学　位　論　文

Studies on induction of effective anti-tumor immunity and regulatory mechanisms by novel long peptide vaccination

(新規人工合成ロングペプチドによる

効率的な抗腫瘍免疫誘導と作用機序に関する研究**)**

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8. Kazutaka Masuko, Shun Kaneumi, Satoshi Terada, Toshiyuki Kita, Kentaro Sumida, Hidemitsu Kitamura, Takashi Nishimura.
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The 72th Annual Meeting of the Japanese Cancer Association, October 3, 2013; Yokohama, Japan.

INTRODUCTION

Since tumor-specific antigen was discovered, it has been reported that vaccine therapy using major histocompatibility complex (MHC) class I-biding peptide (short peptide) of tumor antigen can induce antigen-specific cytotoxic T lymphocyte (CTL). Although they prolonged the survival of cancer patients, it is hard to induce complete regression. The first successful peptide vaccination was achieved by Aichele et al.[1] who demonstrated that the injection of mice with short peptide encoded by lymphocytic choriomeningitis virus (LCMV) protected mice from subsequent challenge of a live LCMV. Although the finding was confirmed by many other investigators[2, 3], it was also demonstrated that short peptide appeared to be suboptimal because short peptide sometimes induced immunological tolerance rather than protective immunity if it was vaccinated with incomplete Freund adjuvant (IFA)[4]. Meanwhile, it turned out that the first successful experiment of CTL short peptide vaccine against LCMV reported by Aichele et al[1] was due to the fact that their LCMV short peptide (15 amino acid sequence) included a helper epitope recognized by $CD4^+$ T cells and it was longer than the general minimal CTL epitope (8-10 amino acid) though it was considered as a short peptide. Thus, the existence of helper epitope might allow the full activation of CTL dependently upon T helper (Th) cell activation. That is to say, Th peptide-bound professional antigen presenting cells (APC), particularly dendritic cells (DC) interact with Th cells via CD40/CD40L and subsequently induce fully activation of CTL and CTL memory [5, 6].

Some authors made improvements of peptide vaccine design to induce a potent immunogenicity *in vivo*. The most evolutional peptide vaccine is synthetic long peptide (SLP), which is the extended long peptide of MHC class I-binding peptide[7, 8]. Melief et al.[7, 9] demonstrated that SLP are primarily processed and presented by professional APC, especially DC and therefore induce a strong activation of CTL in inflamed draining lymph node (dLN). Moreover, they indicated that SLP was superior to CTL short peptide for inducing sustained antigen presentation in dLN, which has been considered to be critical for clonal expansion and interferon (IFN)-γ production by effector T cells[7]. Interestingly, they also indicated that human papillomavirus (HPV)16 SLP, which is a HPV16-derived 35 amino acid long peptide containing naturally occurring Th epitope peptide in addition to CTL epitope peptides, induced strong HPV-specific $CD4^+$ and $CD8^+$ T cell immunity and eradicated established HPV-positive tumor cells[10]. The other advantage of SLP vaccine is overcoming effect on tolerance induction by IFA and short CTL peptide[11]. Thus, the existence of Th epitope peptide and the length of SLP appeared to be key factors for

designing therapeutic peptide vaccine.

The conjugation of Th short peptide and CTL short peptide was reported as another peptide vaccine design. It has been reported that a single linear hybrid peptide conjugating both Th and Tc epitope peptide exhibited more efficient vaccine effect to induce CTL against hepatitis C virus in comparison to the vaccine including the mixture of Th and CTL short peptides[12]. However, it remains unclear whether such hybrid peptide vaccine works as a superior vaccine to protect mice from tumor and virus *in vivo*. Compared with SLP using natural occurring sequence of antigen, the hybrid peptide has a merit to easily constitute artificial long peptide vaccine using newly identified Th epitope, CTL epitope and linker peptide.

Immune system, consisted of various cells such as DC, macrophages (Mϕ), natural killer (NK) cells, NKT cells, $CD4^+$ T cells and $CD8^+$ T cells, plays critical roles in host defense mechanism. Particularly, reguratory T cells (Treg), myeloid derived suppressor cells (MDSC). IL-17-producing γδ T cells suppress immune response. These suppressive cells accumulate in the tumor immunosurveillance. To conquer tumor immunosurveillance including regulatory cells such as Treg or MDSC, it is critical to induce Th1-dominant immunity. Recently, we have proposed that the introduction of Th1-dominant immunity is critical for inducing fully activated CTL and CTL memory in tumor-bearing mice[13-15]. Therefore, we hypothesized that we could develop a superior peptide vaccine useful for immunotherapy of tumor and infectious diseases, if we synthesized a single linear long peptide containing both Th and CTL epitope peptides.

In the present work, to overcome current limitations of cancer vaccine therapy using short peptides, we have prepared a cancer vaccine, referred to as helper/killer-hybrid epitope long peptide (OVA-H/K-HELP), consisting of both CTL and Th epitopes of ovalbumin (OVA) model tumor antigen with glycine linker. Also, immune adjuvant such as unmethylated cytosine-phosphorothioate (CpG-ODN), which stimulated DC through toll-like receptor (TLR) 9 and promoted type 1 immunity is necessary to induce Type 1 immunity. Thus, we demonstrated therapeutic activity against tumor and the regulatory mechanism by H/K-HELP vaccination with CpG-ODN in a mouse tumor model with OVA-expressing EG7.

ABBREVIATIONS

APC: antigen presenting cells
CFSE: carboxyfluorescein succinimidyl ester
CPG-ODN: unmethylated cytosine-phosphorothioate
CTL: cytotoxic T lymphocytes
DC: dendritic cells
dLN: draining lymph node
ELISA: enzyme-linked immunosorbent assay
FACS: fluorescence activated cell sorting
f.p.: footpad
HCV: hepatitis C virus
H/K-HELP: Helper/Killer-Hybrid Epitope Long Peptide
HPV: Human papillomavirus
i.d.: intradermally
IFA: Incomplete Freund's adjuvant
IFN: interferon
LCMV: lymphocytic choriomeningitis virus
LN: lymph node
long peptides-Mix: mixture of long CTL and Th peptide epitopes
mAb: monocronal antibody
MDSC: myeloid derived suppressor cells
MHC: major histocompatibility complex
Mϕ: macrophages
NK: natural killer
OVA: ovalbumin
OVA-H/K-HELP: H/K-HELP based on the model antigen OVA
SD: Standard deviation
short peptides-Mix: mixture of short CTL and Th peptide epitopes
SLP: synthetic long peptide
Th: T helper
Treg: reguratory T cells
WT: wild type

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from Charles River Japan (Yokohama, Japan). OT-I and OT-II TCR transgenic mice were provided by F.R. Carbone (University of Melbourne, Victoria, Australia). C57BL/6-background Ly5.1 mice were purchased from RIKEN Bioresource Center. Ly5.1 OT-I and OT-II mice were bred in our facility. All mice used in the present studies were 5-8 weeks old and were maintained in specific pathogen-free conditions according to the guidelines for animal care at our institute. All mice were used in accordance with the guidance of an institutional committee at Hokkaido University.

Antibodies

APC-conjugated anti-CD11c monoclonal antibody (mAb) (HL3), anti-IFN-γ mAb (XMG1.2), anti-CD8 mAb (53-6.7), PE-conjugated anti-CD3 mAb (145-2C11), anti-CD19 mAb (1D3), FITC-conjugated anti-CD44 (IM7), PE-Cy7-conjugated anti-CD4 mAb (RM4-5), and anti-CD45.1 mAb (A20) were purchased from BD bioscience (SanDiego, USA). 7-Amino-actinomycin D (7AAD) was purchased from Beckman coulter (Miami, USA). H-2K^{b} OVA tetramer-SIINFEKL-PE (OVA tetramer) was purchased from MBL (Nagoya, Japan).

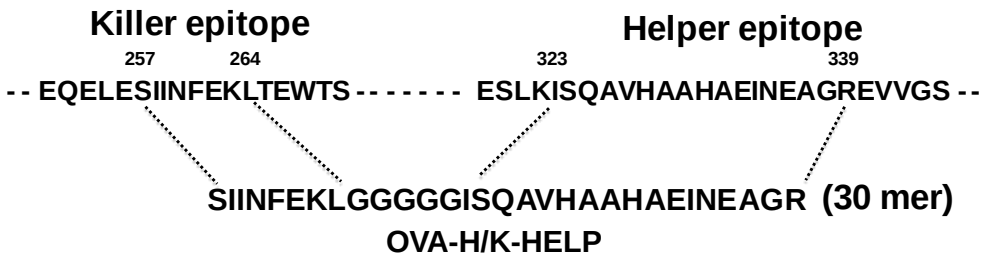
Reagents

Anti-CD4, anti-CD8 and anti-APC mAb-conjugated microbeads for the MACS system were purchased from Miltenyi Biotec (Bergisch, Gladbach, Germany). RPMI-1640 medium (Wako, Osaka, Japan). penicillin G (Meiji-Seika, Tokyo, Japan). streptomycin (Meiji-Seika, Tokyo, Japan). G418 (Sigma-Aldrich, Tokyo, Japan). FCS (Nichirei Bioscience, Tokyo, Japan). Mytomicin C. CpG-ODN 1668 (5'-TCCATGACGTTCCTGATGCT-3') (Hokkaido System Science, Sapporo, Japan). Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen). Brefeldin-A (Sigma-Aldrich, Tokyo, Japan). 4% paraformaldehyde phosphate buffer solution (Wako, Osaka, Japan). OptEIA^{TM} mouse IFN-γ ELISA kits (BD Bioscience)

Synthetic peptides

As illustrated in **Table. 1**, we have developed a novel tumor vaccine peptide: helper/killer-hybrid epitope long peptide (H/K-HELP), which artificially conjugates CTL ($OVA_{257-264}$) and Th ($OVA_{323-339}$) peptides to stimulate both CTL and Th1 cells *in vivo*. We have used the following minimal CTL peptide epitope: $OVA_{257-264}$ SIINFEKL. The minimal

Th peptide sequence of OVA was as follows: $OVA_{323-339}$ ISQAVHAAHAEINEAGR. In addition, the following long peptides deduced from the natural sequence of each protein were used: CTL peptide $OVA_{241-270}$ SMLVLLPDEVSGLEQLESIINFEKLTEWTS (note that this peptide does not contain the C-terminal Th epitope $OVA_{265-280}$); and Th peptide $OVA_{317-346}$ SSAESLKISQAVHAAHAEINEAGREVVGSAE. Moreover, we synthesized modified OVA-H/K-HELP by substitution of glycine-linker with other peptide-linker. The chemical structure of these synthetic peptides was illustrated in **Table 1**.



ClassI short peptide: NH_2 - SI INF EKL - COOH
ClassII short peptide: NH_2 - IS QAV HAA HAE INE AGR - COOH
ClassI long peptide: NH_2 - SM LVL LPD EVS GLEQL ESI INF EKL TEW TS - COOH
ClassII long peptide: NH_2 - SS AES LKI SQA VHA AHA EIN EAG REV VGS A - COOH
H/K HELP OVA(G): NH_2 - SI INF EKL GGG GGI SQA VHA AHA EIN EAGR - COOH
H/K HELP OVA(A): NH_2 - SI INF EKL AAA AAI SQA VHA AHA EIN EAG R - COOH
H/K HELP OVA(P): NH_2 - SI INF EKL PPP PPI SQA VHA AHA EIN EAG R - COOH

Table. 1 List of peptide vaccines used in this paper. The chemical structure of peptide vaccines used in the experiments was summarized. The details were described in Materials and Methods.

Peptide vaccination

C57BL/6 wild type (WT) mice were foot pad (f.p.) vaccinated with 10 nmol of (each) peptide admixed with 10 μg of CpG-ODN in a total volume of 30 μl PBS. Ten days after immunization, induction of antigen-specific immune responses were analyzed.

Tumor cell culture

EG-7 tumor cells expressing the full-length OVA antigen were cultured in RPMI-1640 medium containing 10% FCS plus penicillin G (200 U/ml) and 0.1 % streptomycin (complete medium) supplemented with 100 μg/ml G418 at 37°C, 5% CO_2. EG-7 cells were used for *in vitro* stimulation of lymphocytes from immunized animals as above.

EG7 co-culture for *in vitro* stimulation

Ten days after vaccination, lymph nodes were removed and single lymphocyte suspensions of 1 x 10^7 cells/ml were prepared. EG-7 cells were incubated with 60 μg/ml of mytomicin C in complete medium at 37 °C for 1 h, and washed four times with medium. Lymphocytes were incubated at a 10:1 ratio with EG-7 cells. Five days later, viable cells were stained with H-2K^b tetramer (TM)-OVA$_{257-264}$ complexes and anti-CD8 mAb.

Tumor challenge for preventive vaccine experiments

C57BL/6 mice were intradermally (i.d.) immunized with OVA-H/K-HELP (10 nmol/mouse) plus CpG-ODN (10 μg/mouse). Twenty-one after peptide vaccination, the immunized mice were i.d. inoculated with EG-7 cells (2 x 10^6) and the growth of the tumor was monitored.

Tumor challenge for therapeutic vaccine experiments

EG-7 cells (2 x 10^6) were i.d. inoculated into C57BL/6 mice. When the tumor mass became large (7-8 mm), the tumor-bearing mice were vaccinated with each peptides plus CpG-ODN. The antitumor activity was determined by measuring the tumor size in perpendicular diameters as described previously[15]. Tumor volume was calculated by the following formula: tumor volume = 0.4 x length (mm) x [width (mm)]^2. Tumor-bearing mice that survived for >60 days after therapy were considered completely cured.

CFSE labeling of T cells and adoptive T cell transfer

Single-cell suspensions were made from spleen and peripheral lymph nodes (LN) of Ly5.1 OT-I or OT-II mice. Erythrocytes were eliminated with 155 mM NH_4Cl, and 1 x 10^7 cells/ml were incubated with 1 μM CFSE at 37 °C for 10 min. The cells were washed three times with PBS, and 1 x 10^6 Tg cells were injected into the tail vein (in 200 μl of PBS) or used for *in vitro* experiments.

***Ex vivo* detection of antigen (Ag)**

Mice were vaccinated with either short peptide mix or H/K-HELP with CpG-ODN. 36 h later, the draining LN and the nondraining LN were isolated and incubated for 30 min with collagenase (Sigma) at 37 °C. Single-cell suspensions were made and the cells were incubated with mAb against CD11c (DC), CD19 (B cells) and CD3 (T cells) and sorted on a MACS system according to the manufacturer's protocol or sorted on a fluorescence activated cell sorting (FACS) Aria cell sorting system (Biosciences). These sorted cells were used as

stimulator cells in co-cultures with naïve CFSE-labeled OT-I $CD8^+$ or OT-II $CD4^+$ T cells for 3 days, after which proliferation of the OT-I or OT-II T cells was evaluated by flow cytometry on the basis of CFSE dilution. Co-cultures contained 1 x 10^5 OT-I or OT-II T cells and one of the following types and numbers of stimulator cells: $CD11c^+$ cells (5 x 10^4), $CD19^+$ (4 x 10^5), or $CD3^+$ (8 x 10^5). These relative numbers were based on their ratio as typically found in the lymph nodes examined.

Cytotoxicity assay

The cytotoxicity mediated by tumor-specific CTLs was measured by a 4 or 6 h ^{51}Cr-release assay, as described previously[16]. Briefly, ^{51}Cr-labeled target cells were co-cultured with various numbers of effector cells for 4 or 6 h in V-bottomed microtiter plates. Released ^{51}Cr in the culture supernatants were measured by a gamma counter (Packard Cobra II gamma counter, Meriden, CT, USA) and the percentage of specific lysis was calculated.

Intracellular cytokine staining

Lymphocytes were incubated for 1 h with 2 μg/ml minimal CTL and Th peptides before 5 μg/ml Brefeldin-A was added. The next day, the stimulated cells were first stained with anti-CD4 and anti-CD8 mAbs and then fixed with 4% paraformaldehyde phosphate buffer solution. After treatment with permeabilizing solution [50 mmol/l NaCl, 5 mmol/l ethylenediaminetetraacetic acid, 0.02 % NaN_3, and 0.5 % Triton X-100 (pH 7.5)], the cells were stained with anti-IFN-γ mAb.

Enzyme-linked immunosorbent assay (ELISA)

IFN-γ levels in the culture supernatants were measured by OptEIA™ mouse IFN-γ ELISA kits according to the manufacturer's instructions.

Statistical analysis

All experiment was independently repeated at least three times. Mean values and Standard deviation (SD) were calculated for *in vitro* data. Significant differences in the results were determined by the two-tailed Student's *t*-test. The $P < 0.05$ was considered as significant in the present experiment.

RESULTS

OVA-H/K-HELP was superior to short peptides-Mix to activate functional Tc1 and Th1 cells.

We synthesized OVA-H/K-HELP **(Table. 1)** and demonstrated induction of anti-tumor immunity using a mouse vaccination model. To measure CTL responses, we treated C57BL/6 mice f.p. with saline, CpG-ODN, mixture of short CTL and Th peptide epitopes (short peptides-Mix) plus CpG-ODN, or OVA-H/K-HELP plus CpG-ODN and determined the induction of OVA-tetramer-reactive CTL. Although detectable OVA-tetramer$^+$ CTL were induced *in vivo* following primary vaccination with OVA-H/K-HELP **(Fig. 1)**, higher numbers of CTL were expanded from unfractionated dLN cells following secondary re-stimulation with mitomycin C-treated OVA-expressing EG-7 tumor cells. OVA-H/K-HELP vaccination induced a higher percentage (76.9 %) and larger numbers of tetramer$^+$ OVA-reactive CTL, which exhibited strong cytotoxicity when compared with the CTL induced by short peptides-Mix vaccination **(Fig. 2A-C)**. Neither saline nor CpG-ODN alone induced OVA-tetramer$^+$ CTL.

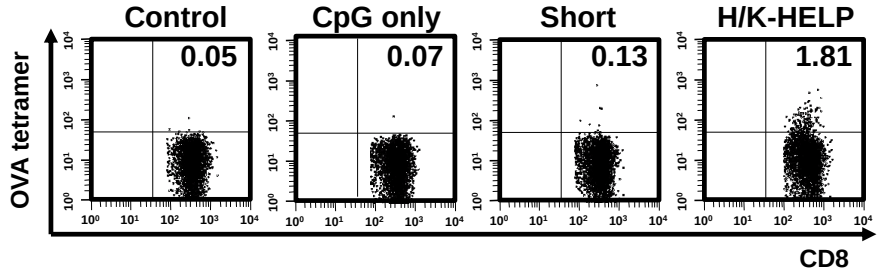


Fig. 1 Induction of OVA-specific CTL following primary vaccination with H/K-HELP. Mice were vaccinated f.p. with the short peptides-Mix or H/K-HELP in combination with CpG-ODN. Ten days later, whole lymphocytes were collected from popliteal dLN. Frequency of OVA-specific CTL was examined by flow cytometry as described in Methods. The percentages of OVA tetramer-positive CD8$^+$ T cells were determined by FACS analysis. Data are shown as the means ± SDs of mice (n = 5) in each experimental group. Similar results were obtained in three separate experiments. Significant differences (*P<0.05) were determined by two-tailed t-test.

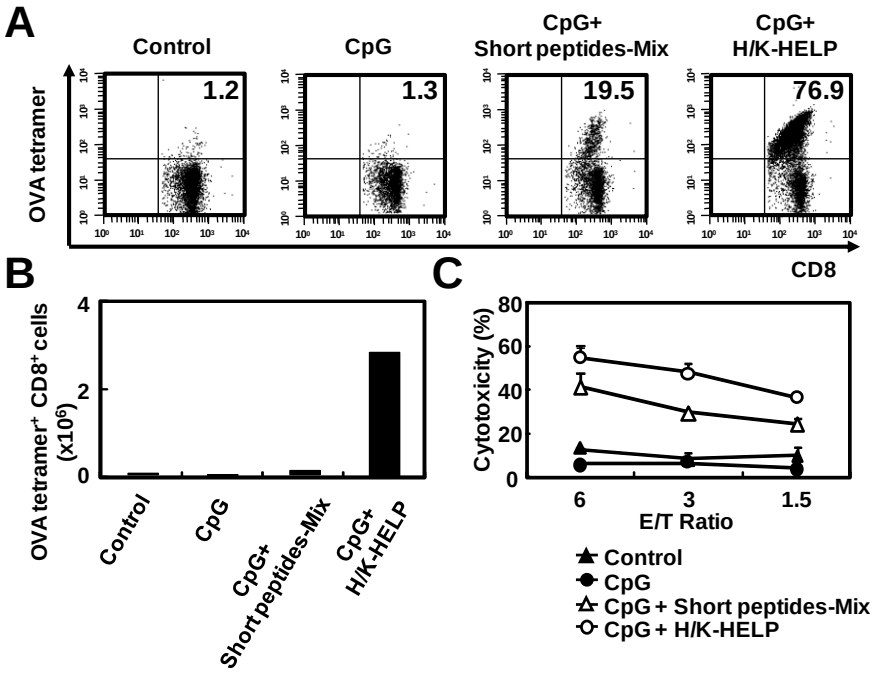


Fig. 2 H/K-HELP is superior to short peptides-Mix to induce antigen specific CTL. Mice were vaccinated f.p. with the short peptides-Mix or H/K-HELP in combination with CpG-ODN. Ten days later, whole lymphocytes were collected from popliteal dLN and cocultured with OVA-expressing EG-7 cells for 5 days. Frequency of OVA-specific CTL was examined by flow cytometry as described in Methods. (**A**) The percentages and (**B**) total cell numbers of OVA tetramer-positive $CD8^+$ T cells were determined by FACS analysis. (**C**) Cytotoxicity of the lymphocytes isolated from the dLN of mice vaccinated with saline (▲), CpG-ODN (●), CpG-ODN + short peptides-Mix (△) or CpG-ODN + H/K-HELP (○) against EG-7 cells was evaluated in a 4h ^{51}Cr-release assay. The means and SDs of representative data are indicated in the figure. Similar results were obtained in three separate experiments. Significant differences (*$P<0.05$) were determined by two-tailed t-test.

Using intracellular staining, we further showed that OVA-H/K-HELP vaccination was superior to short peptides-Mix vaccination for inducing $CD4^+$ IFN-γ-producing Th1 cells **(Fig. 3A)**. When dLN cells from OVA-H/K-HELP-vaccinated mice were re-stimulated with either class I-binding $OVA_{257-264}$ or class II-binding $OVA_{323-339}$ peptide, marked amounts of IFN-γ production were detected by ELISA **(Fig. 3B)**. These results indicated that OVA-H/K-HELP vaccination properly stimulated both class I-restricted IFN-γ-producing CTL and class II-restricted Th1 cells *in vivo*.

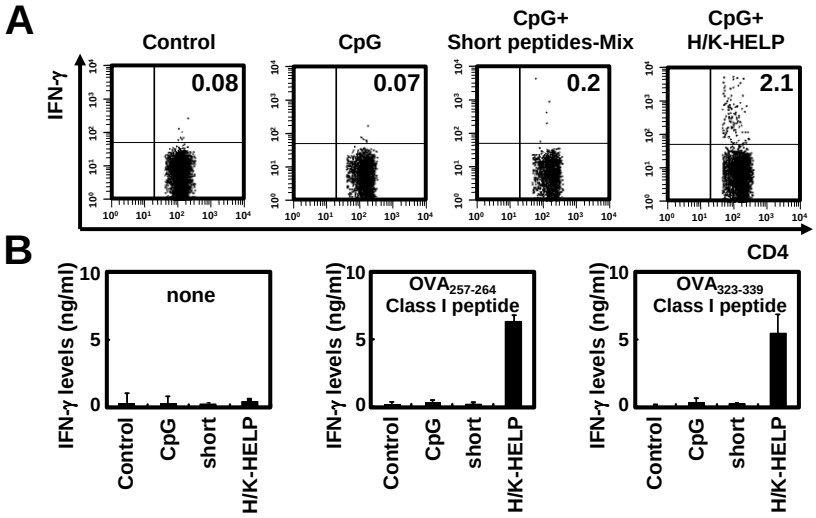


Fig. 3 H/K-HELP is superior to short peptides-Mix to activate functional Tc1 and Th1 cells. Mice were vaccinated f.p. with the short peptides-Mix or H/K-HELP in combination with CpG-ODN. Ten days later, whole lymphocytes were collected from popliteal dLN. (**A**) The collected cells were stimulated with OVA class I and class II peptides (2 μg/ml) for 18 h. OVA-specific IFN-γ-producing $CD4^+$ T cells were detected by intracellular staining. (**B**) The collected cells were cultured in the presence of OVA class I or class II peptides (1 nmol/ml) for 96 h. IFN-γ production levels in the culture supernatants were determined by ELISA. The means and SDs of representative data are indicated in the figure. Similar results were obtained in three separate experiments. Significant differences (*P<0.05) were determined by two-tailed t-test.

OVA-H/K-HELP is selectively presented by professional DC in the inflamed draining LN.

It has been reported that sustained presentation of antigenic peptide by MHC class I or class II molecules on appropriately activated DC but not T or B cells is crucial for inducing robust $CD4^+$ and $CD8^+$ T cell responses, which are essential for tumor eradication[17, 18]. Therefore, it was crucially important issue to investigate whether there are some differences in antigen-presentation mechanisms between H/K-HELP and short peptides-Mix. For this purpose, we isolated $CD11c^+$, $CD11c^-$, $CD19^+$ or $CD3^+$ APC from dLN or ndLN of mice 36 h after vaccination with OVA-H/K-HELP or short peptides-Mix and determined the antigen-presenting capability of these distinct APC subsets to stimulate CFSE-labeled $CD8^+$ OT-I T cells. As a result, OVA-H/K-HELP was selectively presented by professional $CD11c^+$ APC (DC) in inflamed dLN but not by $CD11c^-$ non-professional APC **(Fig.4)**. Also, DC that infiltrated the inflamed dLN but not the non-inflamed ndLN was able to present OVA-H/K-HELP to peptide-specific OT-I T cells. In sharp contrast, short peptides-Mix was

presented by both professional DC in inflamed dLN and non-professional APC (B and T cells) in non-inflamed ndLN. Moreover, short peptides-Mix-loaded $CD11c^+$ professional APC (DC) and $CD11c^-$ non-professional APC populations (T and B cells) were widely distributed in both dLN and ndLN of vaccinated mice.

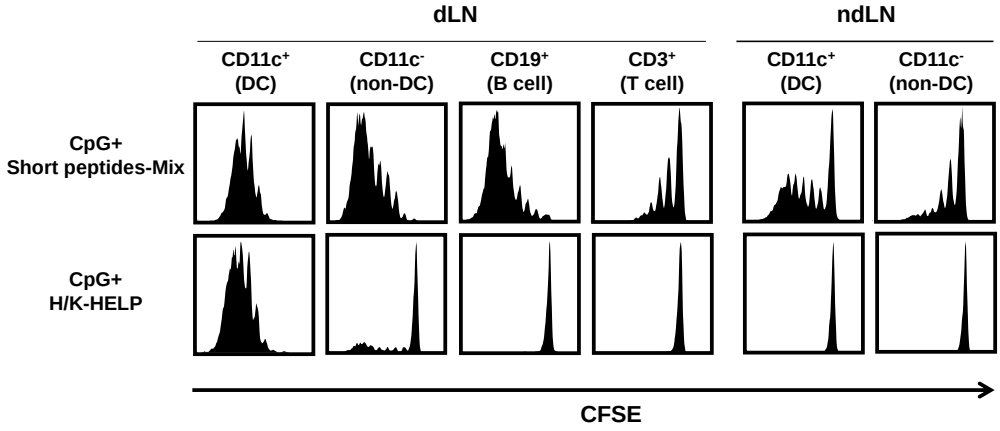


Fig. 4 H/K-HELP but not short peptides-Mix is selectively presented by professional APC in inflamed dLN at the vaccine inoculation site. Mice were vaccinated f.p. with the indicated peptides in combination with CpG-ODN dLN (popliteal) and ndLN (axillary) were collected at 36 h after vaccination. $CD11c^+$ DC, $CD19^+$ B cells and $CD3^+$ T cells were then isolated from the dLN cells and $CD11c^+$ DC and $CD11c^-$ cells were isolated from ndLN of the vaccinated mice. The sorted $CD11c^+$ cells (5×10^4), $CD19^+$ cells (4×10^5), $CD3^+$ cells (8×10^5) or $CD11c^-$ cells (8×10^5) were cocultured with naïve CFSE-labeled OT- I/Ly5.1 $CD8^+$ T cells (1×10^5) for 3 days. Proliferation of OT-I/Ly5.1 $CD8^+$ T cells was evaluated as dilution of CFSE by flow cytometry. Similar results were obtained in three separate experiments. Significant differences ($^*P<0.05$) were determined by two-tailed t-test.

Both B cells and T cells loaded by short peptides-Mix triggered the proliferation of OT-I $CD8^+$ T cells as effectively as peptide-loaded professional DC **(Fig. 5A)**. However, short peptides-Mix-loaded B cells and T cells were inferior to peptide-loaded DC with the respect to induction of IFN-γ-producing CTL (Tc1 cells), which are crucial for tumor eradication *in vivo* **(Fig. 5B)**. Thus, we demonstrated that OVA-H/K-HELP was selectively presented by DC in inflamed dLN and that OVA-H/K-HELP-pulsed DC efficiently induced superior Tc1-dependent immunity.

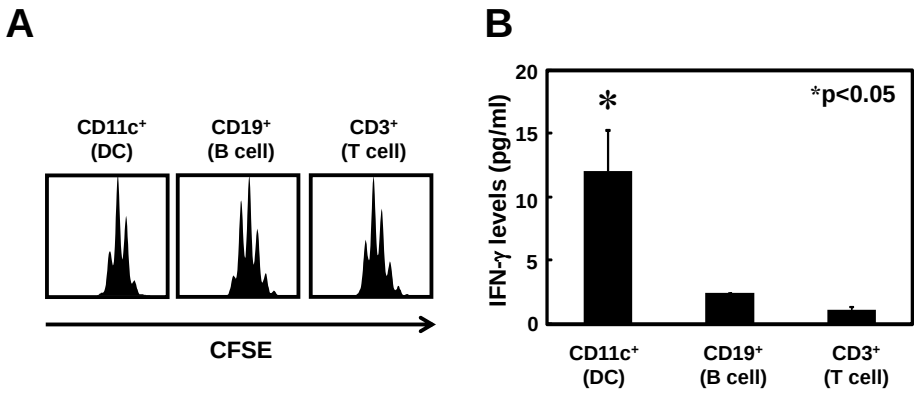


Fig. 5 Class I short peptide induces CD8$^+$ T cell tolerance through presentation by B or T cell. (**A, B**) CD11c$^+$, CD19$^+$ and CD3$^+$ cells isolated from wild type mice were pulsed with class I OVA peptide (1 nmol/ml). The peptide-loaded cells (5 x 10^4) were cocultured with naïve CFSE-labeled OT-I/Ly5.1 CD8$^+$ T cells (1 x 10^5) for 3 days. (**A**) Proliferation of OT-I/Ly5.1 CD8$^+$ T cells was evaluated as dilution of CFSE by flow cytometry. (**B**) IFN-γ production levels in the supernatants were determined by ELISA. Similar results were obtained in three separate experiments. Significant differences (*P<0.05) were determined by two-tailed t-test.

OVA-H/K-HELP-loaded DCs exhibited long term-duration of antigen presentation to induce antigen-specific CTL and Th cell responses *in vivo*.

After vaccination of C57BL/6 mice with short peptides-Mix or OVA-H/K-HELP, the duration time of antigen presentation by APC *in vivo* were determined by infusing of CFSE-labeled OT-I or OT-II T cells as detector cells. In the case of short peptides-Mix vaccination, CFSE-labeled CD8$^+$ OT-I T cells extensively proliferated in response to APC from dLN of vaccinated animals up to 10 days after vaccination **(Fig. 6A)**. In contrast, OVA-H/K-HELP vaccination resulted in sustained antigen-presentation by APC (DC), which triggered the proliferation of OT-I T cells until 60 days after vaccination. Similar long-lasting antigen presentation by DC was also demonstrated when the mice were vaccinated with OVA-H/K-HELP, followed by infusion of $OVA_{323-339}$-specific CD4$^+$ OT-II T cells **(Fig. 6B)**. Strong antigen presentation was detected at 36 h after vaccination with short peptides-Mix, but this was substantially decreased by 10 days after vaccination. However, if the mice were vaccinated with OVA-H/K-HELP, the antigen presenting capability of DC lasted until 60 days. These data indicated that antigen-specific CTL and Th1 cells could be effectively induced by H/K-HELP vaccination *in vivo*, because the duration of antigen presentation was sustained in the case of H/K-HELP vaccination.

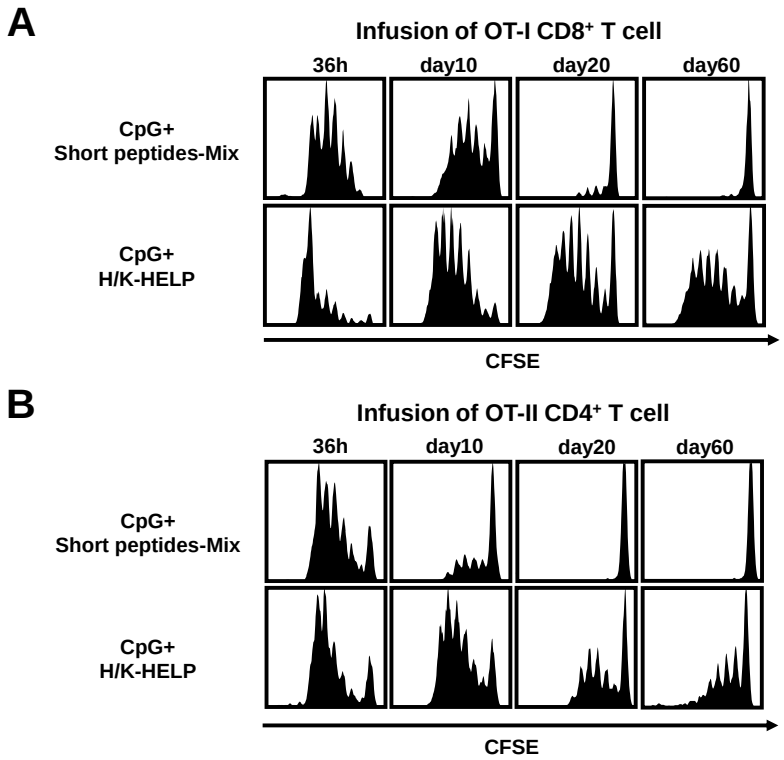


Fig. 6 H/K-HELP-loaded DC presents antigens and induces tumor-specific CTL and Th cells for an extended time period. (**A, B**) C57BL/6 mice were vaccinated f.p. with the short peptides-Mix or H/K-HELP in combination with CpG-ODN. Naïve CFSE-labeled OT-I/Ly5.1 $CD8^+$ T cells (**A**) or OT-II/Ly5.1 $CD4^+$ T cells (2 x 10^6) (**B**) were infused at 36 h, 10, or 20 days after the vaccination. Three days later, proliferation of OT-I/Ly5.1 $CD8^+$ or OT-II/Ly5.1 $CD4^+$ T cells in the dLN was evaluated by flow cytometry. Similar results were obtained in three separate experiments. Significant differences (*$P<0.05$) were determined by two-tailed t-test.

Critical role of the peptide-linker for the efficacy of an OVA-H/K-HELP vaccination.

OVA-H/K-HELP was artificially synthesized by conjugation with glycine-linker **(Table. 1)**. As described above **(Fig. 1-4)**, OVA-H/K-HELP was efficiently processed and presented by DC to stimulate Th and CTL. Next, we determined whether substitution of the glycine-linker to another amino-acid peptide-linker impacts vaccine efficacy. To address this question we synthesized OVA-H/K-HELP using a proline- or alanine-linker, which has a similar chemical structure as the glycine-linker. Interestingly, OVA-H/K-HELP conjugated with the glycine-linker exhibited superior immunostimulating activity for inducing OVA-specific $CD8^+$ T cells in both primary responses *in vivo* and secondary expansion *in vitro* **(Fig. 7A-D)**. Thus, the glycine peptide-linker is a critical factor for preparing an

effective H/K-HELP vaccine. Especially, glycine-linker seemed to be a suitable peptide-linker for preparing artificially synthesized long peptide by conjugating both helper and killer epitope peptides in this study.

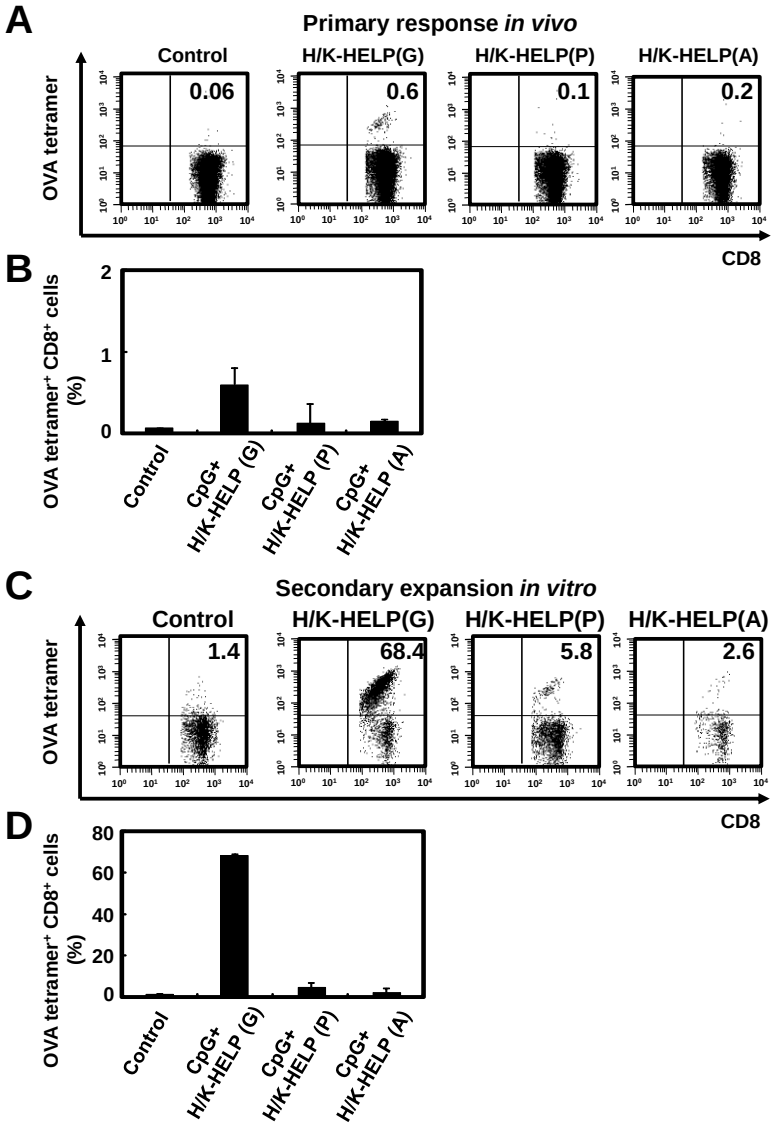


Fig. 7 The peptide-linker for conjugating helper and killer epitope peptides is critical role for the efficacy of H/K-HELP vaccine. Mice were vaccinated f.p. with H/K-HELP a glycine-, proline- or alanine-linker to conjugate the class I and class II epitopes, in combination with CpG-ODN. Ten days later, (**A, B**) whole lymphocytes were isolated from popliteal dLN and (**C, D**) cocultured with OVA-expressing EG-7 cells for 5 days. Frequency of OVA-specific CTL was examined by flow cytometry as described in Methods. The

percentages of OVA tetramer-positive $CD8^+$ T cells were determined by FACS analysis. Three independent experiments were carried out and means and SDs of the representative data are indicated in the figure.

OVA-H/K-HELP induced stronger protective activity against EG-7 tumors.

To determine protective activity by peptide vaccination, immunized mice were prepared by vaccination with CpG-ODN plus OVA-H/K-HELP or short peptide-Mix. Twenty-one days after vaccination, OVA-expressing EG7 tumor cells (2×10^6 cells) were inoculated into the immunized mice to detect the protective efficacy of each vaccine 7 days after tumor inoculation **(Fig. 8A)**. The immunized mice with OVA-H/K-HELP plus CpG-ODN caused the increased induction of OVA-$tetramer^+$ CTL in parallel with the activation of IFN-γ-producing Th1 and CTL **(Fig. 8B)**. Moreover, it was demonstrated that OVA-H/K-HELP-immunized mice exhibited augmented generation of EG7-specific CTL and completely eradicated challenged EG7 tumor cells **(Fig. 8B-D)**. However, C57BL/6 mice immunized with short peptides-Mix and CpG-ODN revealed lower activation of antitumor immunity and could not reject EG7 tumor cells. These data suggested that H/K-HELP vaccination could effectively induce memory response and protective activity against tumor.

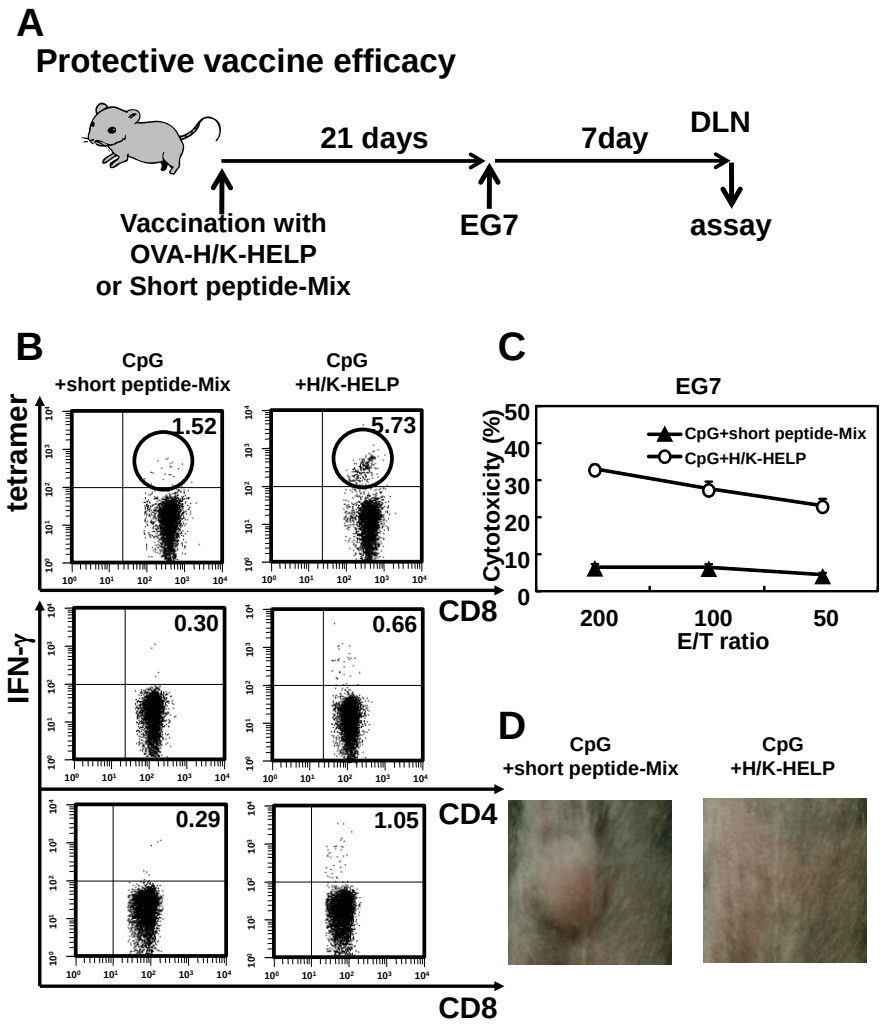


Fig. 8 H/K-HELP induced stronger protective activity against EG-7 tumors. (**A**) C57BL/6 mice were intradermally (i.d.) injected with CpG-ODN + short peptide or CpG-ODN + H/K-HELP near the right axillary lymph nodes. Twenty-one days after vaccination, EG7 cells ($2x10^6$ cells) were i.d. inoculated into vaccinated mice at the right flank. Seven days after tumor injection, lymphocytes were prepared from draining lymph node (DLN). Protective activity against EG-7 tumors were estimated the percentages of OVA-specific CTL by FACS analysis (**B**), cytotoxicity by ^{51}Cr-release assay (**C**) and rejection tumor (**D**) by measuring the tumor size, respectively. Three independent experiments were carried out and means and SDs of the representative data are indicated in the figure.

Established EG-7 tumors were completely eradicated by OVA-H/K-HELP but not short peptides-Mix vaccination in combination with CpG-ODN.

C57BL/6 mice were i.d. inoculated with 2 x 10^6 EG-7 tumor cells expressing OVA as a

model tumor antigen[15, 19]. When the tumor mass became palpable (7-8 mm), the tumor-bearing mice were vaccinated by i.d. injection of OVA peptides in combination with CpG-ODN near the DLN of the tumor. In a pilot study, we confirmed that the growth of EG-7 tumor cells was not strongly inhibited by vaccination with a short CTL peptide epitope ($OVA_{257-264}$) alone or a short Th peptide epitope ($OVA_{323-339}$) alone **(Fig. 9)**. Therefore, we injected mice with a mixture of short peptides-Mix. Further, we synthesized OVA-H/K-HELP by conjugating Th and CTL peptide epitopes via a glycine-linker **(Fig. 10A)**. While a single i.d. injection of short peptides-Mix plus CpG-ODN caused significant inhibition of tumor growth, this inhibitory effect was not higher than that of CpG-ODN alone. However, profound inhibition of tumor growth was caused by vaccination with OVA-H/K-HELP plus CpG-ODN. As a result, 80 % of the vaccinated mice were completely cured of their tumor **(Fig. 10B and 10C)**.

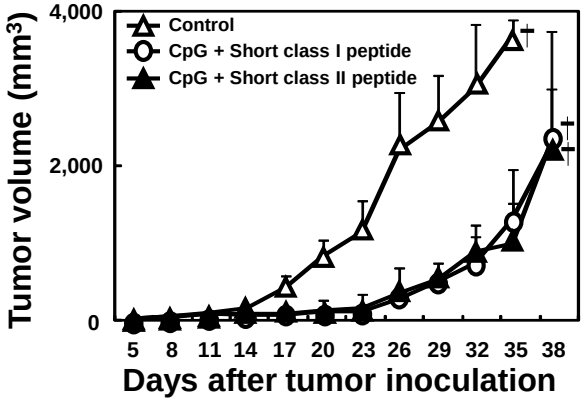


Fig. 9 Efficacy of short class I or class II peptides as therapeutic tumor vaccines. EG-7 cells (2×10^6) were intradermally (i.d.) inoculated into C57BL/6 mice. When the tumor mass became palpable (7-8 mm), the tumor-bearing mice were i.d. injected near the dLN with saline (△), CpG-ODN + class I short peptide (○) or CpG-ODN + class II short peptide (▲). The anti-tumor effect was determined by measuring the tumor size. Three independent experiments were carried out and means and SDs of the representative data are indicated in the figure.

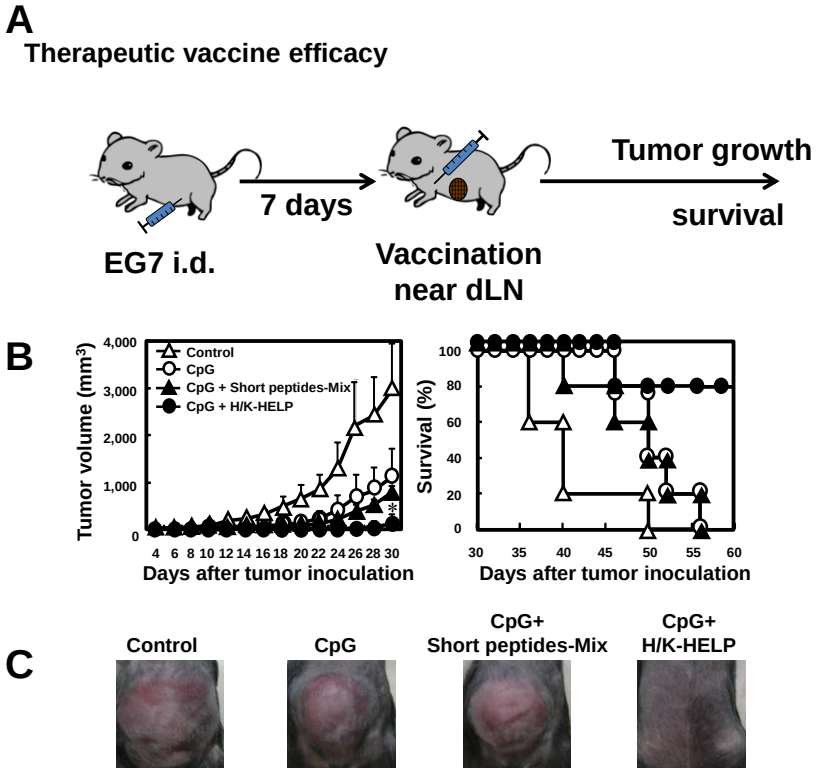


Fig. 10 Therapeutic vaccination with H/K-HELP but not short peptides-Mix completely eradicates established EG-7 tumors. (**A**) OVA-H/K-HELP was artificially synthesized by conjugating $OVA_{257-264}$ class I epitope (SIINFEKL) with $OVA_{323-339}$ class II epitope (ISQAVHAAHAEINEAGR). (**B, C**) EG-7 cells (2×10^6) were intradermally (i.d.) inoculated into wild-type C57BL/6 mice. When the tumor mass became palpable (7-8 mm), the tumor-bearing mice were i.d. injected near the dLN with saline (△), CpG-ODN (○), CpG-ODN + short peptides-Mix (▲) or CpG-ODN + H/K-HELP (●). The anti-tumor effect was determined by measuring the tumor size and by assessing the survival ratio. Tumor volume was calculated as described in Methods. Data are shown as the means ± SDs of mice (n = 5) in each experimental group. Similar results were obtained in three separate experiments. Significant differences (*P<0.05) were determined by two-tailed t-test.

OVA-H/K-HELP induces stronger CTL and Th cell responses than 30 amino acid peptides containing extended class I or class II OVA epitopes.

It has been reported that peptides containing extended class I epitopes are superior to peptides containing minimal class I epitopes to induce effective CTL responses[11]. It is therefore critical to determine the mechanism for the superior vaccine properties of OVA-H/K-HELP. For this purpose, we synthesized extended 30 amino acid long class I ($OVA_{241-270}$) and class II ($OVA_{317-346}$) peptide **(Table. 2)**. We compared the vaccine efficacy of short class I peptide, long class I peptide, short peptides-Mix, long class I and class II

peptides mix (long peptides-Mix) or OVA-H/K-HELP. Compared with short peptides-Mix, the extended class I and class II peptides mixture (long peptides-Mix) exhibited enhanced vaccine efficacy. Indeed, OVA-H/K-HELP, which is a single long peptide, induced antigen specific CTL as effectively as extended long class I peptide or long peptides-Mix **(Fig. 11A, C)**. However, cell number of antigen specific CTL was superior H/K-HELP or long peptides-Mix vaccination compared with class I long peptide vaccination **(Fig. 11B)**. Finally, we compared the therapeutic efficacy of OVA-H/K-HELP, extended long peptides-Mix, short peptides-Mix, extended long class I peptide and short class I peptide **(Fig. 12)**. Rejection of EG-7 tumor cells was induced by OVA-H/K-HELP vaccination as effectively as long peptides-Mix compared with class I long peptide vaccination. These data indicated that it is critical for both the peptide length and conjugation of helper/killer epitopes to contribute to the superior therapeutic vaccine effect.

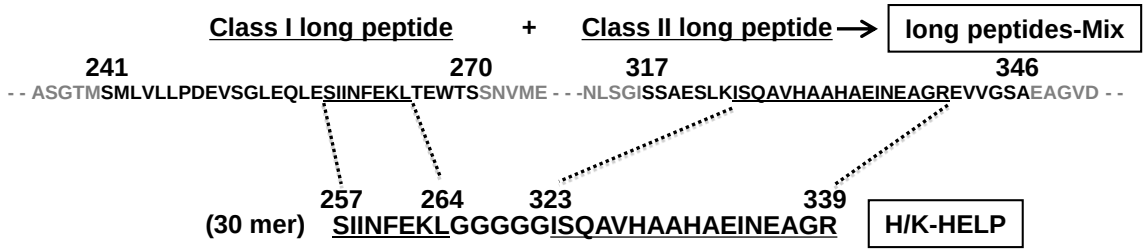


Table. 2 Long $OVA_{241-270}$ class I or $OVA_{317-346}$ class II peptides that contain cognate class I and class II epitopes were synthesized.

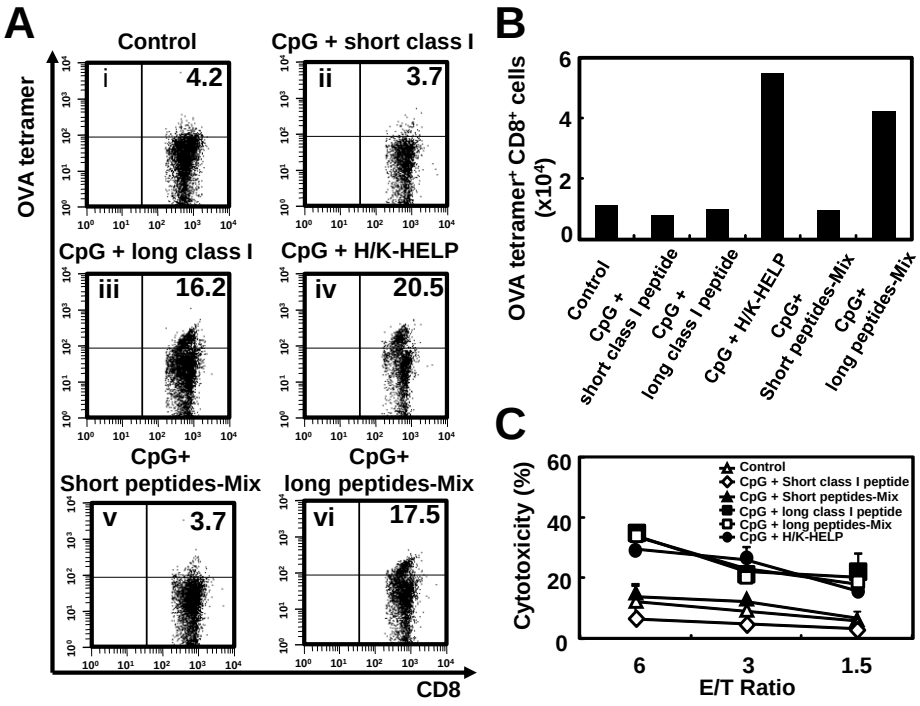


Fig. 11 Activation of CTL and Th cells by OVA-H/K-HELP is comparable to the responses elicited by extended OVA class I and class II peptides. Mice were vaccinated f.p. with the class I short peptide, short peptides-Mix, class I long peptide, long peptides-Mix or H/K-HELP in combination with CpG-ODN. Ten days later, whole lymphocytes were collected from popliteal dLN and cocultured with OVA-expressing EG-7 cells for 5 days. Frequency of OVA-specific CTL frequency was examined by flow cytometry. (**A**) The percentages and (**B**) total cell numbers of OVA tetramer-positive $CD8^+$ T cells were determined by FACS analysis. (**C**) Cytotoxicity of the lymphocytes from the dLN of mice vaccinated with saline (△) or CpG-ODN (○) with class I short peptide (◇), short peptides-Mix (▲), class I long peptide (■), long peptides-Mix (□), or H/K-HELP (●) against EG-7 cells was examined in a 4 h ^{51}Cr-release assay. Data are shown as the means ± SDs of mice (n = 5) in each experimental group. Similar results were obtained in three separate experiments. Significant differences (*P<0.05) were determined by two-tailed t-test.

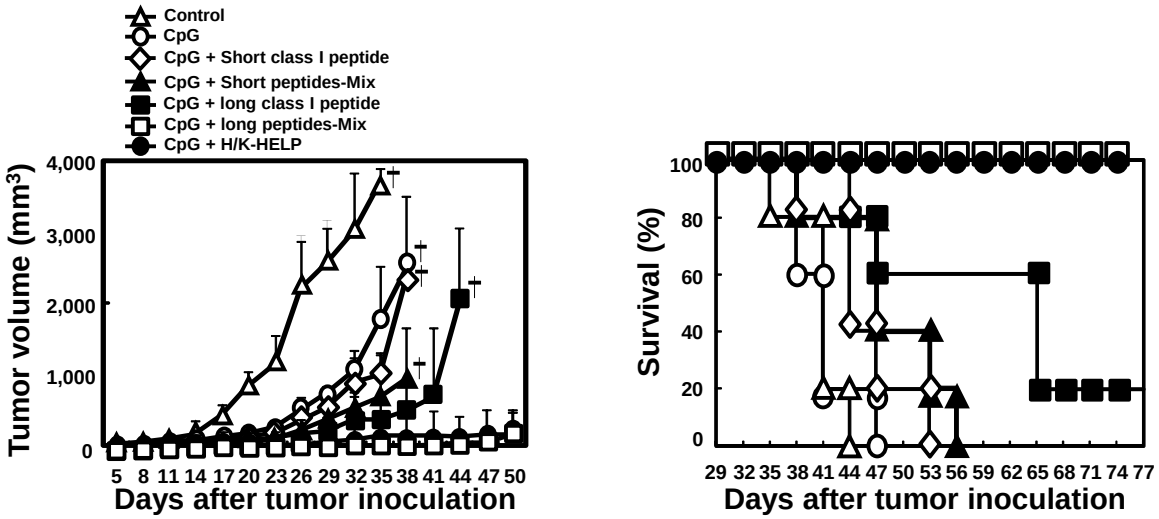


Fig. 12 Both the peptide length and conjugation of helper/killer epitopes appeared to contribute to the superior therapeutic vaccine effect. The tumor-bearing mice were i.d. injected near the dLN with the indicated peptides and/or CpG-ODN. The anti-tumor effect was determined by measuring the tumor size. Tumor volume was calculated as described in Methods. Data are shown as the means ± SDs of mice (n = 5) in each experimental group. Similar results were obtained in three separate experiments. Significant differences (*P<0.05) were determined by two-tailed t-test.

DISCUSSION

In this work, to address the underlying mechanisms for the antitumor efficacy of the H/K-HELP vaccination, we synthesized a 30 amino acid H/K-HELP of the model tumor antigen OVA. We demonstrated that OVA-H/K-HELP vaccination of mice combined with CpG-ODN resulted in the sustained antigen presentation by DC in the dLN and potently activated IFN-γ-producing CTL and Th1 cells **(Figs. 2-4)**. Moreover, OVA-H/K-HELP vaccination with CpG-ODN indicated efficient preventive and therapeutic vaccine effects against tumor **(Figs. 8 and 10)**. Indeed, therapeutic cancer vaccination with OVA-H/K-HELP plus CpG-ODN caused complete eradication of OVA-expressing tumors in 80 % of mice **(Fig. 12)**.

Some reports have been performed to develop a therapeutic vaccine that can combat established cancers and infectious diseases[20-23]. However, the development of therapeutic vaccines has been hampered by difficulties to trigger T cell-mediated immunity as compared with a prophylactic vaccine aimed at inducing neutralizing antibodies[24]. A therapeutic vaccine for treatment of established cancers requires the capacity to induce sustained antigen presentation by DC, which are critical for inducing fully activated tumor antigen-specific CTL and Th1 cells[15]. Since cancer antigenic peptide was discovered[25], short MHC-class I-binding peptides derived from tumor-associated antigenic proteins have been used as a therapeutic vaccine. However, the clinical trials with such peptides have been generally disappointing[26, 27]. This might be because that MHC class I-binding short peptide emulsified in IFA (or Montanide) was processed by non-professional APC (T cells and B cells) in non-inflamed LN, which provided optimal environments to induce $CD8^+$ T cell tolerance as reported previously[7, 11, 28]. We also confirmed that short peptide induced the proliferation of T cells but downmodulated the activation of IFN-γ-producing CTL **(Fig. 5)**. Another reason for the failure of vaccine therapies based on minimal CTL epitopes might be due to the lack of Th1 cell immunity, which is critically important for the induction of fully activated CTL and memory T cells in the immunosuppressive environment of the tumor-bearing host[13-15]. To overcome these problems, Melief *et al.*[7] recently developed an evolutional peptide vaccine, SLP and demonstrated that SLP corresponding to tumor antigens was superior to a short peptide for delivery to DC and for the activation of tumor-specific CTL in only inflamed dLN[7, 8]. They also demonstrated that vaccinations with the HPV16-derived 35 amino acid long peptide $E7_{43–77}$, containing both a CTL epitope and a Th epitope, resulted in the induction of far more robust E7-specific $CD8^+$ T cell responses than vaccinations with the minimal CTL epitope only[10]. Moreover, they reported that vaccination with HPV-SLP with

CpG-ODN resulted in the eradication of large, established HPV16-expressing tumors[10]. The discovery of SLP vaccine indicated a new direction of therapeutic vaccine design useful for the therapy of cancer and infectious diseases. Indeed, in that clinical trial, Kenter et al.[29] demonstrated that vaccination of high-grade vulvar intraperitoneal neoplasia patients with 13 SLPs mixture containing the entire length of the two oncoproteins E6 and E7 of HPV-16 resulted in inducing complete responses (47 % of patients) concomitantly with the activation of IFN-γ-producing $CD4^+$ T cells and E6-specific CTL. Thus, it was clarified that (i) the long peptide vaccine is superior to short peptide because long peptide but not short peptide is selectively processed and presented by professional antigen presenting DC; (ii) the existence of Th epitope in addition to CTL epitope in SLP revealed a strong and efficient therapeutic vaccine efficacy. These findings encouraged us to develop more efficient long peptide vaccine, which could induce CTL and Th cell responses in patients by vaccination with a single long peptide conjugating with both Th and killer epitopes. It has been reported that hybrid peptide conjugating Th and CTL epitopes is superior to short peptide to induce hepatitis C virus (HCV)-specific CTL *in vivo*[12]. However, the first clinical trial using a synthetic 15 amino acid helper and killer epitopes of $gp100_{175\text{-}189}$ exhibited no significant impact for therapeutic efficacy of melanoma[26]. Therefore, it still remains unclear whether the artificial long hybrid peptide conjugating Th and CTL epitope exhibits superior vaccine efficacy against tumor and infectious diseases *in vivo*.

In this experiment, we showed a superior preventive and therapeutic vaccine, which could activate both peptide-specific Th1 cells and CTL by OVA-H/K-HELP conjugating Th and CTL epitope peptides with glycine-linker. Compared with a short peptide vaccine, OVA-H/K-HELP possessed several advantages for induction of long-lasting tumor-specific T cell immunity, which was able to prevent mice from tumor challenge and cure mice of established tumors **(Fig. 6, 8 and 10)**. OVA-H/K-HELP is superior to short peptide in the following points; (i) selective presentation by only DC in vaccinated dLN; (ii) efficient induction of Th1-dependent immunity preferable for CTL generation (iii) long-lasting antigen presentation; and (iv) enhanced activation of antigen-specific CTL and Th cells. Moreover, OVA-H/K-HELP exhibited stronger antitumor activity compared with recently developed extended class I long peptide consisted of 30 amino acid **(Fig. 11, 12)**.

Recently, it was reported that persisting CTL short peptide vaccine depots in IFA induce specific T cell sequestration, dysfunction and deletion at the vaccination site[28]. However, Bijker *et al.*[11] demonstrated that extended long peptides of natural CTL epitopes promoted sustained induction of CTL. Moreover, these investigators showed that addition of a minimal Th peptide epitope to a minimal CTL peptide epitope in IFA prevented the downmodulation

of CTL responses[11]. Therefore, it might be possible to consider that OVA-H/K-HELP conjugating Th and CTL epitope by glycine-linker might be able to overcome the disadvantages associated with short class I-binding peptide vaccines. We have already demonstrated that the tumor antigen-specific immune responses were induced in cancer patients after vaccination with MAGE-A4- and Survivin-H/K-HELP in our clinical trial[30]. As shown in **Fig. 11 and 12**, we demonstrated that (i) OVA-H/K-HELP and long peptides-Mix were able to induce higher numbers of OVA-tetramer$^+$ CTL and superior therapeutic efficacy compared with short peptides-Mix and class I long peptide; (ii) The class I long peptide induced a higher percentage of CTL and antitumor therapeutic activity than class I short peptide and short peptides-Mix, but exhibited lower antitumor activity compared with OVA-H/K-HELP. Based on these results, we concluded that the peptide length, glycine linker, and conjugation of both helper and killer epitopes were critical for the superior therapeutic vaccine efficacy of OVA-H/K-HELP.

CONCLUDING REMARKS

New discoveries from this research

・OVA-H/K-HELP vaccination enhanced activation of antigen-specific CTL and Th1 cells.

・H/K-HELP exhibited stronger antitumor activity compared with the extended class I long peptide or short peptides-Mix in the mice EG7 tumor model.

・OVA-H/K-HELP induced stronger protective activity against EG-7 tumors.

・OVA-H/K-HELP but not short peptides-Mix was selectively presented by only DC in vaccinated dLN and long-lasting antigen presentation.

・The glycine peptide-linker but not proline- or alanine-linker is a critical factor for preparing an effective H/K-HELP vaccine.

The immunotherapeutic significance of the discovery and future perspectives

The therapeutic efficacy of cancer vaccine therapy using short MHC class I-binding CTL epitopes has been limited. The development of an effective therapeutic cancer vaccine needs for address several challenges to overcome strong immunosuppression and tumor escape mechanisms in tumor-bearing hosts.

It has been reported that the lack of Th cell activation might be a critical deficiency of the current therapeutic cancer vaccines. Also, SLP corresponding to tumor antigens was superior to a short peptide for delivery to DC and for activation of tumor-specific Th1 and Tc1 cells. We constructed novel strategy of artificial long peptide (H/K-HELP) vaccine that conjugated helper and killer epitope peptides of an antigenic tumor protein via a glycine-linker. In this study, we demonstrated OVA-H/K-HELP exhibited stronger antitumor activity as effectively as long peptides-Mix compared with recently developed extended class I long peptide consisted to 30 amino acid. Therefore, we concluded that both the peptide length and peptide conjugation of helper/killer epitopes were critical for the superior therapeutic vaccine efficacy of OVA-H/K-HELP. Furthermore, the chemical structure of the peptide-linker

appeared to be a critical factor for vaccine efficacy. These data suggested that the peptide-linker might be affect antigen ingestion and cleavage by DC.

In the near future, we will investigate that which amino acid sequence is identified as suitable for use with linker of H/K-HELP. Also, to clear why H/K-HELP is long lasting antigen presentation, we will demonstrate uptake mechanism of H/K-HELP by DC. Moreover, we consider that H/K-HELP potency would be established by examining whether anti-tumor activity is increased by H/K-HELP consisting of natural tumor antigen.

Finally, we demonstrated that vaccination of mice with OVA-H/K-HELP combined with CpG-ODN resulted in the sustained antigen presentation by DC in the dLN and potently activated IFN-γ-producing CTL and Th1 cells. Indeed, OVA-H/K-HELP vaccination with CpG-ODN indicated an efficient preventive and therapeutic vaccine effect against tumor. Therefore, we believe that H/K-HELP will become an innovative vaccine design useful for developing preventive and therapeutic vaccine against tumor and infectious diseases in future.

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