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Development of a cancer vaccine: peptides, proteins, and DNA

Abstract Genetic changes leading to protooncogene activation qualitatively and/or quantitatively alter their gene products and are exclusively or largely restricted to transforming cells and their precursors. The overexpression of HER2 is among those changes and is often detected in adenocarcinomas such as breast, ovarian, lung, and gastric cancer. This provides a rationale for exploring the possibility that HER2 is a target of host immune responses against cancer cells. We have recently demonstrated that HER2 can be a target for tumor-rejecting immune responses against syngeneic murine $HER2^+$ tumor cells. We defined two different peptides, HER2p63–71 and HER2p780–788, with a K^d anchor motif that can induce $CD8^+$ cytotoxic T lymphocytes (CTLs). The growth of $HER2^+$ syngeneic tumors was suppressed in mice immunized with HER2p63–71 or p780–788. Since murine K^d and human HLA-A24 share a similar anchor motif for peptides, HER2p63–71 and HER2p780–788 were examined for induction of CTLs in HLA-$A24^+$ individuals. $CD8^+$ CTL clones specific for these peptides were established and they lysed $HER2^+$ tumor cells in a human leukocyte antigen (HLA)-A24-restricted manner. To elicit specific $CD8^+$ T cell immune responses against cancer, the development of efficient devices to deliver tumor antigen peptides to the major histocompatibility complex (MHC) class I pathway constitutes a central issue. We have developed a novel formula of hydrophobized polysaccharide nanoparticles which can deliver a HER2 oncoprotein containing an epitope peptide to the MHC class I pathway. We designed a simple protein delivery system: cholesteryl group-bearing polysaccharides, mannan or pullulan (CHM or CHP, respectively), complexed with the truncated HER2 protein containing the 147 *N*-terminal amino acids. These complexes were able to induce $CD8^+$ CTLs against $HER2^+$ tumors. CTLs were MHC class I restricted and specifically recognized HER2p63–71, a part of a truncated HER2 protein used as an immunogen. The complete rejection of tumors also occurred when CHM-HER2 was applied early after tumor implantation. In the effector phase of in vivo tumor rejection, $CD8^+$ T cells played a major role. The results suggest that this unique hydrophobized polysaccharide may help soluble proteins to induce cellular immunity. Such a novel vaccine may be of potential benefit in cancer prevention and cancer therapy.

Key words Hydrophobized polysaccharides · HER2/neu/c-erbB2 oncoprotein · Dendritic cells · Tumor vaccine

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Introduction

Recently obtained insights into tumor rejection antigens (TRAs), the recognition of tumor antigen-derived peptides by $CD8^+$ cytotoxic T lymphocytes (CTLs), and the detailed mechanisms of antigen presentation to T

lymphocytes have sparked new enthusiasm for the concept of cancer vaccines and provided the knowledge required to design novel immunotherapy strategies. The major focus has been on peptide or protein vaccines in association with major histocompatibility complex (MHC) molecules to elicit cytolytic immunity. Several investigators chose the approach of using a soluble protein or a peptide with an anchor motif as a tumor immunogen to elicit cellular immunity with or without adjuvants [3, 5–7, 11, 15–17, 19].

In general, exogenous soluble proteins cannot induce $CD8^+$ CTLs, because they rarely enter the MHC class I pathway and generally are internalized in the endosome. To overcome this problem, researchers are trying to find suitable adjuvants. We previously reported that a soluble truncated hybrid protein of *gag* and *env* of the human T lymphotropic virus type 1 induced specific $CD8^+$ T cell-dependent immunity when reconstituted into mannan derivative-coated liposomes [15]. Considering the increasing evidence for receptors that can specifically bind polysaccharides, we consider coating by polysaccharides as more important than liposomes for the delivery of proteins. Therefore we designed a novel and simple protein delivery system for cancer immunotherapy: cholesteryl group-bearing polysaccharides mannan (CHM) and pullulan (CHP) complexed with the truncated protein encoded by the HER2 protooncogene, which is overexpressed with high frequency in breast, stomach, ovarian, bladder, and other cancers [4, 18, 20, 21].

In the present study, we investigated whether the HER2 protein alone or complexed with CHM or CHP can induce responses by $CD8^+$ CTLs and tumor rejection specific for HER2-expressing tumor cells. The humoral immune response against HER2 generated by this novel vaccine was also investigated. Its usefulness in the prevention of tumor growth and as therapy for established tumors was evaluated.

Materials and methods

Mice

Six- to 8-week-old female BALB/c mice were purchased from the Shizuoka Animal Laboratory Center (Shizuoka, Japan) and used in all experiments. The animals were maintained at the Animal Center of Mie University School of Medicine, Tsu, Japan, under conditions fulfilling the institutional regulations governing the treatment of laboratory animals.

Tumor cell lines

CMS7 and CMS17 are 3-methylcholanthrene-induced fibrosarcoma cell lines of BALB/c mouse origin. P1.HTR is a mastocytoma cell line of DBA/2 mouse origin. These cell lines were transfected with the cDNA for human HER2 as described elsewhere [13] and designated CMS7HE and CMS17HE, respectively.

Expression and purification of ^{6}His-HER2 protein

Truncated HER2 cDNA encoding an amino-terminal portion (amino acid position 1–147) was amplified from a plasmid containing full-length cDNA using a 5′-primer (5′-AGC TGC AGT GAT CAC CAT GGA GCT-3′) containing a BclI site and a 3′-primer (5′-TGA ATT CTA TGT GAG ACT TCG AAG CTG CA-3′) containing an inframe stop codon [2]. The blunt-ended polymerase chain reaction (PCR) product was cut with BclI and cloned into BamHI and blunt-ended HindIII sites of pQE11 (Qiagen, Hilden, Germany). JM109 was transformed with the resulting plasmid, pQH5′, and ^{6}His-HER2 ended according to the manufacturer's instructions. The recombinant protein was eluted at low pH in 6 M urea in Tris-buffered saline and immediately neutralized. Typically, purified recombinant protein 20 mg was obtained from confluent culture 500 mL.

Preparation of cholesteryl group-bearing polysaccharide nanoparticles

CHM-85-2.3 and CHP-108-0.9 were identical to those used in our previous work [1, 14]. Mannan (molecular weight 85,000) and pullulan (molecular weight 108,000) were substituted by 2.3 cholesteryl moieties per 100 mannose units of mannan and 0.9 cholesteryl moieties per 100 glucose units of pullulan, respectively. An appropriate amount of CHM or CHP was dissolved in dimethyl sulfoxide (DMSO) and dialyzed against phosphate-buffered saline (PBS) 150 mM, pH 7.9. After dialysis, the suspension was sonicated using a probe-type sonifier (UR-200P, Tomy, Tokyo, Japan) at 40 W for 10 min. The suspension obtained was filtered through three types of membrane filter (Super Acrodics 25, Gelman Science, Ann Arbor, MI, USA; pore size 1.2 μm, 0.45 μm, and 0.2 μm) to make the particle size constant and to remove dust. Finally, an optically clear suspension was obtained. The cholesteryl group-bearing polysaccharides formed nanoparticles by self-aggregation with diameters of 20–30 nm [1, 2].

Preparation of complexes between HER2 protein and cholesteryl group-bearing polysaccharides

The HER2-derived protein described above was dissolved in 6 M urea. Protein solution 2.0 mg/mL was mixed with 2.1 mL of a suspension of cholesteryl-bearing polysaccharides 5.7 mg/mL at room temperature, resulting in the formation of a CHM- or CHP-HER2 complex (CHM or CHP: 5.0 mg/mL, protein: 0.25 mg/mL, 0.75 M urea). CHP- and CHM-carbonic anhydrase II (CAB, Sigma, St. Louis, MO, USA) complexes were prepared as controls using the same method.

Peptide pulsing

HER2-derived p63–71 (TYLPTNASL) and p440–448 (AYSLTLQGL) peptides were synthesized at Chiron Mimotopes (Melbourne, Australia). P1.HTR cells 1×10^6 were washed with plain RPMI medium and then resuspended in plain RPMI 500 μL. Peptide solution 10 μL (1 mg/mL in PBS) was added and incubated for 15 min at room temperature. Then RPMI 500 μL with 20% fetal calf serum (FCS) was added and incubated for 45 min at room temperature, followed by additional incubation at 37 °C for 1 h. Finally, the suspension was washed twice with plain RPMI. Thereafter the pulsed cells were used as target cells for CTLs.

Preparation of T cells and dendritic cells

BALB/c mice were subcutaneously immunized twice with CHP-HER2 complexes (truncated HER2 protein 20 μg and CHP 400 μg) at a one-week interval. Spleen cells were obtained one week after the last immunization. For preparation of T cell subpopulations, spleen cells were enriched using nylon fiber columns followed by treatment with anti-Lyt2.2 (CD8) monoclonal antibody (mAb) or anti-L3T4 (CD4) mAb and low-toxicity rabbit complement (Cedarlane, Ontario, Canada) to obtain $CD4^+$ T cells and $CD8^+$ T cells, respectively.

Bone marrow-derived dendritic cells (DCs) were prepared from normal BALB/c bone marrow as described by Inaba et al. [10] with minor modifications. Briefly, single bone marrow cell suspensions were obtained from femurs and tibias, then depleted of lymphocytes, granulocytes, and Ia^+ cells using a mixture of monoclonal antibodies (mAbs) (anti-CD4, anti-CD8, anti-B220/CD45R, and anti-Ia) for 45 min on ice, followed by incubation with low-toxicity rabbit complement for 30 min at 37 °C. Cells were resuspended at a concentration of 10^6 cells/mL of RPMI 1640 medium supplemented with recombinant murine granulocyte/macrophage colony-stimulating factor (rmGM-CSF) 10 ng/mL and were plated at 3 mL per well in six-well plates. Floating cells were removed on days 3 and 5 of culture by gentle pipetting and fed with fresh medium. On day 7 of culture, nonadherent and slightly adherent cells were collected for experiments. The phenotype of DCs was analyzed by FACScan flow cytometry.

T cell proliferation assay

Nylon fiber-purified suspensions of $CD4^+$ T cells or $CD8^+$ T cells from immunized mice were plated into 96-well U-bottom microtiter plates at 3×10^5 cells per well and used as responder cells. DCs pretreated with CHP-HER2 complex or control CHP-CAB complex for 3 h, or untreated DCs were added as stimulator cells at an R:S ratio of 40:1 followed by 18-h culture. After incubation in RPMI 1640 supplemented with 10% FCS and 2-mercaptoethanol 5×10^{-5} M at 37 °C in a 5% CO_2 atmosphere for 90 h, cells were labeled with ^{3}H-thymidine 1 μCi per well during the last 18 h of culture, and proliferation was determined using microplate scintillation counter. The results are presented as the mean of duplicate experiments.

Results

Induction of HER2-specific $CD8^+$ CTLs by in vivo priming with CHM- or CHP-HER2 complexes

We have recently demonstrated that HER2 can be a target for tumor-rejecting immune responses against syngeneic murine $HER2^+$ tumor cells [13]. We defined two different peptides, HER2p63-71 and HER2p780-788, that bind with K^d, one of which is of murine MHC class I that can induce $CD8^+$ CTLs. The growth of $HER2^+$ syngeneic tumors was suppressed in mice immunized with HER2p63-71 or -p780-788. Based on these findings, we prepared a truncated HER2 protein with 147 *N*-terminal amino acids which contain murine K^d-restricted CTL epitopes of HER2p63-71.

BALB/c female mice were subcutaneously immunized twice at a one-week interval with HER2 protein 20 μg complexed with cholesteryl group-bearing polysaccharides. Spleen cells were obtained from immunized and control animals one week after the second immunization and sensitized in vitro with mitomycin C-treated CMS17HE as described in the Materials and methods section. Killer cell activity specific for HER2-expressing target cells was induced from spleen cells of the animals immunized with complexes of cholesteryl group-bearing polysaccharide (CHM or CHP), and HER2 protein. No HER2-specific killer activity was induced from spleen cells derived from the mice immunized with complexes of cholesterol-bearing polysaccharides and control protein CAB, CHM or CHP alone, HER2 protein alone, or CAB protein alone [2]. The specific killer activity induced in animals immunized with the CHM-HER2 complex was blocked in vitro with mAbs against CD3, CD8, or K^d. In contrast, mAbs against CD4, I-Ad, Ld, or Dd did not show any significant blocking activity. The results suggest that the induced killer cells are $CD8^+$ CTLs with K^d restriction.

We asked whether CTLs induced in BALB/c mice primed with the CHM-HER2 complex can also recognize HER2p63-71 peptide, since the truncated HER2 protein consists of 147 amino acids from the *N*-terminus. P1.HTR cells pulsed with peptides were subjected to a ^{51}Cr release assay using CTLs derived from mice immunized with CHM-HER2. The CTLs specifically lysed P1.HTR pulsed with HER2-derived peptide p63-71, but not P1.HTR pulsed with other peptides with K^d binding motifs or nonpulsed target cells [8].

Immunization with the CHP-HER2 complex is therapeutically effective against HER2-expressing tumors

BALB/c mice inoculated with HER2-expressing CMS7HE tumor cells 2×10^6 were given weekly immunizations with protein of CHP-HER2 complex 20 μg starting on the day of the challenge or 3, 7, or 14 days after tumor challenge. Complete tumor rejection was observed when the immunization was initiated either on the day of tumor challenge or on day 3 after primary tumor challenge (Fig. 1). When the immunization was started 7 or 14 days after tumor inoculation, only marginal suppression of tumor growth was observed without complete rejection.

$CD8^+$ T cells are the major in vivo effector cells for the rejection of $HER2^+$ tumors

We sought to determine whether $CD8^+$ T cells were also the effector cells in vivo for the prevention of HER2-expressing tumor cell growth. After the second immunization with CHM-HER2, the mice were administered mAbs against CD4, CD8, or their combination one day before tumor challenge. The suppression of tumor growth was abolished by the administration of mAbs against CD8, or the combination of mAbs against CD4 and CD8, but not by the administration of mAbs against CD4 alone.

DCs can incorporate CHP-HER2 complex and specifically stimulate $CD8^+$ $CD4^+$ T cells

To investigate whether bone marrow-derived DCs could incorporate CHP-HER2 complex and stimulate T cells by providing the cognate target peptides, BALB/c mice were immunized twice with CHP-HER2 complex at a one-week interval. One week after the last immunization, $CD4^+$ and $CD8^+$ T cell subpopulations were

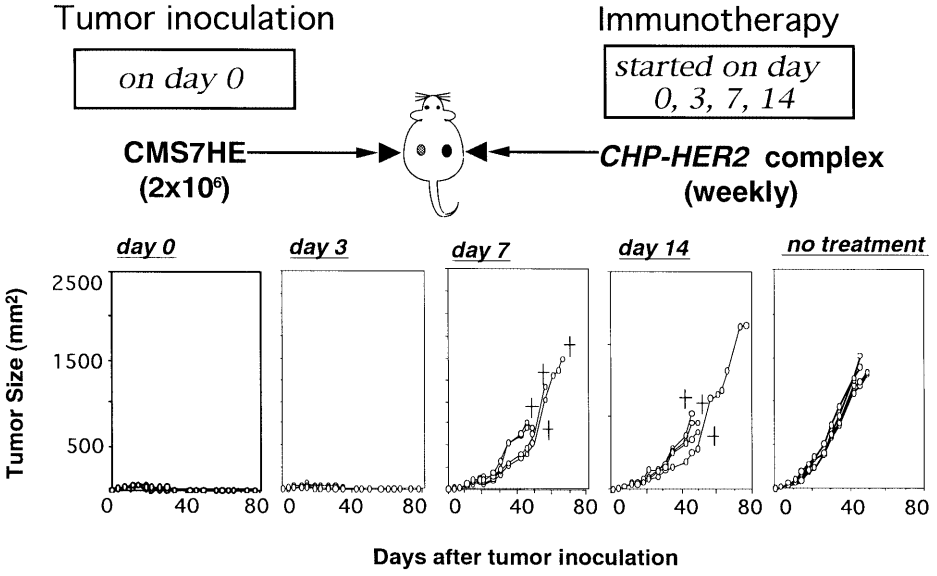

Fig. 1 Therapeutic effect of CHP-HER2 vaccine. BALB/c mice were challenged with CMS7HE 2×10^6 sc and weekly received CHP-HER2 complex containing protein 20 μg starting on the day of challenge, or 3, 7, or 14 days after. Each group consisted of four mice. Each line represents a single mouse

prepared. DCs cultured with CHP-HER2 complex or a control CHP-CAB complex for 3 h, or untreated DCs were used as antigen-presenting cells to stimulate T cells. Both $CD4^+$ and $CD8^+$ T cells showed a significantly stronger response to DCs treated with CHP-HER2 complex than to DCs treated with CHP-CAB or to DCs without prior treatment (Fig. 2). The result shows that DCs can incorporate CHP-HER2 complex and present cognate peptides to both $CD4^+$ and $CD8^+$ T cells after appropriate processing.

Experimental cell therapy using DCs pretreated with CHP-HER2 complex

We further examined whether DCs treated ex vivo with CHP-HER2 complex could be used as a vaccine against HER2-expressing tumor cells. CMS7HE cells 2×10^6 sc were inoculated into BALB/c mice. Ten days after inoculation, vaccination with DCs 4×10^5 sc pretreated with CHP-HER2 complex or CHP-CAB control complex, or DCs without treatment was started on a weekly basis. In the group of four mice vaccinated with CHP-HER2 complex-pretreated DCs, complete eradication of tumor was observed in all (Fig. 3). In contrast, tumor growth in mice in groups either treated with DCs pretreated with CHP-CAB control complex or DCs alone was similar to the tumor growth observed in mice without vaccination.

Discussion

Lysis of tumor cells by $CD8^+$ CTLs has been demonstrated to be an important effector mechanism of tumor defense and rejection. They destroy target cells by

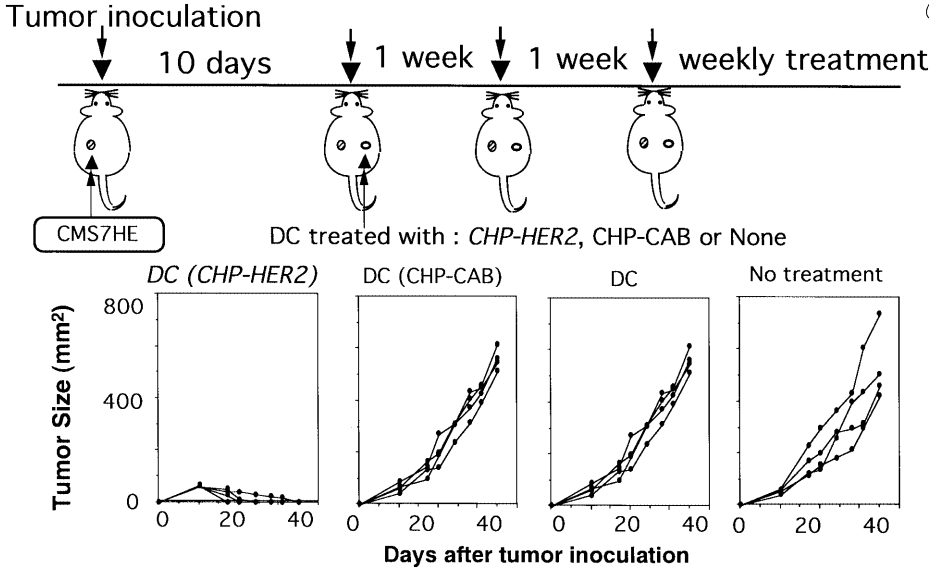

Fig. 2 Bone marrow-derived DCs demonstrate a potent antigen-presenting cell function. DCs pretreated with CHP-HER2 complex, control CHP-CAB complex, or untreated DCs were used as stimulator cells. Responder $CD4^+$ and $CD8^+$ T cells were obtained from nylon fiber-purified spleen cells of mice immunized with CHP-HER2 complex. ^{3}H-TdR proliferation assay was performed. Both $CD4^+$ and $CD8^+$ T cells responded significantly only to CHP-HER2 complex-pretreated DCs

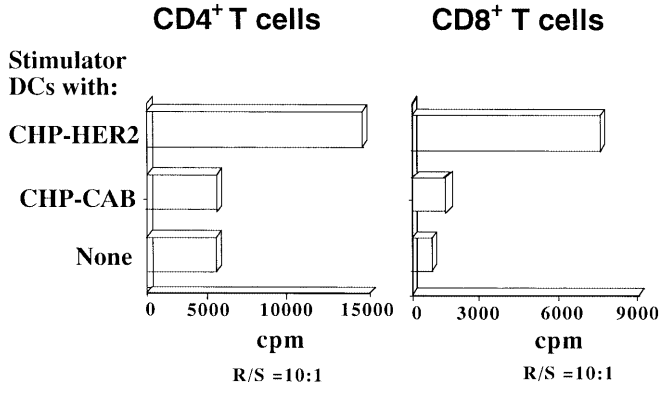

Fig. 3 Therapeutic effect of DCs pretreated with CHP-HER2 complex. BALB/c mice were challenged with CMS7HE 2×10^6 sc. Vaccination with 4×10^5 DCs pretreated with CHP-HER2 complex, CHP-CAB control complex, or DCs with no prior treatment was started on day 10 after tumor challenge and continued on a weekly basis. Strong tumor suppression was only observed in the group of mice vaccinated with CHP-HER2 complex-pretreated DCs. Four mice were used for each experimental group. Each line represents a single mouse

recognizing epitope peptides in a MHC class I-restricted manner. In certain murine tumor systems, epitope peptides recognized by $CD8^+$ CTLs have been identified, and the feasibility of vaccination with those peptides either for the prevention or treatment of tumors has been demonstrated [9, 12, 13].

However, immunization with a single epitope containing one anchor motif occasionally results in weak and limited immunological responses [16]. Soluble proteins with epitope peptides may be alternative candidates for vaccines. By receiving more stimulation from various types of potential CTL and T helper epitopes with different anchor motifs, protein antigens may have greater potential to elicit immunological responses in a wider range than single peptides, and therefore the protein-induced immunity may be stronger than the immunity induced by a single-epitope peptide. Although the use of recombinant soluble proteins containing epitope peptide sequences is a promising approach, failure to induce specific $CD8^+$ CTL activity by immunizing hosts with such exogenous protein molecules has been experienced repeatedly [16]. Investigators have been looking for ways to overcome these problems by novel approaches to induce MHC class I-restricted CTL activity using recombinant proteins.

In our present study, the HER2 protein required hydrophobized polysaccharides for the induction of a CTL response and the rejection of HER2-expressing tumor cells in vivo. These CTLs were K^d-restricted $CD8^+$ T lymphocytes specifically recognizing the HER2-derived peptide p63–71, part of the HER2 protein used for the vaccine, indicating that a soluble oncoprotein could enter the MHC class I pathway with the help of CHM or CHP.

The CHP-HER2 complex was revealed to be therapeutically potent. When mice were immunized with either CHP-HER2 complex they generated both $CD4^+$ and $CD8^+$ T cells specifically reactive with DCs pretreated with CHP-HER2 complex. It is interesting that in the animals either immunized with CHP-HER2 complex or HER2 protein alone, DCs pretreated with CHP-HER2 complex, but not HER2 protein alone, strongly stimulated specific $CD8^+$ T cells. These results show that DCs, whether pretreated with CHP-HER2 complex or HER2 protein alone, can incorporate and process the antigen peptides and finally present them to $CD4^+$ T cells.

Having established that bone marrow-derived DCs can efficiently stimulate both $CD4^+$ and $CD8^+$ T cells, we examined their usefulness for immunotherapy of HER2-expressing tumors. After mice were inoculated with CMS7HE 10 days prior to immunization, obvious suppression of tumor growth was observed in the group utilizing DCs pretreated with CHP-HER2 complex. A nonspecific adjuvant effect of CHP seems unlikely because DCs treated with CHP-CAB control complex showed no tumor suppression when compared with mice without immunization. It is of particular interest that in four mice immunized with CHP-HER2 complex-pretreated DCs, complete tumor eradication was observed in all. In our experience, after immunization either with CHP-HER2 complex or CHM-HER2 complex, complete tumor suppression was possible only when immunization was initiated less than 4 days after tumor inoculation.

The present data strongly suggest that CHP-HER2 complex can be used as a cancer vaccine in concert with bone marrow-derived DCs for immunological cell therapy. Since murine K^d and human HLA-A24 share a similar anchor motif for peptides, HER2p63–71 and HER2p780–788 were examined for induction of CTLs in $HLA\text{-}A24^+$ individuals (manuscript in preparation). Human $CD8^+$ CTL clones specific for these peptides were established and they lysed $HER2^+$ tumor cells in an HLA-A24-restricted manner. These results indicate the immediate usefulness of CHP-HER2 complex for clinical cancer immunotherapy, since the identical target antigen peptide HER2p63 is recognized by both murine and human CTLs (manuscript in preparation).

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