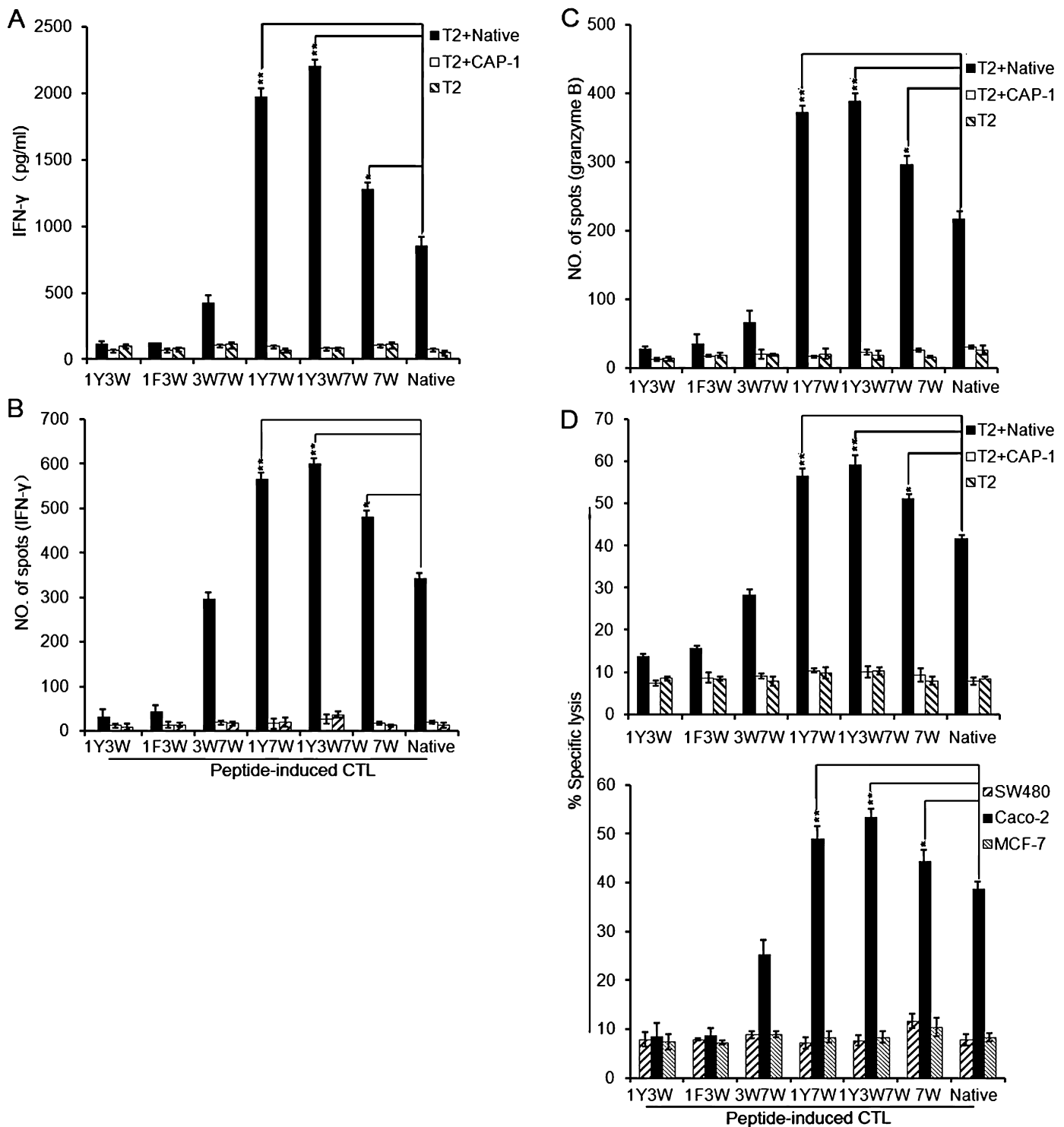
Fig. 4. The 1Y3W7W, 1Y7W, and 7W analogs induce more efficient eEF2-specific CTLs than the native P739–747 epitope in HLA-A2.1/K[b] Tg mice. Splenocytes from HLA-A2.1/K[b] Tg mice (5 mice per group) immunized with native P739–747- or analog-pulsed DCs were stimulated *in vitro* with P739–747 for 7 days. (A) IFN-γ level measured by ELISA; n = 3; **P < 0.01, *P < 0.05. (B) Number of IFN-γ-positive splenocytes by ELISPOT; n = 3; **P < 0.01, *P < 0.05. Cytotoxicity to P739–747-pulsed T2 examined by (C) granzyme B ELISPOT and (D) ^{51}Cr-release assay. In (C), number of granzyme B (GrB)-positive SFCs from 10[5] splenocytes is calculated; n = 3; **P < 0.01, *P < 0.05. In (D), native-peptide–pulsed T2 (T2+Native) and Caco-2 (eEF2[+]/HLA-A2.1^{+}) cells were used as the eEF2-specific HLA-A2.1-restricted target cells. CAP-1 peptide-pulsed T2 (T2+CAP-1), unpulsed T2 (T2), SW480 (eEF2[+]/HLA-A2.1[−]), and MCF-7 (eEF2[−]/HLA-A2.1^{+}) cells were used as control targets. The cytotoxicity of various CTLs against the corresponding target cells was tested at a 10:1 E:T ratio in the granzyme B ELISPOT assay and at a 50:1 E:T ratio in the ^{51}Cr-release assay (n = 3; **P < 0.01, *P < 0.05).

colon cancer to determine whether the analog peptides were capable of vaccinating against tumor growth *in vivo*. To do so, we established a model whereby HLA-A2.1/Kb Tg mouse-derived lymphocytes were adoptively transferred into C57BL/6 nu/nu mice bearing the

Caco-2 colon carcinoma. We observed that 1Y3W7W- and 1Y7W-induced lymphocytes significantly inhibited progressive tumor growth *in vivo* compared with P739–747-inuced lymphocytes, whereas the control groups failed to inhibit tumor development
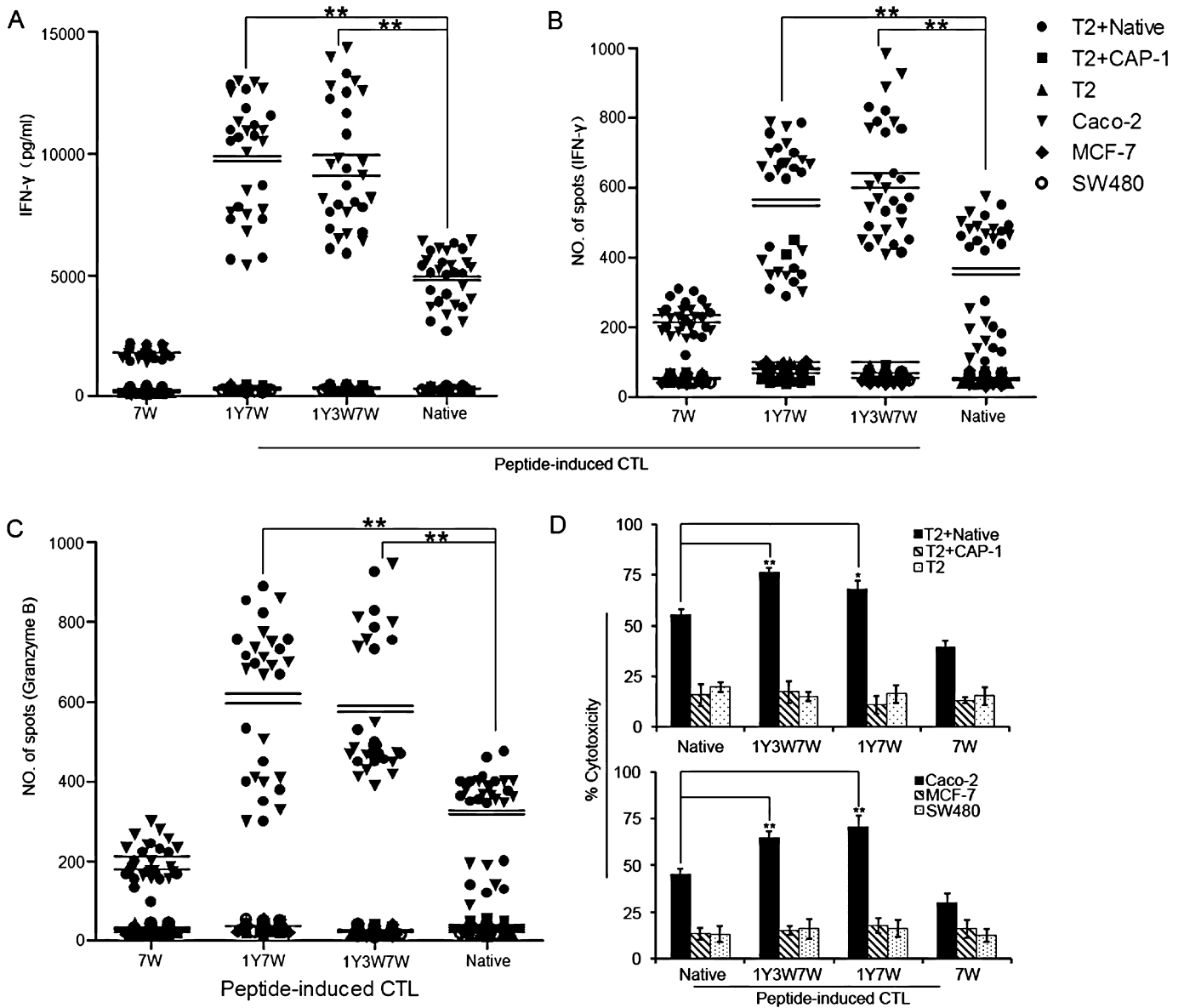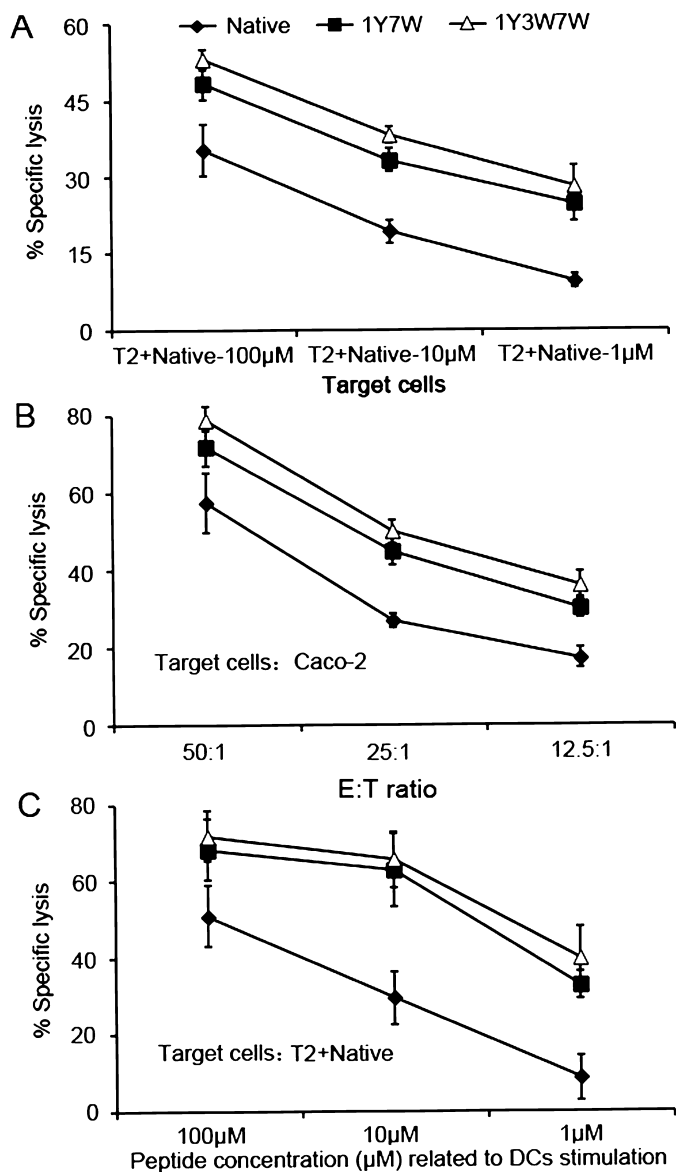
Fig. 5. Enhanced cytotoxicity by modified eEF2 1Y3W7W, 1Y7W, 7W tested with PBLs from HLA-A*0201-positive colon cancer patients. PBLs from HLA-A*0201-positive colon cancer patients were stimulated with 1Y3W7W, 1Y7W, 7W, or native P739–747 peptides three times at weekly intervals as described in the Materials and methods section. On day 7 after the third stimulation, CD8$^+$ T lymphocytes were enriched by positive selection using magnetic immunobeads for test. (A–D) The CTLs in PBLs were then tested for (A and B) IFN-γ release, (C) granzyme B release, and (D) standard ^{51}Cr-release assay against native-peptide–pulsed T2 or eEF2-expressing HLA-A*0201-positive tumor cells. (A) ELISA results are indicated by IFN-γ pg/mL. (B) ELISPOT results are indicated by the number of IFN-γ-positive SFCs/10^6 CD8$^+$ T lymphocytes. (C) Number of granzyme B (GrB)-positive SFCs from 10^5 CD8$^+$ T lymphocytes. Values are expressed as means; n = 3; **P < 0.01 for the number of IFN-γ- or Granzyme B-positive CD8$^+$ T cells in 1Y7W-CTL or 1Y3W7W-CTL and Native-CTL when T2+Native and Caco-2 cells were used as stimulator cells. Each data point represents one individual sample. (D) Native-peptide–pulsed T2 (T2+Native) and Caco-2 (eEF2$^+$/HLA-A2.1$^+$) cells were used as the eEF2-specific, HLA-A2.1-restricted target cells. CAP-1 peptide-pulsed T2 (T2+CAP-1), unpulsed T2 (T2), SW480 (eEF2$^+$/HLA-A2.1$^-$), and MCF-7 (eEF2$^-$/HLA-A2.1$^+$) cells were used as control targets. The cytotoxicity of various CTLs against the corresponding target cells was tested at a 10:1 E:T ratio in the granzyme B ELISPOT assay and at a 50:1 E:T ratio in the ^{51}Cr-release assay (n = 3; **P < 0.01, *P < 0.05).

(Fig. 7A). As shown in Fig. 7B, 36% (4/11) and 45% (5/11) of the 1Y3W7W- and 1Y7W-vaccinated mice, respectively, survived for more than 80 days after tumor inoculation. However, only 18% (2/11) of the mice treated with the P739–747 vaccine survived for more than 80 days. All mice in the control groups succumbed to death between day 25 and day 38 after tumor inoculation. Thus, these results indicated that vaccination with either the 1Y3W7W or 1Y7W analog peptide induced effective *in vivo* anti-tumor responses that were superior to those induced by the native P739–747 peptide.

Discussion

In this study, we show that immunogenic peptide, P739–747 (RLMEPIYLV), can elicit specific CTL responses in both HLA-A2.1/K^b transgenic mice and cell culture with peripheral blood lymphocytes from colon cancer patients. Furthermore, peptide modification (at positions 1, 3, 7) within the P739–747 sequence further enhanced the immunogenicity against eEF2. Analogs containing the auxiliary HLA-A*0201 anchor residues can bind to HLA-A*0201 and enhance CTL responses compared with the native sequence; two
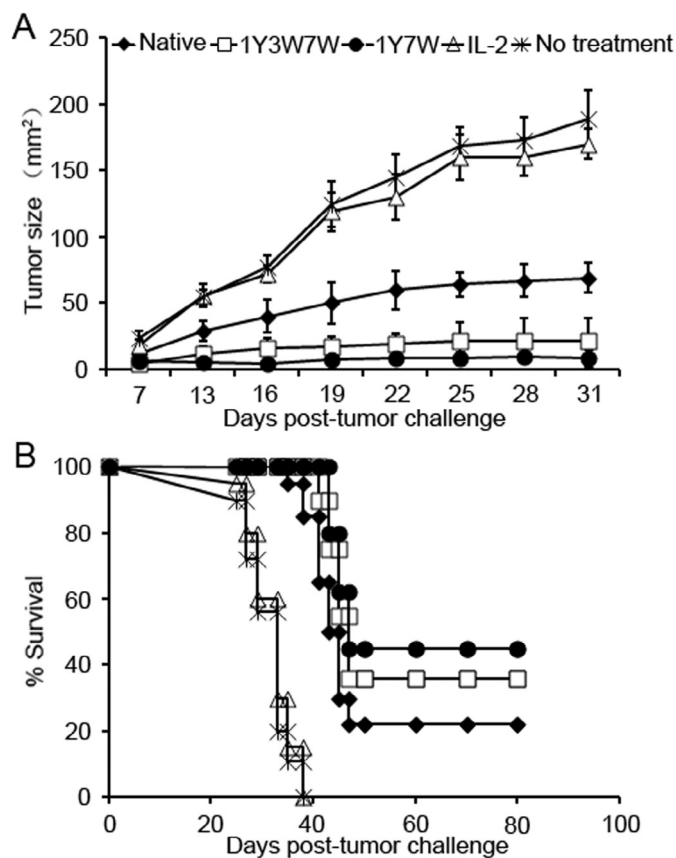
Fig. 6. The 1Y3W7W and 1Y7W analogs induce higher effective CTLs that recognize the native peptide and eEF2-expressing HLA-A*0201-positive tumor cells. CD8+ T cells from different HLA-A*0201-positive colon cancer patients were stimulated *in vitro* after induction by the 1Y3W7W, 1Y7W, or native P739–747 peptides, as described in the Materials and methods section, and tested for cytotoxicity by the standard ^{51}Cr-release assay. (A) Native, 1Y3W7W-, and 1Y7W-induced CTLs were tested against T2 cells pre-pulsed with various concentrations of native peptide (100, 10, or 1 μM) at a 25:1 E:T ratio. (B) CTLs were also tested against Caco-2 cells at various E:T ratios (50:1, 25:1, and 12.5:1). (C) PBLs from HLA-A*0201-positive colon cancer patients were stimulated with autologous DCs loaded with various concentrations (100, 10, or 1 μM) of 1Y3W7W, 1Y7W, or native peptides. Seven days after the third stimulation, purified CD8+ T cells from each individual were tested against T2 cells pulsed with 20 μM native peptide at a 25:1 E:T ratio. n = 3; p < 0.05.

Fig. 7. Adoptive immunotherapy of Caco-2 tumor-bearing nude mice. Caco-2 tumor cells (5×10^6 cells/mouse) were injected into the flanks of C57BL/6 nu/nu mice. Three days later, splenocytes (1×10^8 cells/mouse) from 1Y3W7W-, 1Y7W-, or P739–747 (native)-immunized HLA-A2.1/K^b Tg mice were injected as described in the Materials and methods dection. Control groups received IL-2 alone or did not receive treatment. (A) Tumor growth was observed at regular intervals every 3 days and recorded as mean tumor size (mm^2). (B) Survival of mice after tumor inoculation (n = 11 mice/group).

tested showed increased anti-tumor effects during adoptive immunotherapy *in vivo*. These results provide the first evidence supporting that modification of eEF2-derived peptides is a potential effective approach to enhance the response of colon cancer to immunotherapy.

Adoptive immunotherapy targeting tumor antigens has emerged as one of the most promising strategies for providing clinical benefit to cancer patients [33]. In phase I clinical trials of peptide-based immunotherapy, active anticancer vaccination with a single TAA epitope has been demonstrated to induce peptide-specific CTLs, resulting in cancer regression without damage to normal tissues [3,4].

Oji and Nakamura et al. [13,14] showed that eEF2 was overexpressed in several types of tumor cells and promoted growth of various type of cancer cells, and an HLA-A*0201-restricted, eEF2-derived 9-mer peptides (eEF2 292–300 aa) was shown to be able to elicit CTL responses in peripheral blood mononuclear cells from an HLA-A*02:01-positive healthy donor. However, due to poor immunogenicity of CTL epitopes from natural tumor antigens, modifications of peptides are approached to enhance the ability to bind to MHC class I molecules or improving recognition by the TCR in order to improve their immunogenicity [10,12]. The use of modified peptides in vaccine therapy has now been reported in three clinical trials: the melanoma Ag gp100$_{(209-217)}$ 2M analog, CAP1-6D analog, and MART1/Melan A$_{27-35}$ 1L super-analog [34]. These analogs appear to more potently activate naïve T cells, produce more clinical responses in melanoma patients, and further regress metastases than their respective native peptides. These findings imply that eEF2 may be appropriate candidate for use as a CTL-directed tumor antigen.

In this study, we identified an eEF2-derived epitope, P739–747, with high affinity for HLA-A*0201 based on the prediction of computer-based algorithms and verification by the peptide-MHC binding assay. We demonstrated that P739–747 is an immunogenic, naturally processed, and HLA-A2.1-restricted CTL epitope in HLA-A2.1/K^b Tg mice. In addition, *in vitro*-induced, peptide-specific CTLs in P739-747-stimulated PBLs from HLA-A*0201-matched patients with colon cancer were not only able to lyse target cells pulsed

with the antigenic peptide but also recognize tumor cells endogenously expressing the eEF2 protein in an antigen-specific and HLA-A2.1-restricted manner, indicating that P739–747-based vaccination could potentially generate eEF2-specific CTLs in patients with colon cancer.

Several reports demonstrate that peptides from viral- and tumor-derived proteins that bind with higher affinity to HLA class I molecules elicit strong CTL immune responses [1,22], and that amino acid modifications of natural CTL epitopes that increase the affinity of peptide-MHC binding can enhance their immunogenicity [8,9,12]. Here, we show that synthetic analogs of the native P739–747 peptide were produced by replacing amino acids at positions 1, 3, and 7. These replacements were performed even at anchor positions, which are known to contain critical residues. Most of the synthesized analog peptides exhibited an enhanced binding to the HLA-A2.1 allele as well as enhanced stability of the peptide–MHC complex. For some of these analog peptides with improved binding affinity, we were greatly interested in knowing whether they also showed improved function over the native P739–747 peptide in the CTL assays. In HLA-A2.1/K^b Tg mice, the 1Y3W7W and 1Y7W analog peptides, but not 7W, induced higher CTL responses than the native peptide. These results are in accordance with several reports showing a relationship between MHC class I binding and *in vivo* and *in vitro* immunogenicity [35–39]. Tanaka et al. [40] reported that modified peptides not only increased the binding affinity to HLA-A2 but also improved recognition of native peptide. However, it needs to be further investigated whether or not it can also promote the expansion or proliferation of Ag-specific CTL.

The property of increasing a peptide's affinity for MHC by increasing the density of peptide–MHC complexes on APCs in order to enhance *in vivo* immunogenicity is important for inducing the CTL response. Here, we showed that the 1Y3W7W and 1Y7W sequences generated cytotoxic responses from PBLs isolated from HLA-A*0201$^+$ colon cancer patients and that these CTLs were cross-reactive against the native sequence. Our data further revealed two analog peptides being able to generate stronger immune responses than the native peptide against T2 cells pulsed with a low concentration of native peptide or against eEF2-expressing tumor cells at low E:T ratios. Thus, these CTLs may be more efficient than those raised against the native peptide in addition to being quantitatively greater in number. These results suggest that modified 1Y3W7W and 1Y7W analog peptides have the advantages over the native peptide in therapeutic efficacy for antitumor vaccine, and more immunogenic in inducing CTLs.

Characterizing the *in vivo* immunogenicity of peptides that are to be used as immunogens is an essential step before a large scale of clinical trial can be conducted since high MHC class I binding affinity and CTL recognition *in vitro* are not always sufficient to induce tumor regression. Using the Caco-2 tumor-bearing nude mouse model, we found that 1Y3W7W- or 1Y7W-induced CTLs more efficiently inhibited tumor growth and improved survival of tumor-bearing mice than the native peptides, indicating the potential of quick application of these immunologic peptides in clinic.

Antigens specifically recognized by CD8$^+$ T cells in the context of MHC class I molecules have been selected for the majority of clinical cancer vaccine trials performed so far [41,42]. Overexpressed self-antigens and viral antigens involved in oncogenesis may also represent useful targets for therapeutic and/or preventive immunological intervention. However, as with the majority of non-mutated and frequently overexpressed tumor antigens, the potential use of epitopes within this self-antigen as targets for antitumor immunotherapy raises concerns about self-tolerance and the development of autoimmunity to these epitopes. A previous study investigated the issue of self-tolerance to TAA-associated epitopes with potential utility for antitumor immunotherapy. In a preclinical model that was established based on transgenic mice expressing

a recombinant HLA-A*0201 molecule transplanted with HLA-A*0201-expressing B16 melanoma cells [38], this group demonstrated that CTL populations reactive against melanocyte differentiation protein (MDP)-derived self-antigen could be activated by mature DCs pulsed with MDP epitopes to overcome tolerance and mount effective antitumor immunity. In our present study, we also could induce eEF2-specific CTLs from the PBLs of patients with colon cancer, suggesting that the eEF2-specific CTL repertoire was not completely tolerated to the eEF2 protein.

With regard to the risk of developing autoimmunity after vaccinating with these epitope peptides, some previous preclinical and clinical trials of overexpressed self-antigen-derived peptide vaccinations showed that effective antitumor immunity was achieved without severe adverse events [22,43]. In addition, a phase I/II study demonstrated that peptides derived from overexpressed HER-2/neu or MUC-1 self-antigens induced effective and specific CTL responses without any side effects [44]. However, some reports revealed that active immunization against MDA in melanoma patients induced potent CD8$^+$ T cell responses that then interacted with skin melanocytes in the skin, resulting in vitiligo. Therefore, although eEF2 may be a candidate for peptide vaccination or targeted T-cell therapies to treat colon carcinoma or other eEF2-expressing carcinomas, additional basic and clinical studies are needed to further assess the feasibility and safety of using eEF2-derived peptides as vaccines for cancer treatment. The effectiveness may also be linked to the ways of vaccinating with peptide-pulsed DC. Currently, the major routes of vaccinating mice with peptide-pulsed DC are subcutaneously (s.c.), intradermally (i.d.) [45–47] or intraperitoneally (i.p.) [20,48–50]. It has been shown that antigen-pulsed DC elicited a higher response by i.d. or s.c. than i.p. administered mice [51]. Thus, it has been suggested that in clinical trials, in order to obtain enhanced vaccine efficiency, peptide-pulsed DCs are immunized mostly via s.c. or i.d. [52–56]. In further studies, a comparison on i.p. with i.d. or s.c. will be more informative for potential clinical trials.

Conclusion

This study characterized P739–747 as a novel HLA-A*0201-restricted, immunogenic CD8$^+$ T-cell epitope derived from eEF2 naturally processed and presented by colon cancer cells. We found that replacing secondary anchor residues in P739–747 yielded new sequences with enhanced *in vivo* and *in vitro* immunogenicity, as shown in the HLA-A2.1/K^b Tg mouse models and in human cells. These results collectively provide the first evidence indicating that the modified eEF2 peptides are potentially able to enhance CTLs *in vitro* for adoptive-transfer immunotherapy and to function as a key component in vaccines aimed at eliminating tumors.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.08.002.

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