


Novel peptide-based vaccine targeting heat shock protein 90 induces effective antitumor immunity in a HER2+ breast cancer murine model

Jinho Kang ¹, Hye-Jin Lee,¹ Jimin Lee,¹ Jinhwa Hong,¹ Yeul Hong Kim,¹ Mary L Disis,² Jeong-An Gim,³ Kyong Hwa Park¹

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¹Department of Internal Medicine, Korea University College of Medicine, Seoul, South Korea

²Department of Medicine, University of Washington, Seattle, Washington, USA

³Center for Research Support, Korea University College of Medicine, Seoul, South Korea

Correspondence to

Dr Kyong Hwa Park; khpark@korea.ac.kr

ABSTRACT

Background Heat shock protein 90 (HSP90) is a protein chaperone for most of the important signal transduction pathways in human epidermal growth factor receptor 2-positive (HER2+) breast cancer, including human epidermal growth factor receptor 2, estrogen receptor, progesterone receptor and Akt. The aim of our study is to identify peptide-based vaccines and to develop an effective immunotherapeutics for the treatment of HER2+ breast cancer.

Methods HSP90-derived major histocompatibility complex (MHC) class II epitopes were selected using in silico algorithms and validated by enzyme-linked immunospot (ELISPOT). In vivo antitumor efficacy was evaluated in MMTV*neu*-transgenic mice. HSP90 peptide-specific systemic T-cell responses were assessed using interferon gamma ELISPOT assay, and immune microenvironment in tumors was evaluated using multiplex immunohistochemistry and TCRβ sequencing.

Results First, candidate HSP90-derived MHC class II epitopes with high binding affinities across multiple human HLA class II genotypes were identified using in silico algorithms. Among the top 10 peptides, p485 and p527 were selected as promising Th1 immunity-inducing epitopes with low potential for Th2 immunity induction. The selected MHC class II HSP90 peptides induced strong antigen-specific T cell responses, which was induced by cross-priming of CD8⁺ T cells in vivo. The HSP90 peptide vaccines were effective in the established tumor model, and their efficacy was further enhanced when combined with stimulator of interferon genes (STING) agonist and/or anticytotoxic T lymphocyte-associated antigen-4 antibody in MMTV*neu*-transgenic mice. Increased tumor rejection was associated with increased systemic HSP90-specific T-cell responses, increased T-cell recruitment in tumor microenvironment, intermolecular epitope spreading, and increased rearrangement of TCRβ by STING agonist.

Conclusions In conclusion, we have provided the first preclinical evidence of the action mechanism of HSP90 peptide vaccines with a distinct potential for improving breast cancer treatment.

BACKGROUND

Heat shock protein 90 (HSP90) is a chaperone whose main function is to restore

stable conditions necessary for freshly produced proteins to have a normal structure.^{1 2} In cancer cells, several HSP90 client proteins are overexpressed that regulate key biological processes in tumor growth, cell division, and metastasis; such client proteins include protein kinases, transcription factors, cell cycle regulators, and hormone receptors.^{3 4} Various therapeutic stress during cancer treatment also induces the accumulation of immature client proteins, resulting in HSP90 overexpression required to maintain homeostasis of oncogenic proteins. In many cancers, high expression levels of HSP90 are associated with an aggressive phenotype⁵ and poor prognosis.¹ Thus, direct ATPase domain-targeted therapeutics, including geldanamycin, 17-AAG, 17-DMAG, IPI-504, and SNX-5422, have been developed,^{6 7} but their clinical application has been limited due to toxicity and low efficacy in early-phase clinical trials.

Intracellular HSP90 plays an essential role in antigen presentation by antigen-presenting cells (APCs) and the subsequent cross-priming of CD4 T cells.^{8 9} Additionally, extracellular HSP90 forms a complex with substrate peptide antigens and facilitates endocytosis.¹⁰ Due to this immunomodulatory role, HSP90 has been developed mainly as a carrier for tumor antigens in the development of cancer vaccines.¹¹ More recently, tumor-derived extracellular vesicles, including HSP90, have been studied as a new emerging therapeutic option.¹² However, these autologous tumor-derived, customized approaches are cost-intensive, require standardized facilities, and are time-consuming. Therefore, the development of safer and more accessible HSP90-directed immunotherapeutics is warranted.

Cancer vaccines developed on different platforms are now accepted as safe and effective approaches for both cancer prevention and treatment.^{13–14} Immunization with T helper (Th)1-directed vaccines for overexpressed tumor-associated antigens (TAAs) has been shown to be effective in clinical trials.^{15–16} To be used for therapeutic purposes in patients with advanced cancer, rational identification of Th1-directed epitopes and optimal strategies for combination are necessary. There is some evidence showing that HSP90 induces an autologous immune response in patients with cancer.^{17–19} Notably, high levels of HSP90 autoantibodies were observed in the serum of patients with advanced stages of breast and ovarian cancers. Here, we analyzed whether HSP90 is immunogenic in breast cancer and whether active immunization has therapeutic effect in advanced-stage cancer.

MATERIALS AND METHODS

Patient samples

Control serum samples were obtained from healthy female donors (n=25) with no previous history of cancer. Breast cancer samples were donated also by patients treated for three representative subtypes of breast cancers (human epidermal growth factor receptor 2-positive (HER2+), triple-negative, and estrogen receptor-positive (ER+)/human epidermal growth factor receptor 2-negative (HER2–)) at the Korea University Anam Hospital (approval number: 2016AN0090; total n=82: 25 ER+/HER2–, 25 triple-negative, 32 HER2+ samples). All sera were aliquoted and stored at –80°C. For human T-cell response studies, peripheral blood mononuclear cells (PBMCs) were taken with informed consent from 10 patients with breast cancer and 10 healthy donors. The cells were isolated and cryopreserved, as previously described.²⁰ PBMC samples were obtained from the General Clinical Research Center Facility at the University of Washington (approval number: UL1 TR002319).

Scoring system for the prediction of MHC class II binding epitope

To identify HSP90-specific MHC class II epitopes with optimal binding affinity and promiscuity across multiple alleles as previously described,²¹ a combined scoring system using widely available algorithms for predicting class II binding was used. Briefly, the following five algorithms were used for the prediction of class II peptides derived from the HSP90 peptide sequence: SYFPEITHI (Institute for Cell Biology, Heidelberg, Germany), Propred (Institute of Microbial Technology, Chandigarh, India), MHC-Thread (University of Aberdeen, Aberdeen, UK), and average binding matrix method and Rankpep (Harvard, Boston, Massachusetts, USA). Binding predictions for the 15 most common MHC class II alleles: DRB1*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0701, DRB1*0802, DRB1*0901, DRB1*1101, DRB1*1201, DRB1*1302, DRB3*0101, DRB4*0101, DRB5*0101 were generated

using each algorithm. The top 10 HSP90 peptides representing all potential ‘immunogenic hotspots’ were selected as follows: for each available MHC II allele from the five algorithms, 15 peptide sequences were initially selected based solely on the rank order of the predicted binding affinity. Individual amino acids in each selected peptide were assigned a score between 1 and 20, with 20 representing an amino acid contained in a peptide sequence which ranked highest for predictive binding affinity across multiple algorithms. These 10 HSP90 peptides were synthesized by Anygen (Gwangju, Korea).

Assessment of antigen-specific T-cell responses of PBMCs from healthy donor

Human PBMCs from 10 healthy donors were evaluated by enzyme-linked immunospot (ELISPOT) for antigen-specific interferon gamma (IFN- γ) or interleukin (IL)-10 production, as previously described.²² Antigens used for stimulation of PBMCs were the following: 10 $\mu\text{g}/\text{mL}$ of the various HSP90 peptides, 2.5 $\mu\text{g}/\text{mL}$ of CMV (p65), or medium alone in a total volume of 200 $\mu\text{L}/\text{well}$. The mean number of spots and SD from six replicates at each dilution were reported for each antigen. Response to peptide antigens was considered to be positive when the mean number of spots in the experimental wells was statistically different ($p<0.05$) from the mean number from no antigen control wells. Data are reported as the mean number of spots for each experimental antigen minus the mean number of spots detected in no antigen control wells \pm SEM (corrected spots per well (CSPWs) per 2×10^5 PBMC). The best candidate epitopes were selected based on the Th1-inducing response as calculated by the following Th1:Th2 ratio: (IFN- γ incidence \times magnitude)/ (IL-10 incidence \times magnitude) as previously described.²³

Mice and cell line

MMTV neu -transgenic mice (strain: FVB/N-Tg(MMTV neu)202Mul) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). These mice harbor non-mutated, non-activated rat neu under the control of the mouse mammary tumor virus (MMTV) promoter/enhancer. The MMTV neu transgene is expressed at low levels in the normal mammary epithelium, salivary gland, and lungs. Until the age of 8 months, 35% of female MMTV neu -transgenic mice spontaneously develop mammary carcinomas that display high neu expression levels.²⁴ Animal care and in vivo studies were in accordance with institutional guidelines. The mouse mammary carcinoma (MMC) cell line was established from a spontaneous tumor in MMTV neu -transgenic mouse as previously described.²⁵ MMC cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Utah, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine, and were incubated in a humidified 37°C incubator with 5% CO₂. All cell lines were tested for mycoplasma infection both before and after the study. All in vivo data are representative of two independent

experiments, and tumor growth is presented as mean tumor volume ($\text{mm}^3 \pm \text{SEM}$).

HSP90 ELISA

The levels of subtypes with HSP90-specific IgG responses in the sera of patients with HER2+ breast cancer were evaluated using ELISA. Briefly, 50 μL of 20 $\mu\text{g}/\text{mL}$ HSP90 recombinant protein (ADI-SPP-776; Enzo Life Science, New York, USA) diluted in carbonate buffer was coated in each well of a 96-well microplate. Standard sample wells were coated with serial dilutions (0.64–0.005 $\mu\text{g}/\text{mL}$) of human IgG. The plate was incubated overnight at 4°C. Subsequently, 100 μL of diluted antihuman IgG-HRP (I4506; Sigma-Aldrich, St. Louis, Missouri, USA) and mouse anti-human IgG1-HRP, IgG2-HRP, IgG3-HRP, and IgG4-HRP (9054–05, 9080–05, 9210–05 and 9200–05, SouthernBiotech, Birmingham, Alabama, USA) was added to each well and incubated for 45 min at room temperature. After adding TMB solution (T0440, Sigma-Aldrich) and incubating for 5 min, stop solution (2 M H_2SO_4) was added. The optical density of each well was measured at 450 and 540 nm using an iMARK microplate reader (Bio-Rad Laboratories, Hercules, California, USA).

Assessment of antigen-specific T-cell responses in murine model

To evaluate antigen-specific T-cell responses in all experimental mice, the IFN- γ ELISPOT assay was performed as previously described.²¹ Briefly, 1×10^5 and 2.5×10^5 splenocytes/well were stimulated using different antigens: HSP90 peptides (10 $\mu\text{g}/\text{mL}$), tetanus toxoid (TT) peptide (10 $\mu\text{g}/\text{mL}$), concanavalin A (2.5 $\mu\text{g}/\text{mL}$) as positive control, and medium alone as negative control. Anti-mouse IFN- γ antibody (Ab) (AN81, MabTech) for coating and then biotinylated anti-mouse IFN- γ Ab (R46A2, MabTech) were used. The color spots were counted using an AID iSpot reader system (AID). Data are reported as the mean number of spots for each experimental antigen minus the mean number of spots detected in no antigen control wells \pm SD (CSPW per 1×10^5 or 2.5×10^5 splenocytes). Response to peptide antigens was considered to be positive when the mean number of spots in the experimental wells was statistically different ($p < 0.05$) from the mean number from no antigen control wells.

Cross-priming of antigen-specific CD8⁺ T cells

To assess the cross-priming of antigen-specific CD8⁺ T cells,^{26–28} HSP90 peptides pulsed splenic dendritic cells (DCs) (130-100-875; Miltenyi Biotec, California, USA) or splenic DCs were injected intravenously into the tail of MMTV^{neu}-transgenic mice. The mice ($n=3$ per groups) were immunized with 100 μg of each HSP90 peptides or phosphate-buffered saline (PBS) mixed with complete Freund's adjuvant (CFA) for 10 days. Ten days after vaccination, splenocytes were isolated using the CD8a⁺ T-cell isolation kit (130-104-075, Miltenyi Biotec). CD8⁺ T cells from splenocytes were used in the IFN- γ ELISPOT assay.

Data are reported as the mean number of spots for each experimental antigen minus the mean number of spots detected in no antigen control wells \pm SD (CSPW per 2×10^5 splenocytes).

T-cell receptor (TCR) sequencing

Genomic DNA was extracted from the frozen tumor tissues ($n=3$ per groups) by Qiagen DNeasy Blood & Tissue kit (#69504; Hilden, North Rhine-Westphalia, Germany). Sequencing of the TCR β CDR3 region, library preparation, and preliminary bioinformatics analysis were performed by Adaptive Biotechnologies (Seattle, Washington, USA). gDNA was also prepared from separate samples of purified clone cells to validate the TCR β CDR3 sequence. TCR β CDR3 sequence was sequenced at survey-level resolution, and analysis of TCR amino acid sequences was confirmed using the Adaptive TCR ImmunoSEQ analyzer program.

Multiplex immunohistochemistry (mIHC) analysis of the tumor sections (8 μm thickness) was performed at Prism CDX (Gyeonggi, Korea) using an automated multimodal tissue analysis system (Vectra). CD4 (#25229, Cell Signaling Technologies), CD8 (#98941, Cell Signaling Technologies), CD3 (ab16669, Abcam), CD11b (ab133357, Abcam), Foxp3 (14-5773-82, Invitrogen), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (sc-376016, Santa Cruz Biotechnology), Gr-1 (MAB1037, R&D Systems), and 4',6-diamidino-2-phenylindole (DAPI) antibodies were obtained from Prism CDX. All mIHC assays were conducted following the Prism CDX company protocols. Data are from analysis of two separate samples per group and averaged the number of T cells in the total tumor area per square millimetre in the two separate samples.

Statistical analysis

All statistical analyses were performed in GraphPad Prism software V.5.01 (San Diego, California, USA) and R program. Pairwise differences between experimental groups were evaluated using Student's t-test. Differences between multiple experimental groups were examined by one-way or two-way analysis of variance (ANOVA) followed by the Tukey test. Figure 6 was examined by R program followed by the Kruskal-Wallis test. In all cases, $p < 0.05$ was considered significant.

RESULTS

HSP90 can induce antigen-specific IgG responses in metastatic breast cancer

To investigate whether patients with metastatic breast cancer have pre-existing humoral immunity to HSP90, ELISA was used to measure HSP90-specific IgG antibodies in sera from patients with three representative subtypes of breast cancer (HER2+, ER+/HER2-, and triple negative) and healthy donors. Significantly higher levels of HSP90-specific Ab responses were detected in patients with breast cancer (96%) than in healthy donors (20%). As

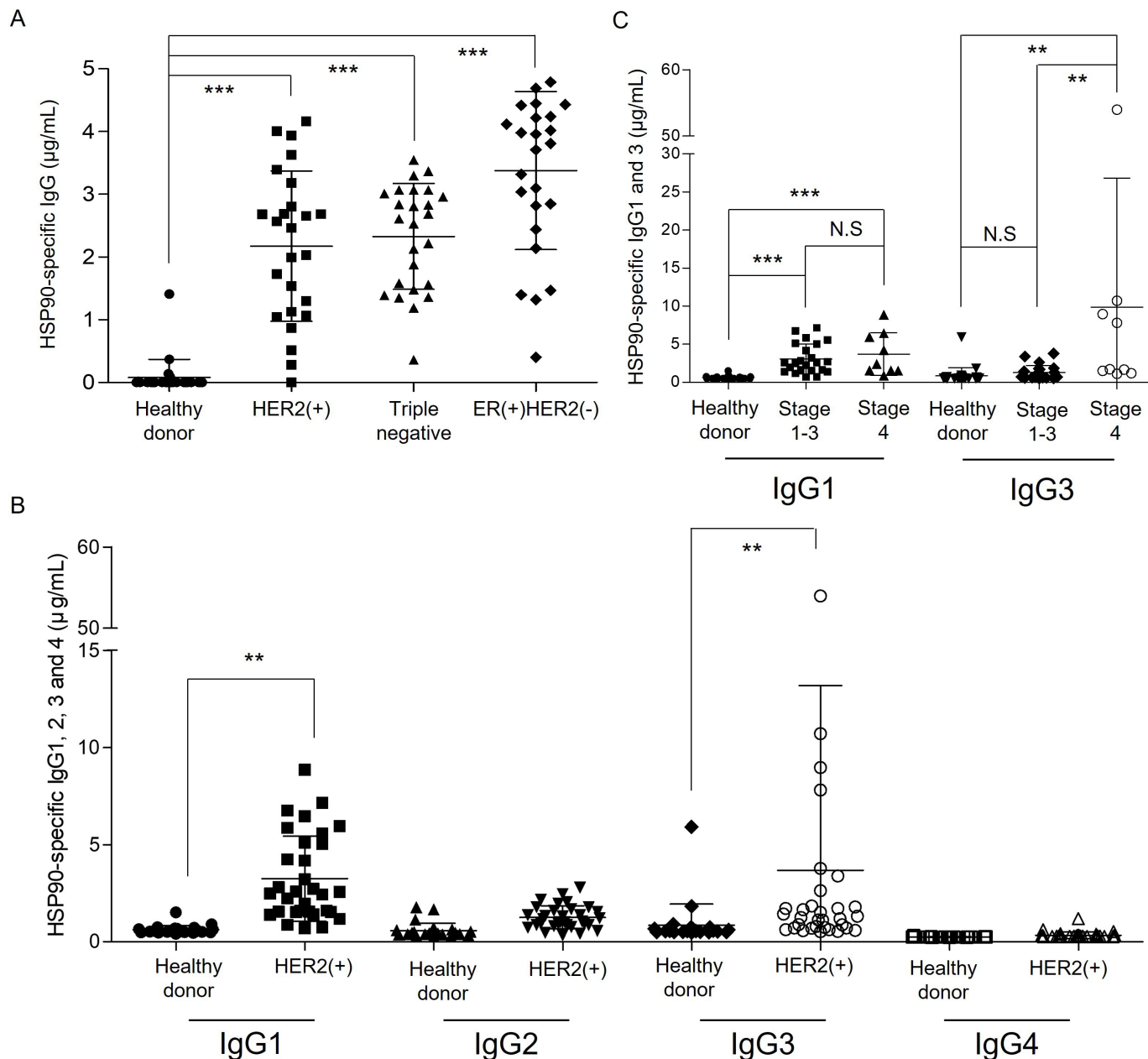


Figure 1 Patients with HER2+ breast cancer can have immunity to HSP90. (A) ELISA was used to examine HSP90-specific IgG immunity in 75 patients with HER2+ breast cancer (n=25, stages 1–3 only), triple-negative breast cancer (n=25) and hormone-positive breast cancer (n=25), and 25 healthy donors. (B) Subtype immunity of HSP90-specific IgG was evaluated in 32 patients with HER2+ breast cancer and 25 healthy donors. (C) In the IgG1 and IgG3 (B), patients with HER2+ breast cancer were classified by stages I–III and IV. Bold lines represent the mean levels of HSP90-specific IgG, IgG1, IgG2, IgG3 and IgG4 in each groups. ** $P < 0.01$, *** $P < 0.001$ in Student's t-test and in Tukey's multiple comparison test of one-way analysis of variance. HER2+, human epidermal growth factor receptor 2-positive; HSP90, heat shock protein 90; N.S., not significant.

shown in [figure 1A](#), the average levels of HSP90-specific IgG were also higher ($p < 0.001$) in patients with the three representative subtypes of breast cancer (ER+/HER2–, 3.37 µg/mL; HER2+, 2.17 µg/mL; and triple negative, 2.32 µg/mL) compared with healthy donors (0.08 µg/mL). When the subtypes of HSP90-specific IgG were evaluated, mean levels of IgG1 (3.24 µg/mL, $p < 0.01$) and IgG3 (3.68 µg/mL, $p < 0.01$) were significantly higher in patients with HER2+ breast cancer compared with healthy donors (0.61 and 0.84 µg/mL), but not IgG2 (1.43 vs

2.09 µg/mL) or IgG4 (0.54 vs 0.74 µg/mL, [figure 1B](#)). Of note, HSP90-specific IgG1 response was similar between patients with early (stages I–III) and advanced (stage IV) cancers ($p = ns$), but significantly higher IgG3 response was observed in the patients with advanced-stage cancer only ($p < 0.01$, [figure 1C](#)). (Patient characteristics of HER2+ breast cancer are available in online supplemental table S1.)

Identification of MHC class II-restricted Th-1 epitopes in HSP90 protein

The binding affinity of the entire HSP90 peptide sequence of most common MHC class II molecules was profiled using a scoring system, and immunogenic hotspots were identified (figure 2A). Ten 15-mer peptides were selected for further evaluation based on a high binding affinity in the scoring system, homology with murine protein, and availability as synthetic peptides. As TAAs are self-proteins that contain sequences of both Th1 antitumor immunity and Th2 immune tolerizing response,²² epitopes for skewed Th1 immunity should be present. Ten 15-mer HSP90 peptides (table 1) were screened for Th1 or Th2 immunity in human PBMCs from 10 healthy donors using IFN- γ and IL-10 ELISPOT assays. As shown in figure 2B, all peptides elicited Th1 immune responses in at least 50% of the volunteers. Th2 immune responses to peptides were also induced in a low proportion of the donors. Among the epitopes with Th1:Th2 ratio higher than 3, p485 and p527 peptides were selected as promising Th1 immunity-inducing epitopes based on the preference for Th1 response and the close positioning in the protein, considering future use as a long peptide vaccine. Immunogenicity of the two peptides was evaluated in the FVB/N mouse model first; indeed, immunization using p485 and p527 peptides could induce significant antigen-specific T-cell responses in vivo (online supplemental figure S1). Based on these results, p485 and p527 peptides were selected as promising Th1 immunity-inducing epitopes with low potential for Th2 immunity induction.

Immunization with selected Th1-directed HSP90 epitopes inhibit tumor growth in MMTV neu -transgenic mice

We evaluated whether immunity induced by the selected Th1-directed HSP90 epitopes affect tumor growth in vivo. In an animal study, MMC cells established from spontaneous tumors in MMTV neu -transgenic mice were used for implantation. As shown in figure 3B, the HSP90 polypeptide vaccine significantly inhibited tumor growth compared with the control group on day 27 (1896 ± 383 mm³ vs 774 ± 43 mm³, $p<0.05$). HSP90-specific IFN- γ -secreting T-cell responses were significantly elevated in the HSP90 peptide group (102 ± 67 spots; CSPW, range 0–22) compared with the control group (0 ± 1 spot; CSPW, range 0–1; $p<0.0001$; figure 3C). These results indicate that immunization with peptides derived from HSP90 affects in vivo tumor growth through the induction of HSP90-specific T-cell response.

Additionally, to investigate the correlation between the roles of CD4⁺ or CD8⁺ T cells and antitumor effects, we depleted CD4⁺ and/or CD8⁺ T cells prior to and during HSP90 peptide immunization of MMTV neu -transgenic mice. Ab-mediated CD4⁺ T-cell depletion had greater impact on the antitumor immunity of HSP90 peptides, while CD8⁺ T-cell depletion had a partial offsetting effect. Combined CD4⁺ T and CD8⁺ T-cell depletion showed the greatest effect. The mean tumor size in mice with CD4⁺ T-cell depletion was 812 ± 52 mm³ ($p=0.149$), significantly

larger than that in mice with CD8⁺ T-cell depletion (558 ± 57 mm³, $p=0.006$), control only (407 ± 59 mm³, $p=0.001$), and undepleted HSP90 (191 ± 64 mm³, $p<0.0001$; figure 3D) on day 26. Immunohistochemistry (IHC) analysis was conducted to verify immune cell depletion in tumors from each experimental mouse (online supplemental figure S2). These results suggest that the antitumor immunity of class II epitopes from HSP90 protein is dependent on CD4⁺ T and CD8⁺ T cells.

Furthermore, to determine whether the class II HSP90 epitopes can be cross-primed to activate antigen-specific CD8⁺ T cells, HSP90 peptide vaccine pulsed splenic-DCs or splenic-DCs only were injected into the tails of the mice. As shown in the IFN- γ ELISPOT assay (figure 3F), a significantly higher number of HSP90-specific CD8⁺ T cells was induced in HSP90 peptides pulsed splenic-DCs with HSP90 peptide vaccine group compared with the splenic-DCs with HSP90 peptide vaccine group. These results suggest that the selected Th1-directed HSP90 epitopes elicit cross-priming of CD8⁺ T cells in vivo.

Stimulator of interferon genes (STING) agonist in combination with HSP90 peptide vaccine enhances HSP90-specific immunity and extends survival time of tumor-bearing MMTV neu -transgenic mice

HSP90 expression is usually adaptive. Thus, it is more likely to be targeted in advanced, treatment-resistant settings where a multitude of cellular stressors have accumulated.²⁹ To apply the HSP90 vaccine in advanced clinical settings as a therapeutic, a combination strategy to augment the antitumor immunity of HSP90-specific T-cell responses is necessary. Recent evidence indicates that STING is involved in the generation of spontaneous anti-tumor T-cell responses and potentiates vaccine-induced immunity by producing high type I interferon (IFN) and CD8⁺ T cells in a murine model.^{30 31} Combination therapy using HSP90 peptides and STING agonists was attempted in an established tumor model (figure 4A). The mean tumor volume in the HSP90 peptides and STING agonist combination group was significantly lower than that in the control or the single HSP90 peptides or STING agonist groups ($p<0.001$). As shown in figure 4B, the mean tumor volumes on day 28 differed based on treatment, with the volume being higher in the control group (mean \pm SD, 971 ± 194 mm³), followed by the HSP90 peptide group (mean \pm SD, 442 ± 48 mm³), STING agonist group (mean \pm SD, 372 ± 39 mm³), and the HSP90 peptide and STING agonist combination group (mean \pm SD, 253 ± 57 mm³).

Notably, therapeutic immunization against the established tumors significantly impacted the survival of mice. The median overall survival times for the control, HSP90 peptides, and STING agonist groups were 56.5, 75.5, and 79.0 days, respectively. As shown in the Kaplan-Meier curve (figure 4C), animals in the group treated with the HSP90 peptide and STING agonist did not reach median survival, with 80% surviving 90 days after tumor implantation. In pairwise comparisons, survival of animals in all

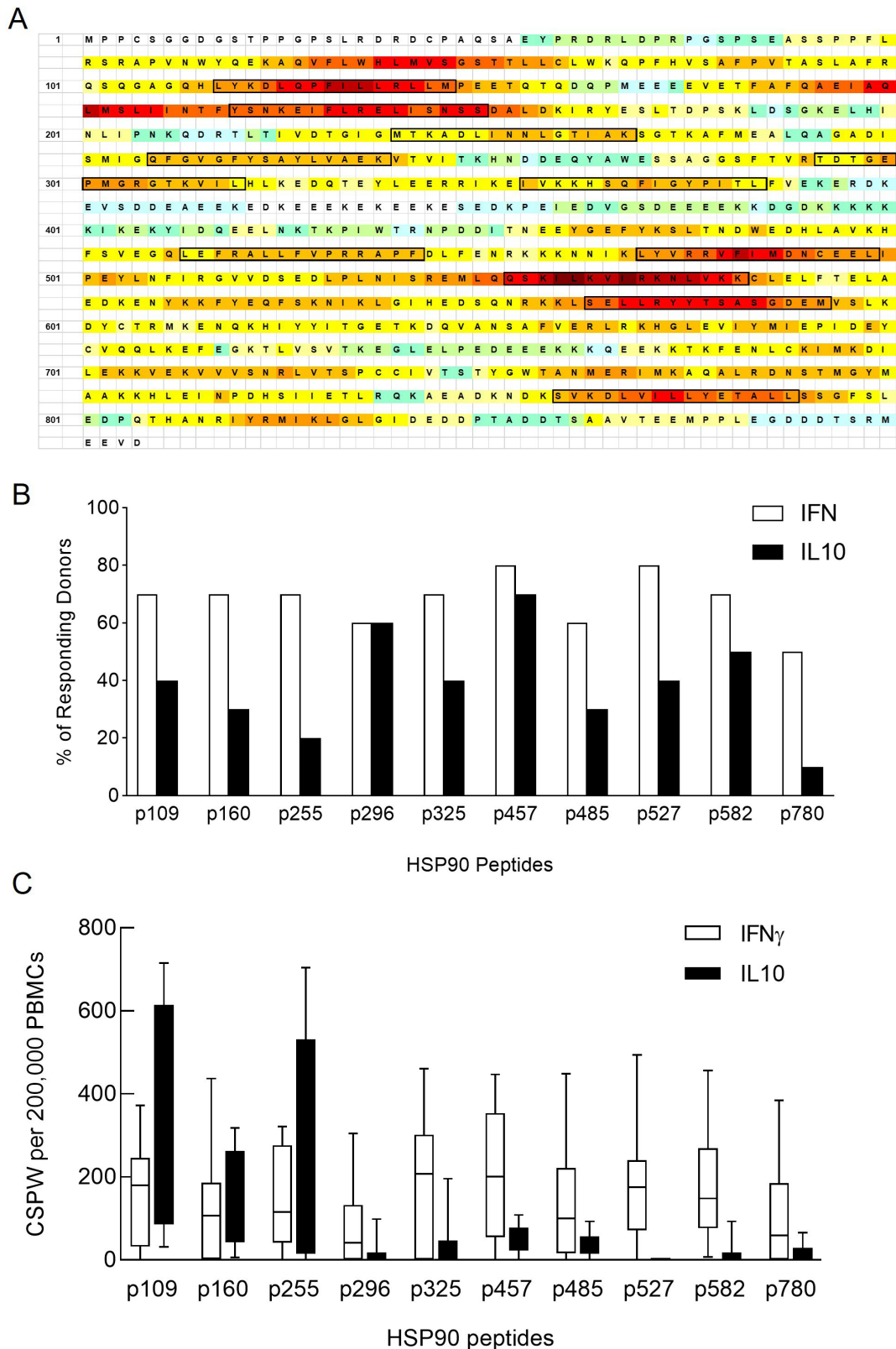


Figure 2 Human T-cell responses specific to HSP90 peptides can be recognized. (A) HSP90 peptides with the highest binding affinities across multiple MHC class II alleles. Colors represent final scores from five algorithms for each peptide sequences from dark red to light blue in the order of rank scores. Color strata are as follows: dark red: >9000, red: 8000–9000, orange: 7000–8000, light orange: 6000–7000, gold: 5000–6000, yellow: 4000–5000, light yellow: 3000–4000, light green: 2000–3000, and light blue: 1000–2000. (B,C) HSP90 peptides were profiled antigen-specific responses in human PBMCs from 10 healthy donors using IFN and IL-10 enzyme-linked immunospot assay. Per cent responding donors to HSP90 epitopes (B) and CSPW (C). White columns, IFN- γ ; black columns, IL-10; horizontal bars indicate mean CSPW. CSPW, corrected spots per well; HSP90, heat shock protein 90; IFN, interferon; IFN- γ , interferon gamma; IL, interleukin; PBMC, peripheral blood mononuclear cell.

Table 1 Human sequences and sequence homology of peptides derived from HSP90

HSP90 peptides	Sequences	% Homology with mouse HSP90
p109-123	LYKDLQPFILLRLLM	50
p160-174	YSNKEIFLRELISNS	100
p255-269	QFGVGFYSAYLVAEK	100
p296-310	TDTGPEMGRGTKVIL	100
p325-340	IVKKHSQFIGYPITL	100
p457-471	LEFRALLFVPRRAPF	100
p485-499	LYVRRVFMIDNCEEL	100
p527-541	QSKILKVIKLNLVKK	100
p582-596	SELLRYYSASGDEM	100
p780-794	SVKDLVILLYETALL	100

treatment groups was significantly extended compared with that of the control group ($p < 0.001$ based on log-rank test). Survival of animals treated with a combination of HSP90 peptides and STING agonist was significantly prolonged compared with animals treated with a single agent ($p < 0.001$). However, differences between HSP90 peptides and STING agonist were not significant.

The tumor-inhibitory effect was associated with enhanced HSP90-specific IFN- γ -secreting T-cell responses in the combination group compared with HSP90 peptide immunization only (figure 4D, $p < 0.05$ based on Tukey's multiple comparison test of one-way ANOVA). The intermolecular epitope spreading to human epidermal growth factor receptor 2 (HER2) peptide and to other oncogenic proteins (HIF-1 α and c-MET) was additionally investigated based on the results from previous work and biological relevance in breast cancer.²³ Indeed, IFN- γ -secreting T-cell responses to the other peptides, HER2 and c-MET were significantly higher ($p < 0.05$) in the HSP90 peptides and STING agonist combination groups but not in the STING agonist only group (figure 4E). Furthermore, to study the properties of the T-cell repertoire in tumor microenvironment, we performed TCR sequencing of the CDR3 variable region of the TCR β chain using tumors (figure 4F). The mean productive rearrangement, a measure of T-cell diversity, was significantly higher in the combination of HSP90 peptides/STING agonist group (median \pm SD, 411 \pm 133, *** $p < 0.005$ in HSP90 peptides) and STING agonist group (median \pm SD, 277 \pm 97, * $p < 0.05$ in HSP90 peptides), but not HSP90 peptide group (median \pm SD, 158 \pm 19, $p = 0.19$), compared with control group (median \pm SD, 195 \pm 71). The productive Simpson clonality in the treated groups was numerically higher than the control group, but comparison was not possible due to small sample size. Altogether, these results suggest that the combination of STING agonist with HSP90 peptides increases the survival of animals by enhancing HSP90-specific T-cell immunity and epitope spreading in an advanced mouse model.

Combination of anti-CTLA-4 Ab with HSP90 peptide vaccine and STING agonist showed therapeutic effect in advanced-stage cancer model of MMTV*neu*-transgenic mice

Anti-CTLA-4 Ab has been shown to potentiate anticancer immunity in multiple preclinical studies by enhancing cross-priming of T cells by APCs.³² Intradermal DNA vaccination combined with dual CTLA-4 and PD-1 blockade provides robust tumor immunity in murine melanoma.³³ To achieve the best antitumor response in an advanced clinical setting, HSP90 peptides were evaluated in combination with the STING agonist and anti-CTLA-4 Ab in the MMTV*neu*-transgenic mouse model. The mean volumes of implanted tumors after three rounds of treatment were measured in all experimental groups; HSP90 peptide and anti-CTLA-4 Ab combination was significantly more effective at suppressing tumor growth compared with HSP90 peptides only or the control (mean \pm SD; 515 \pm 171, 843 \pm 165 and 1003 \pm 150 mm³ in HSP90 peptides/anti-CTLA-4 Ab, HSP90 peptides only, and control group, respectively). The best tumor control was achieved by the combination of HSP90 peptides, STING agonist, and anti-CTLA-4 Ab followed by the STING agonist and anti-CTLA-4 Ab combination, but there was no statistical difference between the two groups (figure 5B). The IFN- γ ELISPOT assay showed that strong HSP90-specific T-cell responses were induced in all HSP90 peptide-immunized animals. However, intermolecular epitope spreading to HER2 was observed in the triple combination therapy consisting of HSP90 peptides/STING agonist/anti-CTLA-4 Ab group only (figure 5C,D). mIHC was used to further investigate the tumor microenvironment, and the distribution of T cells was assessed by IHC staining of the tumor tissues. The number of infiltrated CD4⁺ and CD8⁺ T cells was higher in the triple combination therapy with HSP90 peptides/STING agonist/anti-CTLA-4 Ab (online supplemental figure S3). These results suggest that combination therapy with STING agonist and anti-CTLA-4 Ab enhances the antitumor activity of HSP90 peptides by potentiating HSP90-specific T-cell responses.

Combination therapy with HSP90 peptides/STING agonist/anti-CTLA-4 Ab increases the levels of tumor-infiltrating lymphocytes (TILs) of tumor microenvironment in MMTV*neu*-transgenic mice

Detailed quantification of the immune cell population was performed using mIHC (figure 6). Each immune cell population and proportional assessment of TILs in the two representative tumors from each experimental mice are presented (figure 6B,C, and online supplemental figure S4). The proportion of CD8⁺ T cells among the total T cells was significantly higher in tumors from the triple combination group (32.4%) compared with other groups (16.6%, 13.2%, 17.9%, and 11.9% in the control, HSP90 peptides, HSP90 peptides/anti-CTLA-4, and STING agonist/anti-CTLA-4, respectively; figure 6C). Compared with the control group, the total T-cell proportion showed a significant difference only in the HSP90

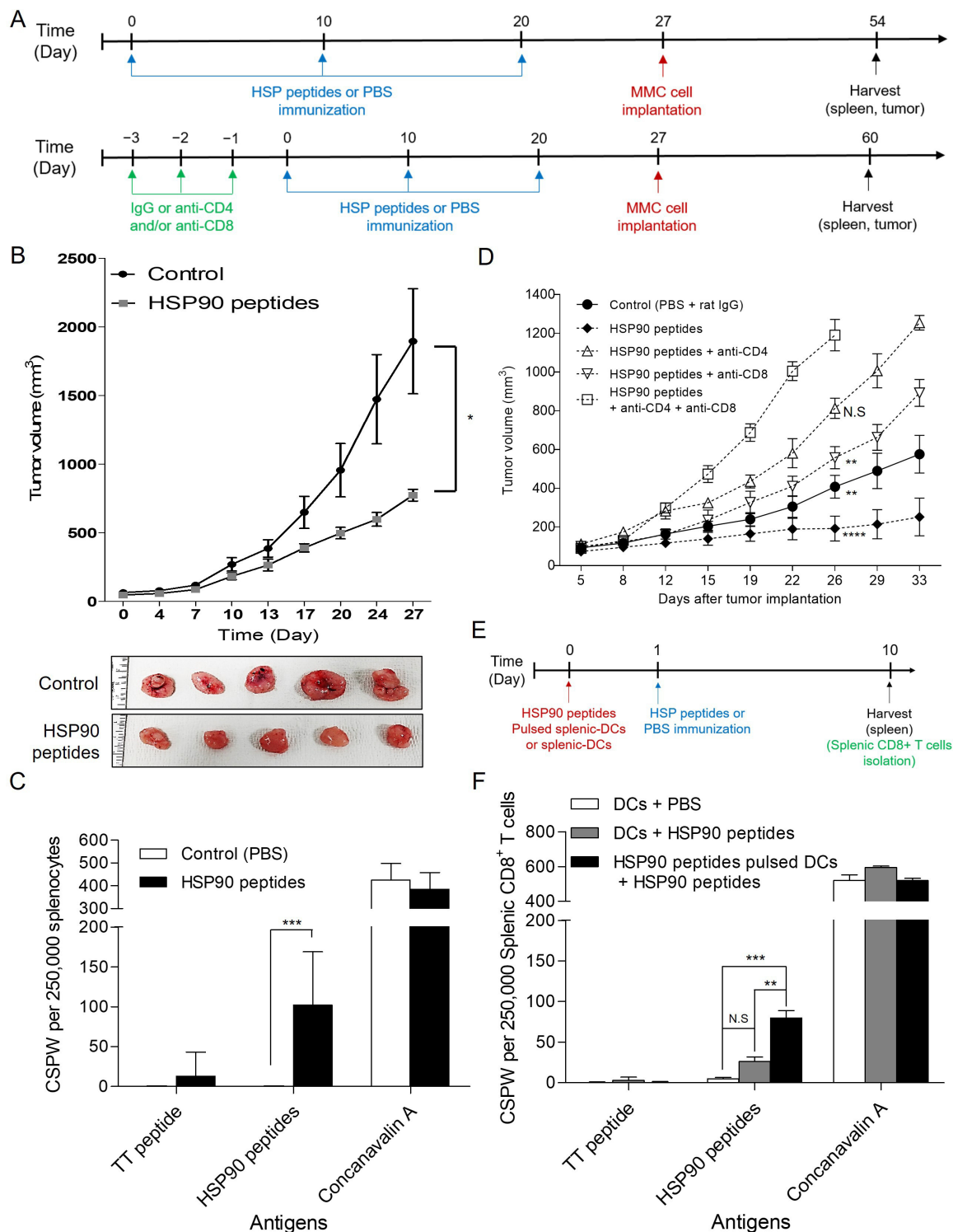


Figure 3 HSP90-specific immunity inhibits tumor growth in MMTV*neu*-transgenic mice. (A) Experimental design and in vivo administration schedules for tumorigenesis (n=5 per groups) and T-cell depletion (n=5 per groups) in mouse models. (B) Images of the tumor and the mean tumor volume in control (PBS) and HSP90 peptide (p485 and p527) groups. (C) Two selected HSP90 peptides were evaluated HSP90-specific T-cell responses using IFN- γ ELISPOT assay. (D) Tumor growth is shown for individual mice in control (PBS+rat IgG), HSP90 peptides, HSP90 peptides+anti-CD4, HSP90 peptides+anti-CD8 and HSP90 peptides+anti-CD4 + anti-CD8 groups. Error bars represent SD. (E) Experimental design (n=3 per group) and schedule for cross-priming CD8⁺ T cells with HSP90 peptides. (F) HSP90 peptides pulsed splenic DCs with HSP90 peptides were evaluated using the cross-priming responses of antigen-specific CD8⁺ T cells using IFN- γ ELISPOT assay. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 in Student's t-test and in Tukey's multiple comparison test of one-way and in Bonferroni post-tests of two-way analysis of variance. CSPW, corrected spots per well; DC, dendritic cell; ELISPOT, enzyme-linked immunospot; HSP, heat shock protein; HSP90, heat shock protein 90; IFN- γ , interferon gamma; MMC, mouse mammary carcinoma; N.S, not significant; TT, tetanus toxoid.

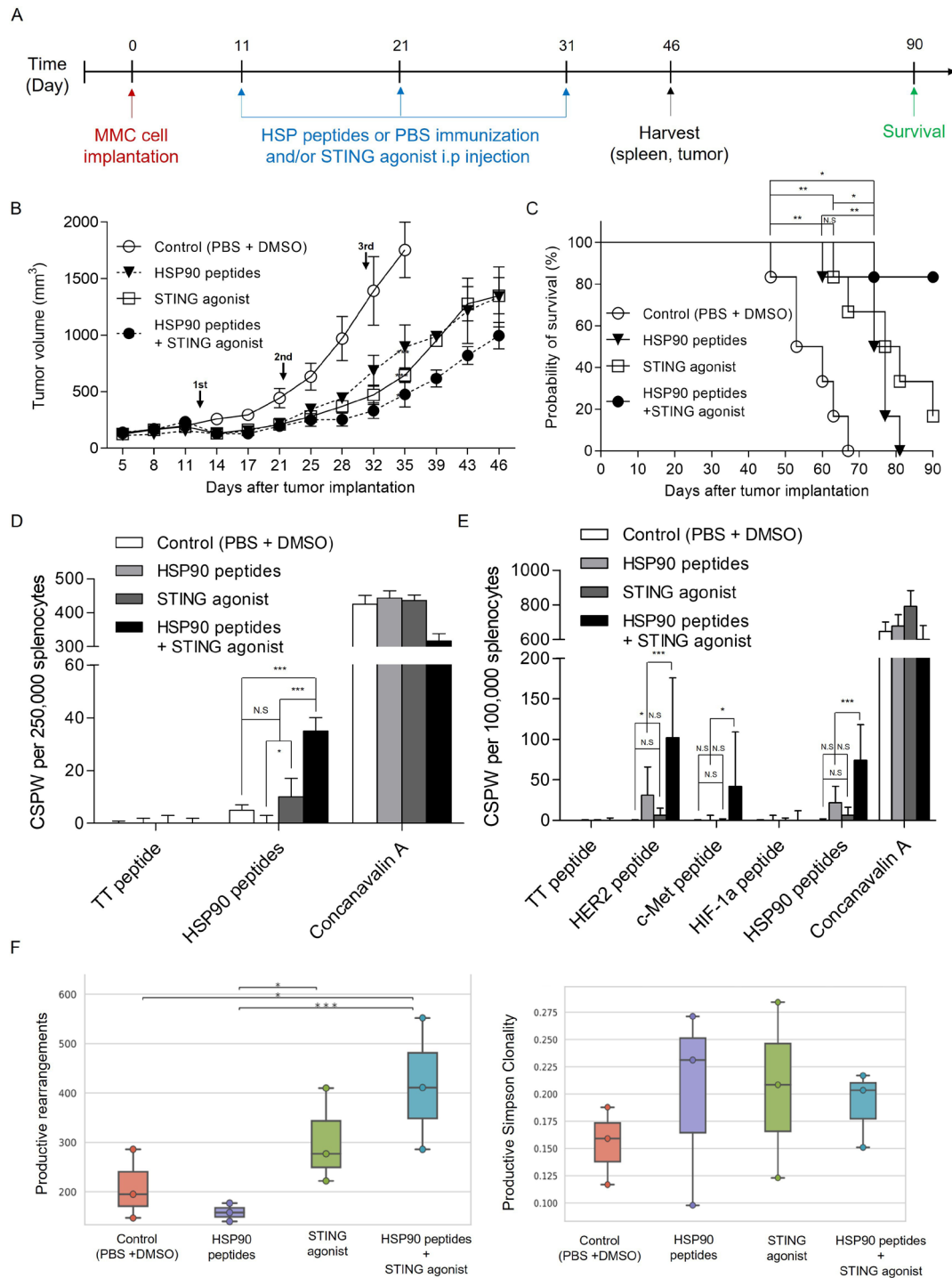


Figure 4 Combination effect of HSP90 peptides and STING agonist improves antitumor effect and survival in MMTV*neu*-transgenic mice. (A) Experimental design showing *in vivo* administration schedules for therapy ($n=3$ per groups) and survival ($n=6$ per groups) due to the combination effect in mouse model. (B) Tumor volume in the control (PBS+dimethyl sulfoxide (DMSO), HSP90 peptides, STING agonist, and HSP90 peptides+STING agonist groups. (C) Kaplan-Meier survival curves of mice (six per groups) implanted with MMC cells and treated with the combination of HSP90 peptides and STING agonist. Statistical analysis of Kaplan-Meier survival curves was performed by log-rank (Mantel-Cox) test in comparison of survival curves using Prism program. (D) HSP90-specific T-cell responses and (E) potential epitope-spreading responses of HER2, c-MET and HIF-1 α peptide were evaluated by enzyme-linked immunospot using splenocytes from experimental groups. (F) Intratumoral TCR repertoire analysis; mean productive rearrangements (left) and productive Simpson clonality of TCR by sequencing of TCR β CDR region from genomic DNA of frozen mice tissues ($n=3$ per groups). Bars represent CSPW and error bars represent SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in Bonferroni post hoc tests of one-way and two-way analyses of variance. CSPW, corrected spots per well; HER2, human epidermal growth factor receptor 2; HSP, heat shock protein; HSP90, heat shock protein 90; MMC, mouse mammary carcinoma; N.S, not significant; STING, stimulator of interferon genes; TCR, T-cell receptor; TT, tetanus toxoid.

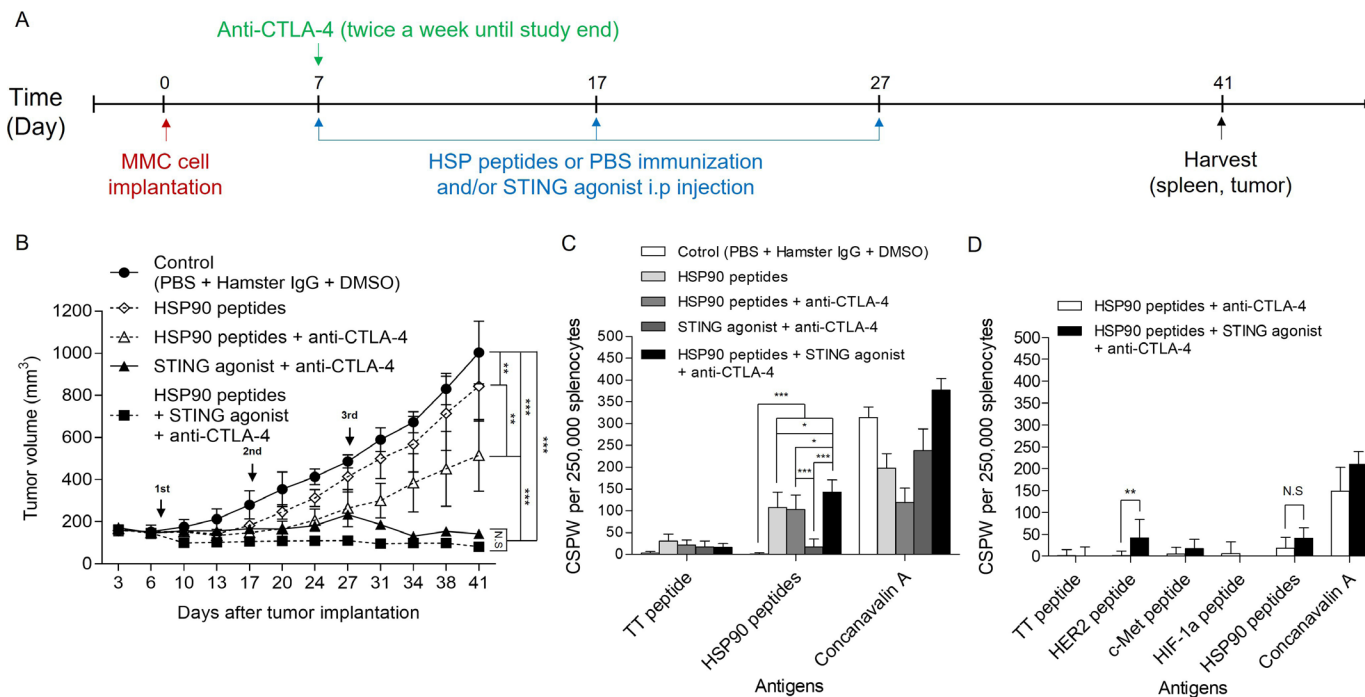


Figure 5 Combination effect of HSP90 peptides/STING agonist/anti-CTLA-4 Ab induces immune environment and epitope spreading by effective antitumor immunity. (A) Experimental design ($n=4$ per groups) and schedule of the combination therapy of HSP90 peptide in the mouse model. (B) Tumor growth is shown for individual mice in control (PBS+hamster IgG+DMSO), HSP90 peptides, HSP90 peptides+anti-CTLA-4 Ab, STING agonist+anti-CTLA-4 Ab and HSP90 peptides+STING agonist+anti-CTLA-4 Ab groups. (C) HSP90-specific T-cell responses and (D) potential epitope-spreading responses of HER2, c-MET and HIF-1 α peptide were evaluated by enzyme-linked immunospot using splenocytes from experimental groups. Bars represent CSPW and error bars represent SD. N.S., not significant. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ in Tukey's multiple comparison test of one-way ANOVA and in Bonferroni post-tests of two-way ANOVA. Ab, antibody; ANOVA, analysis of variance; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; HER2, human epidermal growth factor receptor 2; HSP, heat shock protein; HSP90, heat shock protein 90; MMC, mouse mammary carcinoma; STING, stimulator of interferon genes; TCR, T-cell receptor; TT, tetanus toxoid.

peptides/anti-CTLA-4 Ab/STING agonist treated group ($p<0.001$).

Immunization with HSP90 vaccine increased the numbers of both CD8 T cells and regulatory T (Treg) cells, but the ratio of CD8⁺ T cell:Treg was lower than that in the controls (figure 6D). On the other hand, combining anti-CTLA-4 Ab with HSP90 vaccine significantly decreased the Treg population in the tumor microenvironment, leading to an increase in the CD8⁺ T cell:Treg ratio (10.5 counts/mm² in control and 75 counts/mm² in HSP90 peptides and anti-CTLA-4 Ab, $p=0.01$). The number of myeloid-derived suppressor cells was highest in the tumor environment of the control group (5071 counts/mm²) and was lower in the tumors of the treated group (4527 counts/mm², 4433 counts/mm², 4617 counts/mm², and 4880 counts/mm² for HSP90 peptides, HSP90 peptides/anti-CTLA-4, STING agonist/anti-CTLA-4, and HSP90 peptides/STING agonist/anti-CTLA-4, respectively), but the difference was not statistically significant (figure 6E). Taken together, these results suggest that addition of anti-CTLA-4 Ab to HSP90 peptides decreased vaccine-induced Tregs, and STING agonist enhanced the proportion of TIL in the tumor microenvironment of the MMTV neu -transgenic mouse model.

DISCUSSION

Among the TAAs, HSP90 has an advantage for targeting by a therapeutic cancer vaccine because of its dynamic overexpression while cancer progresses and develops resistance to treatment. In the current study, we identified HSP90 as a valuable tumor antigen in breast cancer, and discovered epitopes that induce strong Th1-directed immunity. Moreover, combining HSP90 vaccine with STING agonist and/or anti-CTLA-4 Ab showed a promising therapeutic effect in an established tumor model.

We focused on HSP90 because accumulating evidence suggests that it plays a pivotal role in breast cancer biology.^{1,2} This molecular chaperone, which has the unique ability to reduce the activity of multiple receptors, kinases and transcription factors known to be involved in human cancer, has been identified as an important therapeutic target in many studies.³⁴ HSP90 inhibitors have been developed and evaluated in clinical trials and shown to be promising cancer therapeutics.^{7,35} However, its clinical development was stopped due to toxicity.^{36,37} Thus, safer strategies such as immunological therapeutics may be a better way of targeting HSP90. Recently, the amplification and higher expression levels of HSP90 were associated with poorer overall survival in patients with a higher

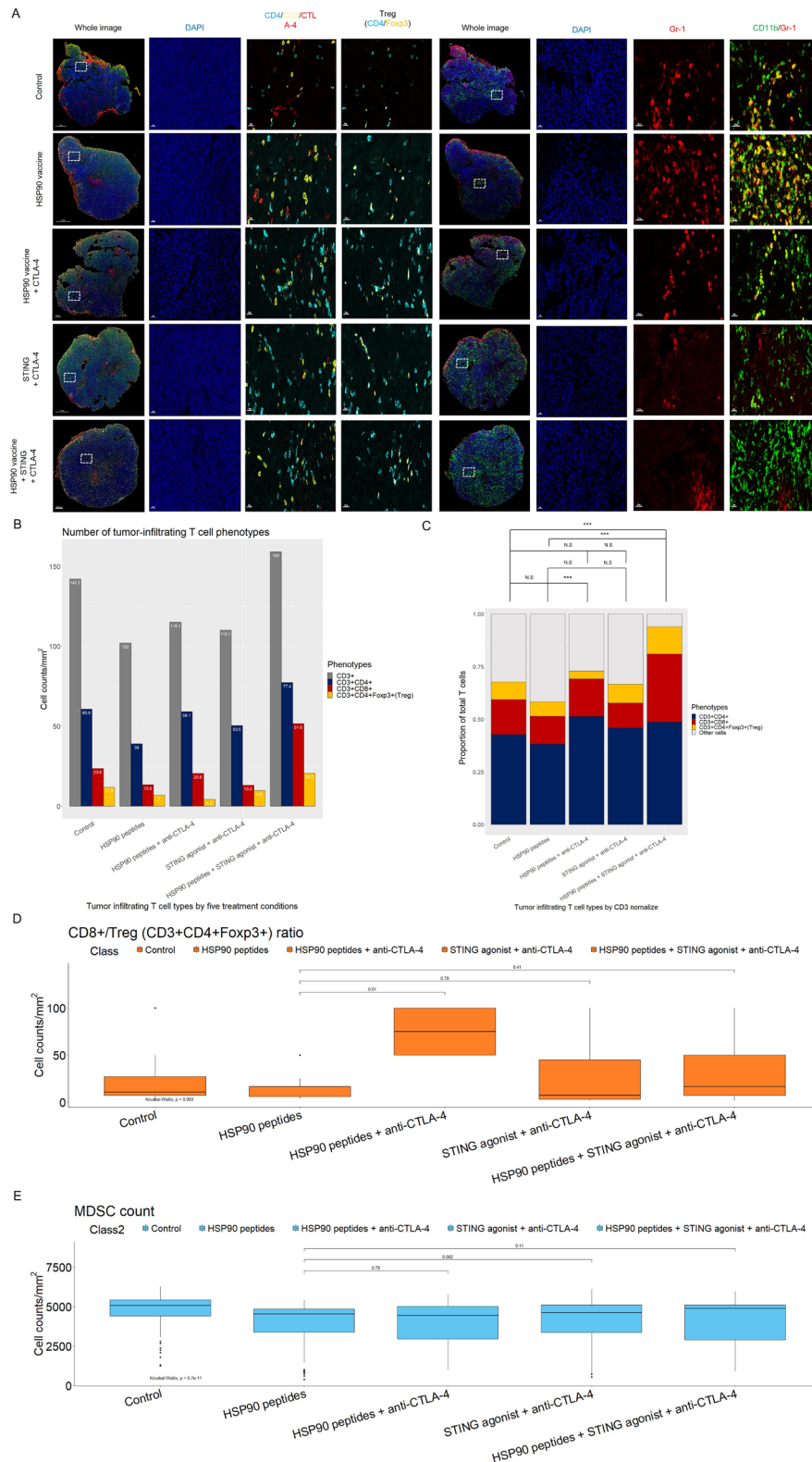


Figure 6 Multiplex IHC reveals immune system distribution of tumor microenvironment in tumor tissues. (A) Representative staining results from multiplex IHC assay of CD4, CD8, Foxp3, CTLA-4, CD11b, and Gr-1 in tumor tissues of mice. (B,C) Counts and percentage of total T cells per area (mm^2) of tumor-infiltrating T-cell phenotypes by multiplex IHC in tumor tissues of mice. (D,E) Boxplots showing median cell counts per area (mm^2) of CD8^+ T cell:Treg ($\text{CD3}^+\text{CD4}^+\text{Foxp3}^+$) ratio and MDSCs ($\text{CD11b}^+\text{Gr-1}^+$) obtained from five treatment conditions. Statistical significance derived from Kruskal-Wallis tests was compared using the stat compare means function, and the overall significance is indicated by the p value. $***P < 0.0001$ in χ^2 test of one-way analysis of variance. CTLA-4, cytotoxic T lymphocyte-associated antigen-4; HSP, heat shock protein; HSP90, heat shock protein 90; IHC, immunohistochemistry; MDSC, myeloid-derived suppressor cell; N.S, not significant; STING, stimulator of interferon genes; Treg, regulatory T.

risk of recurrence of HER2+ and HER2-/ER+ breast cancer subtypes.³⁸

HSP90 has long been studied as a target for cancer therapeutics because of its pivotal role in maturation of client oncoproteins in cancer cells. Moreover, its role in antigen presentation with MHC I on APCs and cross-priming has led to research using a combination of HSP90 and immune checkpoint inhibitors.³⁹ In the development of cancer vaccine, HSP90 has served as a carrier protein in the form of autologous purified HSP vaccines combined with tumor antigens and has shown promising activity in patients with advanced cancer.^{37,40} In our study, we found high levels of HSP90-specific autoantibodies in patients with advanced-stage breast cancer irrespective of subtype (figure 1). Of interest, different subtypes of IgG responses were observed in patients with HER2+ breast cancer; and IgG1 in early stage versus IgG3 in patients with metastatic cancer. Based on the findings, we subsequently identified Th1-directed epitopes using a previously established platform.^{21,22} Identification of promiscuous sequences across the most common MHC II molecules and subsequent exclusion of IL-10-inducing sequences enabled us to select the two promising epitopes for the vaccine. These approaches can overcome the limitation of autologous tumor-derived vaccines, that is, inducing Treg cells.^{37,41} In addition, the use of specific epitopes can solve practical issues associated with immunological monitoring, determination of dosing and schedule in clinical trials.

To investigate whether the HSP90 vaccine can be used for therapeutic purposes in advanced breast cancer, we used a murine model of HER2+ and estrogen receptor-low MMTV^{neu}-transgenic mice with established tumors before immunization. As the growth inhibitory effect and antigen-specific T-cell response by HSP90 peptides alone were not enough in the established tumor model (figure 4B,D), the STING agonist was added for the first combination to enhance the antitumor immunity by vaccine. The combination of HSP90 peptides and STING agonist showed a moderate growth inhibitory effect on tumor growth, with the animals surviving for significantly longer (figure 4). The improved antitumor effect was associated with augmented HSP90-specific T-cell responses, but also T-cell response to another epitope than immunized HSP90: HER2. STING agonists appeared to have a short-term tumor growth inhibitory effect similar to that of vaccines. However, its effect was inferior to that of the combination of HSP90 vaccine and STING agonist in terms of survival. Non-specific immunity by STING agonist alone led to acquired tolerance, leading to a catch-up growth of the tumors (figure 4B,C). On the other hand, animals immunized with HSP90 peptides and STING agonist survived longer by the mechanisms of enhanced HSP90-specific immunity and epitope spreading (figure 4E). In addition to the systemic immune responses, quality of the expanded T cells in tumor microenvironment was further investigated by the TCR sequencing and showed that STING agonist has a significant synergy with HSP90 peptides in increasing

TCR diversity (figure 4F). Previous studies have shown that the STING signaling pathway promotes or induces innate and adaptive immune responses.⁴² Stimulated APCs and upregulated type I IFN subsequently activate immune cells for T-cell priming.^{43,44} Thus, innate immune sensing through the STING signaling pathway is critical for enhancing the optimal antitumor effect of the vaccine. Using a MMTV^{neu}-transgenic mouse model, we showed that combination therapy using HSP90 peptides and STING agonist could survive mice with advanced tumors longer via enhanced efficacy of HSP90 vaccine (figure 4). Our data further verify the favorable role of STING agonist in inducing Th1 immune response for advanced cancer treatment.

Given the synergy between the HSP90 peptide vaccine and the STING agonist, we explored the efficacy of anti-CTLA-4 Ab as part of a triple combination. Although anti-CTLA-4 antibodies are currently being evaluated in clinical trials in breast cancer, we questioned if it can enhance antitumor immunity via better antigen presentation as previously described.³² In the established tumor model, HSP90 vaccine only showed a limited antitumor efficacy, but combination with anti-CTLA-4 Ab showed significant improvement. Of note, the triple combination had the greatest synergy, resulting in significant tumor regression by 40 days of observation (figure 5B). In terms of systemic T-cell immunity, combination with anti-CTLA-4 Ab did not increase antigen-specific immunity to HSP90, but further combination with STING agonist enhanced antigen-specific immunity to HSP90, also to unimmunized epitope, HER-2. On the other hand, anti-CTLA-4 Ab was effective in decreasing Treg cells in tumor microenvironment, then increasing the ratio of CD8⁺ T cells:Tregs (figure 6B,D). Interestingly, the dual combination of STING agonist and anti-CTLA-4 showed good synergy in tumor inhibition, but there was no systemic antigen-specific T-cell expansion or CD8 T-cell recruitment in tumor microenvironment. CTLA-4 is a costimulatory signal expressed by activated T cells and dampens T-cell responses. Thus, anti-CTLA-4 Ab is known to eliminate the immunosuppressive propagation of Treg-cell activation and to enhance antitumor immunity via better cross-priming by APCs, leading to effective antitumor responses.^{32,45} In the present study, induction of effector T cells using the HSP90 peptide vaccine also recruited more Tregs, but the regulatory mechanism was successfully controlled by adding anti-CTLA-4 Ab (figure 6D).

One of the important mechanisms for the synergy between the HSP90 peptides and STING agonist was the phenomenon of 'epitope spreading'. Cancer immunotherapy not only produces immune response against a specific target antigen but can also work partially through epitope spreading.⁴⁶ The expanded immune response is not limited to the molecules derived from the vaccine (intramolecular) but to the other oncogenic proteins (inter-molecular). Previous work has shown that epitope spreading was associated with antitumor effects in peptide-based cancer immunotherapy and has

been found to improve therapeutic effects. In this study, we observed that intermolecular epitope spreading to other oncogenic proteins such as HER2 and c-MET was induced by STING agonist rather than by anti-CTLA-4 Ab. This phenomenon in systemic immune response was concordant with an increase in CD8⁺ T cells and productive TCR β rearrangements of TILs and finally resulted in better antitumor efficacy. Thus, our study provides scientific data for therapeutic strategy in combining of STING agonist and/or anti-CTLA-4 Ab to maximize the effect of HSP90 peptides in advanced HER2+ breast cancer.

In conclusion, we have provided the first preclinical evidence supporting the mechanism of action of HSP90 peptide vaccine with a distinct potential to improve the treatment of breast cancer. Our data also suggest that combination therapy with HSP90 peptides, STING agonist, and/or anti-CTLA-4 Ab is a promising immunological strategy in patients with advanced HER2+ breast cancer and various cancers. A phase I clinical trial using the HSP90 peptide vaccine is currently under way.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human subjects, and the institutional review board approved the use of human PBMC (University of Washington, approval number UL1 TR002319) and sera (Korea University Anam Hospital, Approval number: 2016AN0090) previously collected after informed consent was obtained from human subjects. Animal research ethics was approved by the Institutional Animal Care and Use Committee of Korea University (IACUC, Approval number: KOREA-2016-128). Additional consents were exempted from this study.

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ORCID iD

Jinho Kang <http://orcid.org/0000-0002-1124-5607>

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