Essential roles of tumor-derived helper T cell epitopes for an effective peptide-based tumor vaccine

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Abstract

In this study, we identified a c-erbB-2/HER2/neu (HER2) derived Th epitope (HER216-30) and examined the role of Th epitopes in HER2-specific CD8+ T cell induction and in vivo tumor eradication, with a particular emphasis on the role of tumor cell-derived Th epitopes. Immunization of BALB/c mice using a mixture of Th epitope HER216-30 and CTL epitope HER263-71 administered subcutaneously with murine GM-CSF (mGM-CSF) induced a much higher level of HER263-71-specific CD8+ T cells compared with that obtained with the CTL epitope alone. HER2-unrelated OVA-derived Th epitope (OVA323-339) exhibited a similar enhancing effect on HER263-71-specific CD8+ T cell induction. However, only mice immunized with HER216-30 and HER263-71, but not with a tumor-unrelated OVA323-339 and HER263-71, showed in vivo eradication of CMS5mHE tumor cells expressing HER2 but not OVA. This distinction was observed in preventative as well as therapeutic experimental settings. Conversely, both HER216-30 and OVA323-339 Th epitopes were equally effective in inducing the eradication of CMS5mHEOVA tumor cells which express HER2 as well as OVA. Our results clearly indicate that CTL and Th epitopes of target tumor cell origin should be used for effective induction of in vivo antitumor immunity.

Introduction

CD4+ T cells have been considered to help activate CD8+ CTLs by providing cytokines, such as IL-2 which is essential for the proliferation of CD8+ CTLs. CD4+ Th is thought to encounter CD8+ CTLs via APCs, such as DCs, providing cognate peptides to both CD4+ and CD8+ T cells which are in close contact, even when not in direct interaction (1, 2, 3). A recent report, however, has proposed an alternative view for the CD4+ Th-enhanced activation of CD8+ CTLs. CD4+ Th recognizes MHC class II binding cognate peptides on APCs and their interaction may result not only in the activation and priming of CD4+ T cells, but also in the activation of APCs.
themselves (4, 5, 6). Consequent to these mutual activations, APCs prime and activate CD8+ CTLs specific for the tumor Ag peptides. This new scenario of cellular interaction proposes that CD4+ and CD8+ T cells may not necessarily be in direct or close association. It is still not clear whether these two different models are mutually exclusive, but both are probable. Based on the understanding of these cellular interactions for the priming and activation of CD4+ and CD8+ T cells, it is important to know the nature of the Ag molecules recognized by the CD4+ and CD8+ T cells involved in antitumor immune responses. In fact, there are ongoing clinical trials utilizing either heterologous helper Ags, such as keyhole limpet hemocyanin (KLH), or tumor-derived helper Ags in order to augment antitumor immune responses by CD8+ CTLs directed against tumor-derived CTL epitopes (7, 8, 9, 10). While it is obvious that CD8+ T cells recognize MHC class I binding peptides derived from tumor target cells based on the subsequent tumor-specific destruction, peptide derivation from tumor cells may not be an absolute requirement for CD4+ T cells since they do not directly interact with tumor cells that mostly lack MHC class II expression. This notion has become extremely important in the design of future vaccines aimed at the efficient activation of both T cell populations involved in antitumor immune responses.

HER2 is often overexpressed in a variety of human cancers with limited expression in normal adult tissues, and has thus been studied extensively as a tumor vaccine candidate (11, 12). As reported previously, we identified a HER2-derived CTL epitope, HER263-71, restricted to murine MHC class I Kd and also to human HLA-A2402 that possesses a peptide-anchoring motif similar to Kd (13, 14, 15). This provides an ideal model to examine murine T cell immune responses to HER2 and HER2-expressing tumors in parallel with human responses. We also prepared a truncated HER2 protein containing 146 aa residues from the N-terminus (HER2-146). By complexing this polypeptide with a cholesteryl-bearing hydrophobized polysaccharide pullulan (CHP), we showed that immunization of BALB/c with these CHP-HER2 complexes induced CTLs specific for HER263-71 and IgG Abs specific for truncated HER2 protein (16, 17, 18). We also reported that vaccination of animals with cDNA plasmids encoding the above-mentioned truncated HER2 protein also resulted in the generation of CTLs and IgG Abs with similar specificities (19). The production of IgG Abs specific for the truncated HER2 protein indicates that the Th cells required for the class switch for the production of IgG Ab molecules were induced in both immunization approaches.

The present study is an extension of the above work (13, 14, 15, 16, 17, 18, 19) and is designed to determine the Th epitopes within the 146 aa residues of the N-terminal of the HER2 protein. We then examined the significance of the defined Th epitopes in terms of CD8+ T cell generation and tumor eradication, with particular emphasis on the necessity of tumor-cell derived Th epitopes.

Results

Determination of the HER216-30 Th epitope

BALB/c mice were immunized with CHP-HER2 complexes as described in Materials and Methods. Several CD4+ T cell clones reactive with the CHP-HER2 complex were established from the popliteal LN cells of immunized mice. One clone, A6, was reactive with HER216-40 in the initial screening of peptides (Figure 1A). Further analysis with shorter synthetic peptides indicated that the A6 clone was most reactive with HER216-30 with MHC class II I-Ad restriction (Figure 1, A and B). The cytokine production profile of the A6 clone was compatible with the Th1 type, with production of IFN-gamma but not IL-4 (Figure 1C).
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Figure 1. Identification of an epitope recognized by CD4+ Th clone A6. (A) Determination of HER2_{16-30} recognition by clone A6. A series of synthetic peptides were added together with irradiated BALB/c spleen cells and clone A6. Incorporation of ^3H-thymidine was examined after a 3-day incubation. A 15-aa peptide, HER2_{16-30}, was defined as the minimal T cell epitope. (B) MHC class II restriction of T cell clone A6 recognition. Clone A6 cells were co-cultured with irradiated BALB/c spleen cells and HER2_{16-30} in the presence of various Abs and proliferation assays performed. Clone A6 recognition of HER2_{16-30} was specifically blocked by anti-CD4 and anti-I-A^d mAb. (C) IFN-gamma production by clone A6. Culture supernatants of clone A6 cells stimulated by HER2_{16-30} were tested for IFN-gamma and IL-4 release. Clone A6 is similar to Th1 type with regard to production of IFN-gamma, but not IL-4.

The HER2_{16-30} Th epitope augments HER2_{63-71}-specific CD8+ T cell induction in the presence of mGM-CSF

HER2_{63-71} peptide is a MHC class I K^d binding nonamer CTL epitope reported previously by our laboratory (13). BALB/c mice immunized with HER2_{63-71} or HER2_{16-30} alone generated no HER2_{63-71}-specific CD8+ splenic cells counted in an ELISPOT assay (data not shown). BALB/c mice were therefore pre-immunized twice with HER2_{16-30}, followed by two immunizations with HER2_{63-71} alone or combinations of HER2_{16-30} and HER2_{63-71}. Immunization with HER2_{63-71} alone failed to induce HER2_{63-71}-specific CD8+ T cells (Figure 2). When HER2_{63-71} was used for immunization together with HER2_{16-30} in the absence of mGM-CSF, a few HER2_{63-71}-specific CD8+ T cells were induced. A marked increase in the number of induced HER2_{63-71}-specific CD8+ T cells was observed when mGM-CSF was administered in addition to peptide immunization. Immunization with HER2_{16-30} and HER2_{63-71} in the presence of mGM-CSF resulted in the highest number of HER2_{63-71}-specific CD8+ T cells (Figure 2). The augmentation of HER2_{63-71}-specific CD8+ T cell generation by HER2_{16-30} was totally cancelled by treating the mice with anti-CD4 mAb, indicating that the increase seen with the Th epitope was CD4+ T cell dependent (data not shown).
**Figure 2.** HER2\textsubscript{16-30} augments HER2\textsubscript{63-71}-specific CD8\textsuperscript{+} T cell induction in the presence of mGM-CSF. Peptides (10 µg), either individually or in combination, were administered s.c. on the back of BALB/c mice in the presence (+) or absence (-) of mGM-CSF (1 µg/shot). One week after the last immunization, splenic CD8\textsuperscript{+} T cells were prepared and an ELISPOT assay was performed to determine the number of IFN-gamma positive spots as described in Materials and Methods. Target cells were HER2\textsubscript{63-71} (solid bars) or control HER2\textsubscript{780-788} (open bars) pulsed with P1.HTR. The data shown are the mean ± SEM of three independent experiments.

**OVA\textsubscript{323-339} and HER2\textsubscript{16-30} exhibit equivalent helper effects in the induction of HER2\textsubscript{63-71}-specific CD8\textsuperscript{+} T cells**

An OVA-derived Th epitope, OVA\textsubscript{323-339}, was reported to induce Th with I-A\textsuperscript{d} restriction similarly to HER2\textsubscript{16-30} (20). We therefore examined the helper activity of OVA\textsubscript{323-339} in the generation of HER2\textsubscript{63-71}-specific CD8\textsuperscript{+} T cells. As shown in Figure 3, a comparable number of HER2\textsubscript{63-71}-specific CD8\textsuperscript{+} T cells were induced following immunization of mice with the HER2\textsubscript{63-71} CTL epitope and either of the two Th epitopes.

**Figure 3.** Two Th epitopes augment HER2\textsubscript{63-71}-specific CD8\textsuperscript{+} T cell immune responses equivalently. Pre-immunization of the mice twice with either of the two Th epitopes was followed by two immunizations with the corresponding Th epitope and HER2\textsubscript{63-71}. An essentially equivalent increase in the number of HER2\textsubscript{63-71}-specific CD8\textsuperscript{+} T cells was observed with the two Th epitopes. The data shown are the mean ± SEM of three independent experiments.
The HER2<sub>16-30</sub> Th epitope provides a stronger helper effect in preventing and eradicating HER2 expressing tumors

We then examined the efficacy of the helper effect by using two different Th epitopes in eradicating a HER2-expressing syngeneic tumor, CMS5mHE. BALB/c mice received 4 weekly immunizations with peptides and mGM-CSF before i.v. administration of 1x10<sup>6</sup> CMS5mHE cells (the immunization protocol is described in Materials and Methods). Immunization of mice with HER2<sub>63-71</sub> peptide alone did not reduce the number of metastatic pulmonary nodules. In contrast, mice immunized with HER2<sub>63-71</sub> and HER2<sub>16-30</sub> showed a marked decrease in pulmonary metastases. On the other hand, a much lower reduction in metastasis was seen in mice immunized with HER2<sub>63-71</sub> and OVA<sub>323-339</sub> (Figure 4A). We then tried to eradicate the metastatic nodules by the therapeutic use of peptide immunization. Peptide vaccination was initiated 4 days after inoculation with tumor cells and repeated on a weekly basis. Similar to the above experiments, HER2<sub>16-30</sub> but not OVA<sub>323-339</sub> showed a significant Th effect, as evidenced by the marked reduction in pulmonary metastatic nodules (Figure 4B).

![Figure 4. Augmentation of in vivo CMS5mHE tumor eradication by HER2<sub>16-30</sub>, but not OVA<sub>323-339</sub>.](http://www.cancerimmunity.org/v3p16/031116.htm) (A) Pre-tumor cell injection. One week after the last peptide immunization described in Materials and Methods, mice were challenged i.v. with 1x10<sup>6</sup> CMS5mHE tumor cells. Twenty days after tumor inoculation, lung metastatic foci were counted under a dissecting microscope. The number of metastatic foci in mice immunized with OVA<sub>323-339</sub> or HER2<sub>16-30</sub> in combination with HER2<sub>63-71</sub> was less than in mice immunized with HER2<sub>63-71</sub> alone or in untreated mice. In mice immunized with HER2<sub>16-30</sub> and HER2<sub>63-71</sub>, markedly fewer metastatic foci were counted than in mice immunized with OVA<sub>323-339</sub> and HER2<sub>63-71</sub> alone. (B and C) Post-tumor cell injection. Four days after i.v. inoculation with 1x10<sup>6</sup> CMS5mHE tumor cells, mice were immunized with peptide on a weekly basis. Results similar to A were observed. Animal survival was also monitored up to 50 days following tumor inoculation. Only mice treated with HER2<sub>16-30</sub> and HER2<sub>63-71</sub> survived throughout the observation period. The number of foci represents the mean ± SD of five mice per group in A and B.
In similar experiments carried out to see the therapeutic effects of peptide immunization, the helper effect of HER2<sub>16-30</sub> was also evident in the prolongation of the survival time observed (Figure 4C). No animal died in the HER2<sub>16-30</sub> and HER2<sub>63-71</sub> immunization group. In contrast, the survival time of mice immunized with HER2<sub>63-71</sub> alone, or HER2<sub>63-71</sub> and OVA<sub>323-339</sub>, was not different from that of the control group (no treatment).

The OVA<sub>323-339</sub> and HER2<sub>16-30</sub> Th epitopes exhibit equivalent helper effects in eradicating syngeneic tumors co-expressing HER2 and OVA

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**Figure 5. Protective and therapeutic immunity elicited by peptide vaccination against CMS5mHEOVA.** The immunization protocol was identical to that described in Figure 4 except that CMS5mHEOVA cells were used. Lung weight was measured as an indication of tumor metastasis, and the presence of metastases was checked visually as shown in the photographs. (A) Pre-tumor injection. CMS5mHEOVA tumor cells were challenged after peptide immunization. In mice immunized with OVA<sub>323-339</sub> or HER2<sub>16-30</sub> in conjunction with HER2<sub>63-71</sub>, no increase in lung weight was observed compared with the lung weight of age-matched naive mice, indicating complete prevention of metastasis. In mice immunized with HER2<sub>63-71</sub> alone, lung weight increased moderately. (B) Post-tumor injection. Peptide immunization similar to that described in Figure 4B was performed, followed by CMS5mHEOVA tumor inoculation. The results were essentially similar to those described in A. Data represent the mean ± SD of five mice per group in A and B. (C) Post-tumor injection. Survival was monitored up to 90 days after tumor inoculation in mice that received weekly peptide immunization. All mice treated with OVA<sub>323-339</sub> or HER2<sub>16-30</sub> in conjunction with HER2<sub>63-71</sub> were alive at the end of the observation period. A limited prolongation of survival time was observed in mice treated with HER2<sub>63-71</sub> alone.
Finally, we examined the importance of tumor-derived Th epitopes in eradicating pulmonary metastases. For this purpose, we prepared CMS5mHE cells transduced with the OVA gene, CMS5mHEOVA. The presence of pulmonary metastases was determined by weighing the lungs (since the metastatic nodules formed large masses) rather than counting individual nodules in the CMS5mHEOVA tumors, as shown in the photographs in Figure 5. In the first set of experiments, the mice were immunized with peptides and mGM-CSF as described for the experiments with CMS5mHE, followed by CMS5mHEOVA tumor challenge. In mice immunized with OVA323-339 or HER216-30 in conjunction with HER263-71, no increase in lung weight was observed compared with age-matched naive mice, in contrast to the lack of effect of OVA323-339 or HER216-30 when given alone. Immunization with HER263-71 alone reduced metastases and lung weight to some extent (Figure 5A). In the second set of experiments, mice were immunized with peptide 4 days after tumor challenge, and repeated doses of the vaccine were administered weekly. Similar to the above results, immunization with HER263-71 and either OVA323-339 or HER216-30 was the most effective approach in eradicating pulmonary metastases whereas each peptide was ineffective when used alone. Immunization with HER263-71 alone was marginally effective in eradicating pulmonary masses (Figure 5B). We also studied the therapeutic effects of peptide immunization by monitoring survival time for up to 90 days after tumor inoculation. None of the mice immunized with HER263-71 combined with HER216-30 or OVA323-339 died during the observation period. In contrast, treatment of mice with OVA323-339 or HER216-30 alone had no effect on survival, while limited prolongation of survival time was observed in mice treated with HER263-71 alone (Figure 5C).

Discussion

In this study using HER2 as a tumor Ag in a murine model, we investigated the contribution of the HER216-30 Th epitope in the generation of HER263-71-specific CD8+ T cells. Repeated administrations of HER263-71 alone in conjunction with mGM-CSF at weekly intervals resulted in the generation of HER263-71-specific CD8+ T cells not exceeding approximately 50/2x10^5 CD8+ splenic T cells (data not shown). The addition of the HER216-30 Th epitope to the CTL epitope resulted in a marked increase in HER263-71-specific CD8+ T cells depending on the number of immunizations. The effects of the Th epitope were cancelled by in vivo administration of the anti-CD4 mAb GK1.5, indicating a CD4+ T cell dependency of the Th effects (data not shown). The HER2-unrelated Th epitope OVA323-339 showed a similar degree of enhancement of CD8+ T cell induction. These results indicate that two Th epitopes, regardless of the difference in their molecular origin, exhibit an equivalent helper activity for in vivo CD8+ T cell generation. Quite different outcomes were observed between the two groups of mice immunized with either OVA323-339 or HER216-30 in terms of in vivo tumor eradication. By immunizing mice with the HER216-30 Th and HER263-71 CTL epitopes, both of HER2 origin, before or after inoculation with HER2-expressing CMS5mHE tumor cells, the tumor disappeared completely. In contrast, replacement of the Th epitope with the tumor-unrelated OVA323-339 epitope resulted in persistence of the tumors when immunization was performed either before or after inoculation with tumor cells. More importantly, in similar experiments using the CMS5mHEOVA tumor line that co-expresses HER2 and OVA, Th epitopes derived from both molecules were equally effective in eradicating the tumor. In either tumor system, immunization with the CTL epitope alone resulted in marginal tumor eradication, indicating the need for Th epitopes for effective in vivo tumor eradication. Taken together, effective tumor eradication was achieved only when tumor-derived Th epitopes were used for immunization in conjunction with the cognate CTL epitope.
In this respect, the results of our study contrast sharply with those reported recently by Casares et al. (21). In their study, they demonstrated that a tumor-unrelated Th epitope, the same OVA-derived peptide used in our study, was equally effective in tumor-specific CTL induction as well as in tumor eradication. Among many, a notable difference between the two systems is the adjuvant used. Casares et al. used IFA while we employed mGM-CSF in our study (22). mGM-CSF at the dose used in our study might be short-acting due to its short half-life in vivo and is effective only within a short range. In contrast, Freund's adjuvant was developed to retain Ags locally and to cause persistent stimulation of lymphocytes by the slow release of the corresponding Ags. Thus, in the system employed by Casares et al. (21), OVA-derived Th plus tumor-specific CTL epitopes emulsified in IFA may have continuously stimulated the clonal expansion of the CTLs with the help of OVA-reacting Ths, thus maintaining the CTLs at a sufficient number to reject the Th epitope-unrelated tumor cells. On the other hand, in our model, OVA-specific Ths that had been induced by intermittent stimulation could not be activated after inoculation with HER2-expressing CMS5mHE tumor cells because neither OVA, nor its derivative peptide, nor mGM-CSF was continuously supplied.

Accordingly, when mice were immunized with Th and CTL epitopes of HER2 origin, the HER2\textsubscript{63-71}-specific CD8+ T cells could expand with the aid of HER2\textsubscript{16-30}-reactive Ths until the challenging HER2-expressing tumor cells were rejected. It is assumed that HER2 polypeptide was processed to produce HER2\textsubscript{16-30} peptide and that it was presented on the MHC class II molecules of APCs adjacent to tumor cells. Thus, the HER2-expressing tumor could continually recruit Th and CD8+ T cells as a consequence of the cross-presentation of HER2 molecules until the tumor cells were completely eradicated. However, in mice whose CD8+ T cells had been expanded by HER2-unrelated OVA peptide, without prior expansion by HER2-derived Th epitopes, the tumor cells expressing HER2 alone seemed to be too weak or too late to prime a sufficient number of Ths for the expansion of HER2-specific CD8+ T cells, resulting in lethal tumor metastases. Since both OVA\textsubscript{323-339} and HER2\textsubscript{16-30} Th epitopes are equally effective in the HER2\textsubscript{63-71} CTL epitope-dependent tumor eradication of CMS5mHEOVA, derivation of Th epitopes from tumor cell-derived molecules, HER2 or OVA, but not necessarily from the HER2 molecule providing CTL epitope HER2\textsubscript{63-71}, is sufficient.

The number of human tumor cell-derived Th epitopes is still limited for preparing vaccines that can cover a wide range of cancers (23). Taking these results into consideration, CTL and Th epitopes originating from the targeted tumor cell should be used for peptide-based vaccines for effective antitumor immunity. A widely used serological analysis of recombinant cDNA expression (SEREX) has identified a variety Ag molecules derived from human cancer cells that induce IgG Ab production in patients with cancer and therefore are likely to be recognized by CD4+ Th. We have recently reported that co-immunization of SEREX-defined Ags with CTL epitopes lead to enhanced CTL activity, as well as \textit{in vivo} tumor rejection in a CD4+ T cell-dependent manner (24). Promising candidate peptides for peptide-based vaccines could be selected from such SEREX-defined Ags. A public database that includes SEREX-defined Ags is currently available on the Internet (25).

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**Abbreviations**

HER2, c-erbB-2/HER2/neu; CHP, cholesteryl-bearing hydrophobized polysaccharide pullulan; RT, room temperature
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References


25. Cancer Immunome Database. URL: http://www2.licr.org/CancerImmunomeDB


Materials and methods

Animals and tumor cell lines

Female BALB/c mice (6- to 10-week-old) purchased from the Shizuoka Animal Laboratory Center (Shizuoka, Japan) were used in all experiments. CMS5mHE is a transfectant obtained by transfecting human HER2 cDNA
into CMS5m, a HER2-negative sarcoma line of BALB/c origin (26). CMS5mHEOVA is a cell line co-transfected with HER2 and OVA cDNA. P1.HTR is a subline of the P815 mastocytoma cell line of DBA/2 origin (27).

**Antibodies and cytokines**

Anti-CD4 (GK1.5), anti-CD8 (19/178), anti-H-2Kd (20-8-4), anti-H-2Dd (34-5-8S), anti-I-Ad (MKD-6) and anti-I-Ed (14-4-4S) mAbs were produced in 8- to 10-week-old BALB/c nude mice by injecting the respective hybridoma cells (28). mGM-CSF was kindly produced by Kirin Brewery Co., Ltd. (Tokyo, Japan).

**Generation of Th clones**

The CHP-HER2 complex was prepared as described previously (16). Briefly, the truncated HER2 recombinant protein encompassing the N-terminal 146 aa residues of HER2 was dissolved in 6 M urea. The protein solution (2.0 mg/ml) was mixed with 2.1 ml of a suspension of CHP (5.7 mg/ml) at room temperature (RT), resulting in the formation of a CHP-HER2 complex (CHP: 5.0 mg/ml, protein: 0.25 mg/ml, 0.75 M urea). BALB/c mice were immunized s.c. in the footpads with CHP-HER2 complex (10 µg/mouse) emulsified in CFA (Difco Laboratories, Detroit, MI) as described previously (29). After 10 days, lymphocytes from the popliteal LNs were stimulated in vitro with CHP-HER2 complex in the presence of irradiated (35 Gy) syngeneic spleen cells as APCs. All the CD4+ T cell clones from the limiting dilution cultures were expanded and screened for sequence recognition using a series of 25-aa long peptides, starting from the N-terminus of the truncated HER2 and overlapping by 10-aa residues at their N- and C-terminus. These peptides were synthesized by Sawaday Technology (Tokyo, Japan).

**Proliferation assays**

Proliferation assays were carried out in 96-well flat-bottomed plates using CD4+ T clones as responder cells at 1x10^5/well. Irradiated spleen cells, 5x10^5/well, with 5 µg/ml of peptides or 1 µg/ml of CHP-HER2 complex were used as stimulators. After 3 days, the cultures were labeled with 1 µCi/well ^3H-thymidine during the last 12 h of culture and the incorporated radioactivity was determined by a micro-plate scintillation counter (Packard).

**ELISA**

Flat-bottomed 96-well micro-titer plates (Immuno-NUNC) were coated with 1.5 µg/ml of rat anti-mouse IFN-gamma or IL-4 mAb (PharMingen, San Diego, CA) at RT overnight. The wells were blocked with PBS containing 1% BSA for 1 h at RT. After washing three times with PBS containing 0.05% Tween 20, diluted culture supernatants were supplemented with 0.3 µg/ml of biotin-labeled detection anti-mouse IFN-gamma or IL-4 mAb (PharMingen) and incubated at RT for 2 h. After washing again, horseradish peroxidase-conjugated streptavidin was added to the well at a 1:12,000 dilution in PBS/2%BSA and incubated at RT for 30 min. After the final washing, 100 µl of 3,3’,5,5’-tetramethylbenzidine peroxidase substrate solution (Bio-Rad Laboratories, Hercules, CA) was added to each well. The color reaction was stopped with 0.18 M H_2SO_4, and the absorbance was measured at 405 nm.

**Immunization with peptides and mGM-CSF**

One immunization consisted of the following: BALB/c mice were injected s.c. at the same site on their backs with 1 µg of mGM-CSF for 3 consecutive days. On the second day, the mice were injected with a single peptide or different combinations of peptides (10 µg of each peptide/mouse) dissolved in mGM-CSF. Immunizations were repeated at 1 wk intervals.
Preparation of splenic CD8+ T cells

One week after the last immunization with peptides, CD8+ T cells were obtained by positive enrichment using MACS system (Minimacs; Miltenyi Biotec, Germany). In brief, splenocytes were labeled with CD8alpha (Lyt2) microbeads in PBS containing 0.5% BSA. After washing, the cells were applied on MS columns equipped with a MiniMACS magnet as specified by the supplier. T cell fractions were confirmed to contain more than 95% CD8+ T cells by flow cytometric analysis.

ELISPOT assays

ELISPOT assays were performed as described by Power et al. (30) with minor modifications. Briefly, a 96-well flat-bottomed nitrocellulose plate (MultiScreen-HA Plate, Cat. No. MAHAS4510; Millipore, Bedford, MA) was coated overnight at 4°C with 2 µg/ml anti-mouse IFN-gamma mAb (clone R4-6A2, PharMingen). The wells were washed with PBS and blocked with FCS-containing culture medium for 1 to 3 h at 37°C. Freshly isolated CD8+ T cells (2x10^5/well) from immunized mice and P1.HTR cells (1x10^5/well) pulsed with HER2 63-71 or control HER2 780-788 with the K^o binding motif (13) were plated into each well at a final volume of 200 µl. After incubation for 22 h at 37°C in a CO_2 incubator, the plate was then washed thoroughly with PBS-Tween, supplemented with 1.25 µg/ml biotinylated anti-mouse IFN-gamma mAb (PharMingen) and incubated overnight at 4°C. After washing with PBS-Tween, 1 µg/ml of alkaline phosphatase-conjugated streptavidin (Mabtech AB, Gamla, Sweden) was added to each well and the plate was incubated at RT for 90 min. Spots were developed by adding 100 µl of alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA) and the plate was prepared for microscopic counting. The number of IFN-gamma-positive spots per well was determined as the proportion of CD8+ T cells. Data are presented as the mean value per 2x10^5 CD8+ T cells of triplicate experiments.

In vivo immunization for tumor eradication

To study the preventive effects of peptide immunization, BALB/c mice were pre-immunized twice with one of the two different Th epitopes followed by two immunizations with HER2 63-71 alone or the corresponding Th epitope and HER2 63-71. One week after the last immunization, the mice were challenged by i.v. injection of 1x10^6 CMS5mHE or CMS5mHEOVA tumor cells. To study the therapeutic effects of peptide immunizations, mice were inoculated i.v. with 1x10^6 CMS5mHE or CMS5mHEOVA tumor cells. Four days later, s.c. vaccinations with a single peptide or different combinations of peptides were administrated once a week. Metastases were counted under a dissecting microscope or examined by weighing the lungs. Animal survival was also monitored.

Ethical considerations

The experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of Mie University.
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