Background and Objectives: Since the discovery of tumor antigens by T. Boon, many investigators have performed clinical trials of vaccine therapy using peptides from the antigens to activate tumor-specific cytotoxic T lymphocyte (CTL) in cancer patients. Such vaccines have been thought to induce tumor specific immune responses to eradicate cancers with superior specificity and safety as compared to other anticancer drugs. However, the therapeutic efficacy of cancer vaccine therapy using short MHC class I binding CTL epitopes was limited to induce complete regression. It has been reported that short peptide vaccine induces defect of Type I helper T (Th1) cell activation and tolerance. To overcome the limited antitumor effect, it is needed to induce fully activated tumor specific CTL, which can eradicate tumor cells and induce a complete cure in tumor bearing hosts. Recently, we have demonstrated a critical role of Th1 immunity for inducing fully activated antitumor CTL, which are essential for eradicating the tumor mass. Moreover, synthetic long peptides (SLP) that simply extend class I peptide epitopes exhibited superior vaccine efficacy compared with short peptides. Thus, the existence of Th epitope peptide and the length of SLP appeared to be key factors for designing therapeutic peptide vaccine. In this study, we have developed an innovative peptide vaccine, referred to as helper/killer-hybrid epitope long peptide (H/K-HELP) by conjugating Th and CTL epitopes of OVA model tumor antigen with glycine linker.

Methods: We synthesized OVA-H/K-HELP (SIINFEKLGGGGGISQAVHAAHAINEAGR), OVA_{257-264} CTL epitope (class I short peptide), OVA_{323-339} Th epitope (class II short peptide), OVA_{241-270} CTL epitope (class I long peptide) and OVA_{317-346} Th epitope (class II long peptide) to demonstrate antitumor activity and regulatory mechanism in a mouse tumor-bearing model. We demonstrated therapeutic activity against tumor by H/K-HELP vaccination with CpG-ODN in the model using OVA-expressing EG7 tumor cells. To discuss the mechanism of antitumor effect by H/K-HELP, C57BL/6 mice were vaccinated with short peptides, long peptides or OVA-H/K-HELP plus CpG-ODN adjuvant and measured OVA-tetramer positive CTL or IFN-γ producing CD8⁺ CTL and CD4⁺ Th1 cells by ELISA and FACS analysis. Also, cytotoxicity against EG7 was measured by ⁵¹Cr release assay. To test which cell subsets presented OVA peptide in vivo, we co-cultured subsets isolated from the vaccinated mice and CFSE-labeled OT-I CD8⁺ cells. Moreover, to examine duration of antigen presentation in vivo, we injected CFSE-labeled OT-I CD8⁺ T cells or OT-II CD4⁺ T cells into the mice various days after immunization. The antigen presenting capability of APC subsets were determined by proliferation of CSFE-labeled OT-I CD8⁺ T cells or OT-II CD4⁺ T cells on FACS analysis. Moreover, we determined whether substitution of the glycine linker to proline or alanine peptide linker impacts vaccine efficacy.

Results: In the EG7 tumor bearing mouse model, OVA-H/K-HELP induced rejection of EG-7 tumor cells as effectively as the long peptides-Mix (class I long peptide plus class II long peptide), but not class I short peptide, short peptides-Mix (class I short peptide plus class II short peptide) or class I
OVA specific CTL were induced by long peptides-Mix or OVA-H/K-HELP vaccination compared with class I long peptide vaccination in the tetramer assay. OVA-H/K-HELP vaccination induced IFN-γ producing OVA specific CTL and Th1 cells by re-stimulation with class I and class II short peptide. Also, percentage of cytotoxicity against EG7 was increased by class I long peptide or OVA-H/K-HELP vaccination. However, total cell numbers of antigen-specific CTL were superior OVA-H/K-HELP to class I long peptide vaccination. OVA-H/K-HELP vaccination resulted in sustained antigen presentation in the inflamed draining lymph node (dLN) by dendritic cells (DC), which triggered the proliferation of CD8^+ OT-I T cells and CD4^+ OT-II T cells until 60 days after vaccination. Moreover, glycine-linkered OVA-H/K-HELP exhibited superior antitumor activity compared with proline- or alanine-linkered OVA-H/K-HELP.

**Discussion:** In this study, we synthesized a 30 amino acid OVA-H/K-HELP of the model tumor antigen OVA and addressed the underlying mechanisms for the efficacy of the H/K-HELP vaccine. 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. Moreover, OVA-H/K-HELP vaccination with CpG-ODN indicated marked preventive and therapeutic effects against tumor. Indeed, therapeutic cancer vaccination with OVA-H/K-HELP plus CpG-ODN caused complete eradication of OVA-expressing tumors in 80% of mice. Moreover, OVA-H/K-HELP exhibited stronger antitumor activity compared with recently developed extended class I long peptide consisted of 30 amino acid. We demonstrated that 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 or class I long peptide. Also, 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. These findings suggested 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.

**Conclusion:** In view of previous works, it is need for novel strategy to develop peptide vaccine therapy for cancer patients. In this study, we designed H/K-HELP, which was conjugated Th and CTL epitopes with glycine linker to activate both CD4^+ T and CD8^+ T cells. 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. Here, we evaluated the mechanisms of H/K-HELP vaccination by using a tumor-bearing mouse model. EG-7 tumor was eradicated by H/K-HELP but not short peptides-Mix vaccination with CpG-ODN adjuvant. H/K-HELP was presented by only professional DC in dLN and exhibited long lasting antigen presentation. Moreover, H/K-HELP exhibited stronger antitumor activity as effectively as the long peptides-Mix compared with the extended class I long peptide. In addition, it was confirmed that antigen-specific immunity was different from a kind of amino acids in the linker region. These results indicate that peptide length, type of linkers, and combination of CTL and Th epitopes are critical for inducing strong antitumor effects *in vivo*. Thus, the present design of H/K-HELP vaccine would contribute to develop promising strategies for cancer patients.