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MHC Class I–Presented Tumor Antigens Identified in Ovarian Cancer by Immunoproteomic Analysis Are Targets for T-Cell Responses against Breast and Ovarian Cancer

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Abstract

Purpose: The purpose of this study is to test whether peptide epitopes chosen from among those naturally processed and overpresented within MHC molecules by malignant, but not normal cells, when formulated into cancer vaccines, could activate antitumor T-cell responses in humans.

Experimental Design: Mixtures of human leukocyte antigen A2 (HLA-A2)-binding ovarian cancer-associated peptides were used to activate naive T cells to generate antigen-specific T cells that could recognize ovarian and breast cancers *in vitro*. Combinations of these peptides (0.3 mg of each peptide or 1 mg of each peptide) were formulated into vaccines in conjunction with Montanide ISA-51 and granulocyte monocyte colony stimulating factor which were used to vaccinate patients with ovarian and breast cancer without evidence of clinical disease in parallel pilot clinical trials.

Results: T cells specific for individual peptides could be generated *in vitro* by using mixtures of peptides, and these T cells recognized ovarian and breast cancers but not nonmalignant cells. Patient vaccinations were well tolerated with the exception of local erythema and induration at the injection site. Nine of the 14 vaccinated patients responded immunologically to their vaccine by inducing peptide-specific T-cell responses that were capable of recognizing HLA-matched breast and ovarian cancer cells.

Conclusion: Mixtures of specific peptides identified as naturally presented on cancer cells and capable of activating tumor-specific T cells *in vitro* also initiate or augment immune responses toward solid tumors in cancer patients. *Clin Cancer Res; 17(10); 3408–19. ©2011 AACR.*

Introduction

Despite improvements in systemic therapy, advanced breast and ovarian cancer remain highly lethal, necessitating the development of new therapies such as immunotherapy. The central hypothesis in breast and ovarian cancer immunotherapy is that these cancers express antigens in the form of peptides presented by MHC class I molecules that can be recognized by cytolytic T cells (CTL) leading to tumor destruction (1, 2). Examples of ovarian and breast tumor antigens recognized by CTL include HER2, CA125, and MUC-1 (3–10). These antigens have been incorporated into cancer vaccines currently in clinical trials (11–19). Nonetheless, modest clinical efficacy of these vaccines suggests that these antigens may not be those resident in tumor-expressed MHC molecules as targets for T cells or that targeting single antigens may be inadequate if down-regulation of a single antigen, as an escape mechanism, occurs efficiently. Therefore, for clinically effective immune responses, there is a need to identify a broader array of T-cell epitopes.

A promising, clinically relevant strategy for prioritizing peptide targets for therapeutic application would be to identify those peptides abundantly presented within the MHC class I molecules by tumor cells. Transformed cells present an MHC peptide repertoire distinct from their normal counterparts (20). An unbiased analysis of the peptide repertoire associated with the MHC class I molecules of cancer cells could identify and allow the prioritization of novel tumor antigens for the development of cancer immunotherapy (reviewed in ref. 21). There are a number of approaches for identifying MHC-associated peptides (reviewed in refs. 22 and 23), including screening clones of cells transfected with tumor cDNA for those capable of stimulating CTL lines (24) or scanning sequences of known proteins *in silico* for peptides with consensus binding motifs specific for desired human leukocyte antigen (HLA) haplotypes (25, 26). Recently, our collaborators,

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Translational Relevance

This study tests the hypothesis that choosing peptides, naturally processed and overpresented within MHC molecules by malignant, but not normal cells, would allow functional prioritization of which peptides to formulate into cancer vaccines capable of activating antitumor T-cell responses in humans. The preclinical studies show that immune responses against individual peptides may be induced with peptide mixtures. The clinical studies validate the strategy by using pools of peptides identified as naturally presented on cancer cells in cancer vaccines to initiate or augment immune responses toward multiple antigens and different tumor cell types. This work has clear applications to the current and future practices of medicine in that the results support further testing of this vaccine strategy in phase I/II studies, which if successful, would lead to phase III clinical trials of a novel therapy for breast and ovarian cancer patients.

Ramakrishna and colleagues (27) used an unbiased technique to identify 16 novel HLA-A2–bound peptides from a very large and complex pool of MHC class I–bound peptides purified from HLA-A2+ ovarian cancer cell lines by using liquid chromatography and mass spectrometry. The biological relevance of these peptides was shown by using synthetic versions to activate CTL *in vitro* and using them to recognize the HLA-A2+ ovarian tumor lines SKOV3-A2 and OVCAR3 and primary ovarian tumor line OV2, suggesting that these epitopes are endogenously processed and presented by the tumor cells in sufficient quantity to be recognized by CTLs. We also showed that these CTL could recognize HLA-A2+ breast cancer cells. These peptide-specific CTL lines showed no lysis of normal HLA-A2+ ovarian cells, suggesting that these epitopes are not present on normal cells and are only presented on tumor cells.

In the process of translating these observations into human clinical trials, we first studied whether T cells isolated from ovarian cancer patients could be stimulated with these peptides, whether the peptide-specific T cells were capable of recognizing ovarian cancer cells, and whether *in vitro* stimulation with combinations of peptides was superior to stimulation with a single peptide alone. To show that these peptides are immunogenic in cancer patients, a vaccine with synthetic peptides plus Montanide ISA-51 and granulocyte monocyte colony stimulating factor (GM-CSF) was formulated for clinical use. To study the vaccines in clinical scenarios with less tumor induced immunosuppression, we conducted 2 phase I studies, one of which enrolled high-risk ovarian cancer patients who had undergone surgical cytoreduction and systemic chemotherapy and the other which enrolled breast cancer patients who had no evidence of disease after surgery, chemotherapy, and radiotherapy but who had a high risk of recurrence. We determined tolerability and safety of these vaccines, clinical activity, and circulating antigen-specific T-cell responses pre- and postvaccination.

Materials and Methods

Subjects

Heparinized blood from healthy HLA-A2+ donors was purchased from Research Blood Components, LLC. Patient blood samples were obtained following written informed consent approved by the Duke University Medical Center Institutional Review Board.

Cell lines and primary cells from human tissues

Human ovarian cancer cell lines SKOV3-A2 (kindly provided by Dr. Ioannides, MD Anderson Cancer Center) and OVCAR3, human breast cancer cell lines MDA-MB-231 and MCF7, and the human prostate cancer cell line LNCaP were originally obtained from ATCC. SKOV3-A2, OVCAR3, and LNCaP were maintained in RPMI 1640 medium (Mediatech) supplemented with 10% FBS (Atlanta Biologicals), L-glutamine (300 mg/mL), nonessential amino acids (1 × concentration), penicillin, and streptomycin (1 × concentration, supplements were purchased from Mediatech). MDA-MB-231 and MCF7 were maintained in Dulbecco's modied Eagle's medium (DMEM) medium supplemented with 10% FBS and other supplements listed above. All cell lines were maintained at 37°C in a humidified incubator with 5% CO_2. Nonmalignant kidney and liver tissues from HLA-A2+ human donors were obtained from NDRI, Philadelphia, PA. Tissues were enzymatically digested and cell suspensions were generated per standard methods. Briefly, tissue samples were minced and digested with 2 μg/mL collagenase, 0.1 μg/mL hyaluronidase, and 0.15 μg/mL DNAse in DMEM supplemented with 2 × concentration of antibiotics and antimycotics (all reagents were obtained from Sigma-Aldrich) at 37°C for 3 to 6 hours. Cell suspensions were pelleted and washed several times with PBS and DMEM supplemented with 10% FBS. Cell viability was assessed by trypan blue exclusion, and cells were frozen in 90% FBS and 10% DMSO (dimethyl sulfoxide; Sigma-Aldrich) for future use.

Synthetic peptides

Synthetic peptides corresponding to the HLA-A2 presented ovarian cancer epitopes (Table 1) were supplied by GenScript Corporation and were reconstituted in DMSO. Group I (P1, P2, P3, P6, P7, and P8) and group II peptides (P4, P5, P9, P13, P14, and P15) were formulated by mixing equal amounts of each of the component peptides. We defined low, intermediate, and high binding affinity based on the time for dissociation data published by Ramakrisha and colleagues (27) and used a $t_{1/2}$ of 1,000 minutes as the cutoff separating low/intermediate from high affinity.

In vitro generation of peptide-specific T cells

Peripheral blood mononuclear cells (PBMC) from HLA-A2+ healthy donors and ovarian cancer patients

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Table 1. Peptides used in preclinical and clinical studies and their source proteins

	Sequence	Protein from which peptide sequence is derived
Group I	KIMDQVQQA (P1)	Adenomatous polyposis coli (APC)
	RLQEDPPAGV (P2)	Ubiquitin conjugating enzyme E2, HHR6A
	KLDVGNAEV (P3)	BAP31 or CDM protein, 6c6-AG
	YLMDTSGKV (P6)	Replication protein A
	ILDDIGHGV (P7)	Abl-binding protein 3c
	LLDRFLATV (P8)	Cyclin I
Group II	FLYDDNQRV (P4)	Topoisomerase IIα; topoisomerase IIβ
	ALMEQQHYV (P5)	Integrin β 8 subunit precursor
	LLIDDKGTIKL (P9)	Cell division control protein 2 (CDC2)
	YLIELIDRV (P13)	TACE, ADAM17
	NLMEQPIKV (P14)	Junction plakoglobin, g-catenin
	FLAEDALNTV(P15)	EDDR1, RTK 6, H-RYK

undergoing cytoreductive surgery were purified by using lymphocyte separation medium (Mediatech) by using differential centrifugation according to standard methods. Cells (20×10^6 per well) were cultured in 2 mL RPMI 1640 medium supplemented with 10% FBS, L-glutamine (300 mg/mL), nonessential amino acids, sodium pyruvate, penicillin, and streptomycin (complete medium) in 6-well tissue culture plates (BD) overnight. Nonadherent cells were removed and saved. Plastic adherent cells were pulsed with 50 μg/mL of each of the synthetic peptides and 1.5 μg/mL human β2-microglobulin (Sigma-Aldrich) in complete medium for 2 hours. Nonadherent cells were added back in 5 mL complete medium supplemented with interleukin-7 (IL-7) at 5 ng/mL, keyhole limpet hemocyanin (KLH; Sigma-Aldrich) at 5 μg/mL, GM-CSF at 25 ng/mL and IL-4 at 50 ng/mL (all cytokines and growth factors were purchased from Peprotech). Plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 12 days. Two milliliters of medium was removed from each well and replaced with fresh complete medium supplemented with 10 U/mL IL-2 for 2 days. T cells were restimulated with CD4/CD8 T cell–depleted autologous monocytes pulsed with synthetic peptide at 10 μg/mL and 1.5 μg/mL human β2-microglobulin in complete medium containing 5 ng/mL IL-7 and 5 μg/mL KLH for 5 days. IL-2 treatment and *in vitro* restimulation were repeated thrice at the indicated time intervals prior to use of *in vitro* expanded T cells in ELISpot assays.

ELISpot assays

In vitro activated and expanded T cells were used as effectors in ELISpot assays to assess antigen stimulated IFN-γ release by using a human IFN-γ assay kit or Granzyme B release by using a human Granzyme B assay kit (BD-Pharmingen) according to the manufacturer's instructions. Typically, a fixed number of various target cells (5×10^3 cells per well) and effector cells (2×10^5 per well), at an effector to target ratio of 40:1, were used in ELISpot assays. T2 cells were pulsed with 20 μg/mL synthetic peptides and 1.5 μg/mL human β2-microglobulin in RPMI 1640 medium supplemented with 1% FBS and other additives (as above) overnight for use as targets in ELISpot assays. ELISpot assays were done in replicate wells. Spots were quantitated by using an Immunospot reader from Cellular Technologies Limited. Results are presented as number of IFN-γ producing cells per 1×10^6 cells.

Parallel phase I clinical trials in breast and ovarian cancer: inclusion criteria and treatment

The ovarian study (NCT00437502) enrolled patients with histologically confirmed, stage II to IV epithelial ovarian, tubal, or peritoneal cancer who had undergone a first or second cytoreductive surgery (optimal or suboptimal) and had a complete clinical response to front-line or second-line platin/taxane-based chemotherapy. The breast study (NCT00674791) enrolled patients with histologically confirmed, resected, breast cancer with one of the following characteristics: T3,4NxM0; TxN1M0 with 4 or more positive lymph nodes; TxN2–3M0; ER−PR−HER2− ("triple negative") which is T1c or greater with any N stage or with locally recurrent or metastatic breast cancer with no evidence of disease after surgery, chemotherapy, and radiotherapy. The remainder of the inclusion and exclusion criteria was the same for each study. Patients could not have a history of autoimmune disease, serious intercurrent chronic or acute illness, active hepatitis, serologic evidence for HIV, splenectomy, or be receiving steroid or immunosuppressive therapy. All subjects were 18 years or older and HLA-A2+. In both studies, there were 2 cohorts of patients each treated with different doses [100 μg of each peptide (low dose) and 1 mg of each peptide (high dose)] of the vaccine referred to as IMT-1012 given 6 times (weeks 0, 1, 2 and 4, 5, 6). For the 100 μg/peptide cohorts of each study, the immunizations consisted of 2 pools of 6 peptides each (group 1: peptides P1, P2, P3, P6, P7, P8 100 μg each) and (group 2: peptides P4, P5, P9, P13, P14, P15 100 μg each) diluted in 50% DMSO (RIMSO-50)/50% water, mixed with GM-CSF (100 μg) and Montanide ISA-51 (1 mL) to give a total volume of 2 mL. The group 1 peptides were

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injected into the left thigh and the group 2 peptides into the right thigh as 200 microliters intradermally and 1.8 mL subcutaneously. Subsequent immunizations of each group were given in the same limb as the previous injections spaced apart by at least 5 cm when possible. Within the first few patients, it was apparent that acute injection site pain would be an issue and, therefore, we used ice packs to cool the injection sites before the injections in the majority of the patients. When the protocol was first written, we had intended to give booster injections and the first ovarian patient did receive 4 of these boosters and the second patient received 1 booster, but because of the injection site reactions, subsequent patients received injections on weeks 0, 1, 2 and 4, 5, 6 only.

To reduce the number of total injections, when we moved to the 1 mg/peptide cohort, we modified the mixing protocol so that all 12 peptides were combined. To achieve this combination, P2, P4, P6, P13, and P15 were separately dissolved in 100% DMSO and P1, P3, P5, P7, P8, P9, and P14 were separately dissolved in 0.05 mol/L NaOH and diluted in USP grade water. The 12 peptide solutions were then added to a single tube containing sodium phosphate buffer in the following order: P1, P2, P4, P5, P6, P7, P8, P9, P13, P14, P15, and P3. Once thoroughly mixed and dissolved, the resulting 12 peptide mix was aliquotted into vials containing 1 mL plus overage and tested for stability and sterility. For patient injections, 1 mL of high-dose peptide aliquot was mixed with GM-CSF (100 μg) and Montanide ISA-51 (1 mL; 2 mL total volume) and given into a single injection site as 200 microliters intradermally and 1.8 mL subcutaneously.

All patients were followed for toxicity which was graded by the CTCv3 criteria and for survival and evidence of progression of disease by CT scans and tumor markers where appropriate and at the discretion of their physician.

Immune analysis

For immune monitoring of IMT-1012 vaccinated patients, PBMC collected and frozen at week 0 through week 8 were thawed and used without any *in vitro* stimulation for the ELISpot assays which were done as described above. A positive immune response was defined as a response to specific peptide greater than 40 spots (out of 10^6 PBMC) over prevaccination and/or negative control response by ELISpot.

Results

A mixture of peptides representing a combination of tumor-associated antigen epitopes induces immune responses equivalent to those of single peptides *in vitro*

The purpose of this project was to carry out the translational development of a multipeptide vaccine to test whether naturally presented peptide epitopes could activate therapeutic immune responses in patients with breast and ovarian cancers. One possible challenge is that 1 or more peptides may interfere with presentation and immune responses to other peptides. Therefore, to formulate the vaccine for the human clinical trial, we first investigated *in vitro* whether T cells specific for an individual peptide would be activated when PBMC were stimulated with a mixture of peptides. The group 1 peptides (low and intermediate MHC class I–binding affinity) mixed together were used to stimulate PBMC from healthy volunteers and cancer patients. The activated T cells were tested for their ability to recognize targets expressing the individual peptides and cancer cell lines. As shown in Figure 1A, a greater frequency of T cells generated from a healthy donor against the group 1 and 2 peptide mixtures secreted IFN-γ on recognition of T2 cells pulsed with each of the individual peptides than against unpulsed T2 cells. We observed similar results when the group 2 peptides (high MHC class I–binding affinity) were mixed together and used to stimulate PBMC. Although there was a range in the magnitude of the T-cell response, all peptides and the mixtures of peptides could be recognized by the activated T cells. The peptide mixture-specific T cells could also recognize HLA-A2+ ovarian (OVCAR3, SKOV3-A2), breast (MDA-MB-231), and prostate (LNCaP) cancer cell lines and, to a lesser extent, a different breast cancer cells (MCF7). In contrast, the T cells did not recognize normal liver cells with comparable HLA-A2 expression (data not shown). As shown in Figure 1B, T cells generated from PBMCs of ovarian cancer patients against the group 1 and 2 peptide mixtures secreted IFN-γ on recognition of T2 cells pulsed with each of the individual peptides that were greater than against unpulsed T2 cells. In addition, these T cells could recognize breast, ovarian, and prostate cancer cell lines but not normal liver or kidney cells, indicating that these peptides are only presented by the tumor cells and not presented by these nonmalignant cells. These data indicate that when PBMC from healthy individuals and cancer patients are stimulated by a pool of peptides, immune responses against each of the component peptides may be activated.

To show that the T-cell response against a single peptide is similar whether activated by a single peptide or a mixture of peptides (containing that peptide), we stimulated T cells from a healthy donor (KP07517) and cancer patients (LPA32 and OVAR34) with selected single peptides (P14; Fig. 2A and P13; Fig. 2B) or with mixtures containing the selected peptide and showed responses of a similar magnitude against T2 cells pulsed with the specific peptide. These data taken together suggest that peptides are similarly immunogenic when used individually or combined in mixes, as would be the case in a cancer vaccine. These data supported the design of the vaccine(s) for the human clinical trials.

Pilot Clinical Trials of Peptide Vaccines in Breast and Ovarian Cancer Patients

Demographics

In the ovarian cancer study, 8 patients having had 1 to 2 prior chemotherapy regimens after their cytoreductive

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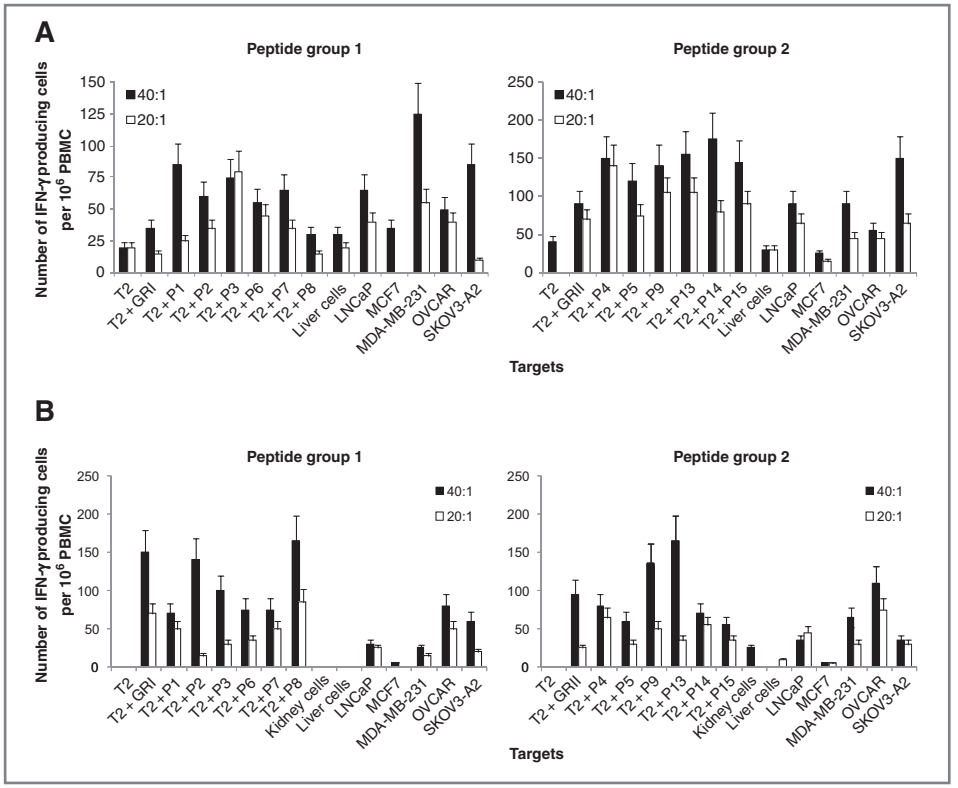

Figure 1. Generation and characterization of T cells specific for ovarian cancer–derived peptides in healthy donors and ovarian cancer patient. PBMC from a healthy HLA-A2+ donor (A) and an HLA-A2+ ovarian cancer patient (B) were *in vitro* stimulated with group I (left) or group II (right) synthetic peptides each consisting of 6 ovarian cancer–derived epitopes (Table 1). These cells were tested in an overnight ELISpot assay by using T2 cells loaded with the group peptides or individual component peptides, normal liver cell suspensions obtained from a HLA-A2+ healthy donor tissue, and HLA-A2+ cancer cells lines (ovarian cancer cell lines SKOV3-A2 and OVCAR3, breast cancer cell lines MDA-MB-231 and MCF7, and a prostate cancer cell line LNCaP). IFN-γ producing cells were quantified by using Immunospot reader. Error bars represent SEM of experimental replicates.

surgery were enrolled [4 at the low dose (100 μg of each peptide) and 4 at the high dose (1 mg of each peptide; Table 2]. Initially, our intention was to give booster doses, but after noting the injection site reactions (see toxicity below), we limited the doses to 6 after the first 2 patients. In the breast cancer study, 7 patients, having had predominantly 1 prior treatment regimen, were enrolled [3 at the low dose (100 μg of each peptide) and 4 at the high dose (1 mg of each peptide)]. All but 1 patient received all 6 injections.

Toxicity

As expected from prior studies by using Montanide, we observed grade 1 or 2 injection site reactions in all participants (Table 3). These began to occur within 24 hours of the injections and reached their maximum by patient report within a week of the immunizations. The induration and erythema persisted beyond the study participation, albeit with slightly less severity, in the majority of the patients (Supplementary Fig. S1). There was no obvious correlation between the dose of peptides and the size or duration of the induration. Other toxicities were mainly grade 1 and were not thought to be related to the study drug. One ovarian cancer patient experienced joint pain and myalgias several months after completing the immunizations. A rheumatologic workup did not show any evidence of an autoimmune disease. One ovarian cancer patient reported presyncopal episodes. At that time, we were alerted by the manufacturer of the GM-CSF to reports of syncope with the liquid formulation being used in our vaccine. We switched to a powdered formulation of GM-CSF and did not have any other reports of these symptoms from other patients. One patient experienced an exacerbation of her chronic psychiatric symptoms (listed as grade 4 psychiatric toxicity) which were consistent with prior exacerbations that had occurred before study enrollment and were thus deemed unrelated to the vaccination; however, these symptoms led her to discontinue study involvement.

Clinical activity

With a median follow-up of 492 days, 4 ovarian patients have relapsed and 3 have died. With a median follow-up of 383 days, 1 breast patient (who had had stage IV disease) has progressed and died (Table 2). The median survival has not been reached on either study.

Immune responses

We conducted ELISPOT analysis on peripheral blood from patients enrolled in the clinical trials to detect immune responses against the individual peptides (as seen in the example in Fig. 3). In majority of patients (4 of the 6 tested ovarian cancer samples and 5 of the 6 tested breast cancer samples), the prevaccination PBMC did not have detectable immune responses to the peptides.

Among the breast cancer patients following the immunizations, there was a trend for the low-dose cohort to have low to no immune responses, whereas all the high-dose

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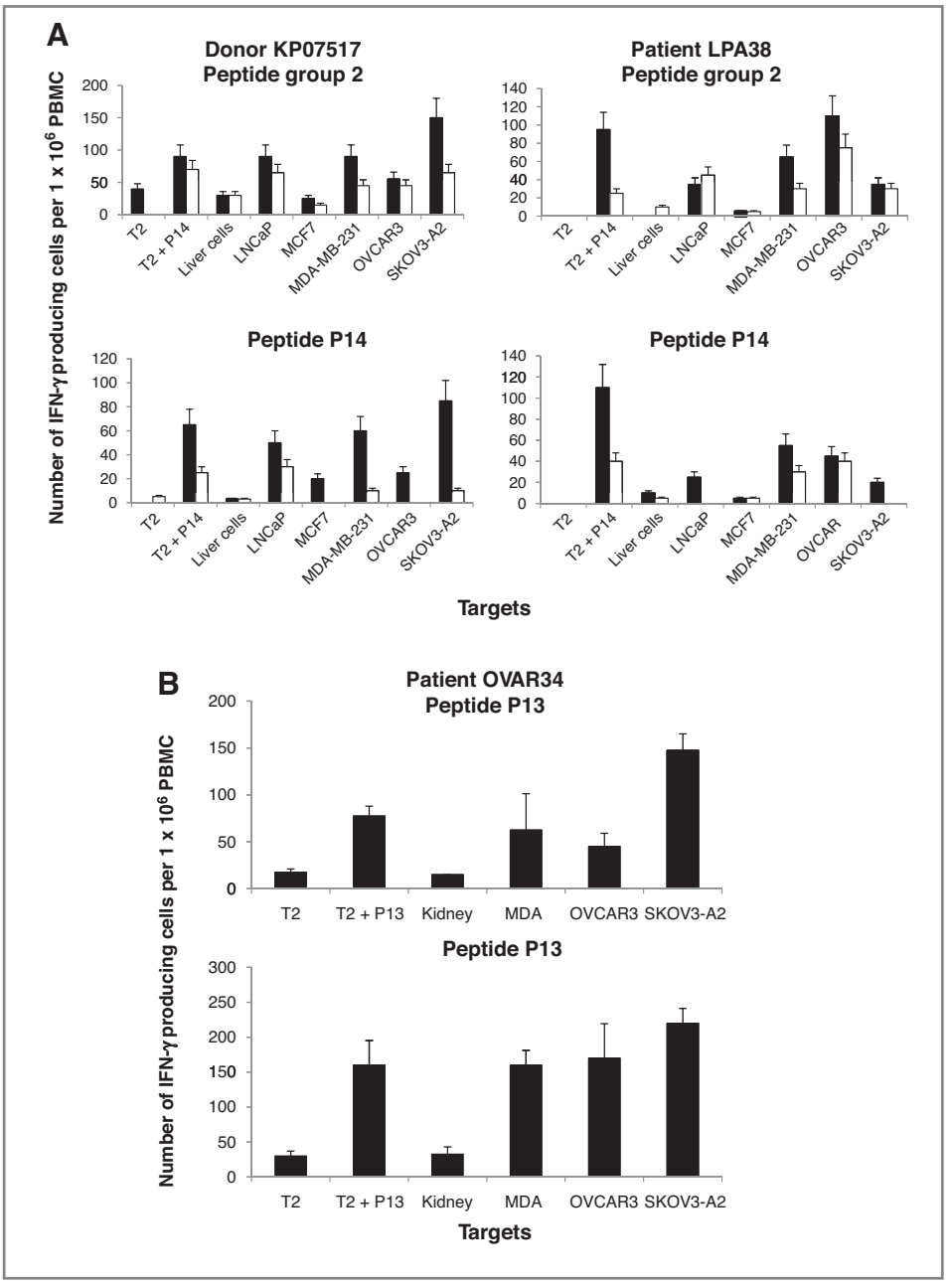

Figure 2. Comparison of CTLs generated from PBMC obtained from healthy donors and ovarian cancer patients. A, PBMC from a healthy HLA-A2+ donor (left) or an HLA-A2+ ovarian cancer patient (right) were *in vitro* stimulated with either group II peptides (top) or a component peptide P14 (bottom). These cells were tested in an overnight ELISpot assay by using T2 cells loaded with the group peptides, normal liver cell suspension obtained from an HLA-A2+ healthy donor and HLA-A2+ cancer cells lines (ovarian cancer cell lines SKOV3-A2 and OVCAR3, breast cancer cell lines MDA-MB-231 and MCF7, and a prostate cancer cell line LNCaP). IFN-γ producing cells were quantitated by using an Immunospot reader. Error bars represent SEM of experimental replicates. B, PBMC from an HLA-A2+ ovarian cancer patient were *in vitro* stimulated with either group II peptides (top) or 2 of the component peptides P13 and P15 (middle and bottom). These cells were tested in an overnight ELISpot assay by using T2 cells loaded with the group peptides, normal kidney cell suspension obtained from an HLA-A2+ healthy donor tissue, and HLA-A2+ cancer cells lines (ovarian cancer cell lines SKOV3-A2 and OVCAR3 and breast cancer cell line MDA-MB-231). IFN-γ producing cells were quantitated by using an immunospot reader. Error bars represent SEM of experimental replicates.

cohort patients who completed the immunizations developed immune responses. For example, breast cancer patients receiving low-dose vaccine had responses to fewer specific peptides (response to only 4 peptides for patient Imm-02-001, 0 for Imm-02-002, and 0 for Imm-02-003) compared with increased responses seen in the breast patients receiving high-dose vaccine (responses to 9 peptides for patient Imm-02-004, 12 for Imm-02-005, and 12 for Imm-02-006; Table 4). Although the vaccine formulation used in the high-dose cohort contained a mixture of peptides with binding affinities ranging from low to high, there does not seem to be a correlation between binding affinity of the peptide and *in vitro* and *in vivo* immune response.

Among the ovarian cancer patients, there were immune responses in both the low- (3 of 4 patients) and high-dose (2 of 4 patients) cohorts. Because of the small number of patients, it is not possible to determine whether these differences between breast and ovarian patients in responsiveness is due to a difference in the potency of the vaccines, or reflects a difference in the immune responsiveness of high-risk breast cancer patients versus high-risk ovarian cancer patients. Furthermore, with short-term follow-up, there is no clear correlation

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Table 2. Study participant demographics and clinical outcome

Patient ID	Diagnosis stage/subtype	Dose	Age	ECOG PS	No. of prior treatments	No. of injections	TTP (d)	Survival (d)
Ovarian cancer study participants								
Imm-01-001	IIIC papillary serous	Low	66	0	2	10	204	735
Imm-01-002	IIIC papillary serous	Low	45	0	2	7	–	1,115+
Imm-01-003	IIIC clear cell	Low	53	0	1	6	78	532
Imm-01-004	IIIC papillary serous	Low	43	0	1	6	–	726+
Imm-01-005	IIIC serous	High	56	0	1	6	442	524+
Imm-01-006	IIIC serous	High	55	0	2	6	65	289
Imm-01-007	IIIC poorly diff adenocarcinoma	High	63	0	1	6	–	451+
Imm-01-008	IIC clear cell	High	47	0	1	6	42	358+
Breast cancer study participants								
Imm-02-001	T3N3 (IIIB), ER+PR-HER2-	Low	53	0	1	6	–	562+
Imm-02-002	IV	Low	58	0	4	6	56	185
Imm-02-003	T2N1 (IIB), triple neg	Low	72	0	1	6	–	595+
Imm-02-004	T1cN1 (IIA), triple neg	High	63	0	1	6	–	383+
Imm-02-005	T2N0 (IIA) triple neg	High	41	0	1	6	–	390+
Imm-02-006	T1bN1a (IIA) ER-PR-HER2+	High	50	1	1	6	–	314+
Imm-02-007	T3N0 (IIB) triple neg	High	51	0	1	3	–	310+

NOTE: TTP indicates no evidence of progression; N/A indicates not available.
For breast cancer, the combination of adjuvant (or neoadjuvant) surgery, chemotherapy and radiation is all counted as 1 treatment.

between immune response and overall or progression-free survival and immune response.

With all patients taken together, there seems to be a dichotomy in response with a subset of patients who had a robust response to almost all (8–12) of the peptides and another subset who did not seem to respond to any of the peptides. There was only a single patient outside of these extremes who responded to 4 peptides. Furthermore, the existence of detectable preexisting immunity did not necessarily correlate with the ability to induce immune responses. The only breast patient with detectable prevaccine immunity (Imm-02-001) had only a minimal response to the vaccinations). The 2 ovarian patients with detectable prevaccine immunity (Imm-01-004 and Imm-01-008) did have augmentation of their immune responses after immunization. Interestingly, patients who had broad immune response to peptides also had T cells that were capable of recognizing both ovarian and breast cancer cells, but not against normal tissues, despite their primary diagnosis of either breast or ovarian cancer (Tables 4 and 5; Fig. 3).

Discussion

The purpose of this study was to extend the previous immunoproteomic identification of peptide antigens from human ovarian cancer cells by showing their relevance in activating tumor-specific T cells *in vitro* and *in vivo*. The broad application of these peptide antigens was supported by the demonstration that peptide-specific T cells could be activated that could recognize HLA-matched ovarian and breast cancer cells, but not normal cells with comparable HLA-A2 expression.

We observed that peptides originally identified from ovarian cancer-derived pools of HLA-A2 associated peptides could be formulated into combinations of peptides that induced immune responses as potent as those induced by single peptides. This observation permitted us to combine peptides into pools, first combining 6 peptides at a time into mixture and then later combining all 12 peptides into a single mixture. We then conducted 2 parallel pilot trials of 2 dose levels of the peptides combined with Montanide and GM-CSF and observed that these peptide vaccines were well tolerated and immunogenic. Potent peptide-specific immune responses were detectable after all vaccinations in 9 of 14 of the patients with available immune data. T cells induced in the cancer patients recognized not only peptide loaded targets but also HLA-A2–matched tumor cell lines with low to no recognition of HLA-A2–matched normal cells.

Among ovarian cancer patients, immune responses were observed in both the low- and high-dose cohorts whereas in breast cancer patients, the immune responses were predominantly in the high-dose cohort, which used a vaccine formulated to contain both low/intermediate and high-affinity peptides. The small sample size makes it difficult to conclude that this difference is due to the immune responsiveness of the breast versus ovarian

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Table 3. Adverse events

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4
Injection site reaction	11	4		
Pain during injection	3	1		
Induration	7	1		
Rash	1			
Pruritis	3	1		
Flu-like syndrome	5			
Fever	1			
Chills	1			
Fatigue	5	1		
Pain-abdominal	1			
Pain-musculoskeletal	2			
Pain-bone		1		
Joint pain	1			
Myalgia	1			
Hypotension	1			
Weight gain	1			
Mouth pain	1			
Cheilitis		1		
Anorexia	1			
Distension/bloating, abdominal	2			
Constipation	1			
Nausea	2			
Incontinence, urinary	1			
Infection-bladder	1			
Headache	2			
Patient odor	1			
Confusion	1			1
Depression				1
Anxiety				1
Agitation				1
Restless leg syndrome	1			
Lymphatics-adenopathy in groin	1			
Elevated creatinine/BUN	4			
Elevated AST/LFTs	2		1	
Jaundice		1		
Decreased leukocytes	2			
Hypercalcemia	2			
Decreased hemoglobin	4	2		
Decreased platelets	1			
Hypoglycemia	2			
Increased serum CO_2	2			
Increased chloride	1			
Hypokalemia			1	
Decreased albumin	1			

cancer patients, or there was an advantage to the vaccine formulation containing all the peptides. Nonetheless, the pilot data showing robust immune responses in cancer patients by using a vaccine containing a mixture of peptides with various affinities allows us to rationally design and conduct future studies to address questions such as these.

Although it was not the intention of this study to determine the clinical activity of the immunizations, we did observe that among the breast cancer patients, only 1 has progressed and that was the sole patient with stage IV disease. Among the ovarian cancer patients with a median follow-up of 492 days, 4 out of 8 had relapsed. The expected relapse rate in ovarian cancer after initial treatment is 75% within 18 to 22 months after completion of therapy (28). Compared with breast cancer, ovarian cancer may be a challenging malignancy in which to apply immunotherapy. Diefenbach (29), utilizing an NY-ESO-1 plus Montanide vaccine also reported that at a median follow-up of 11.3 months, 6 of 9 patients with high-risk epithelial ovarian cancer patients in first clinical remission had recurred.

We chose to use a multiepitope peptide vaccine for our clinical trial. Peptide-based vaccines have a number of potential advantages compared with other vaccine strategies such as full length protein or viral vectors (30). They are relatively simple and inexpensive to manufacture, analyze, and standardize. They lack the risks of infection or genetic integration or recombination. Finally, they can be designed to include multiple epitopes from the same or different proteins and to exclude potentially deleterious sequences. Although there have been numerous peptide vaccine studies, our study has similarities to a few and is unique from many others. Similar to our study, mixtures of antigenic peptides derived from a few proteins with various HLA-binding affinities have been reported to induce individual peptide-specific and tumor-specific T-cell responses in ovarian and melanoma patients (31–33). In these studies, different HLA-specific peptides derived from a small group of proteins that are not involved in critical tumor pathways (e.g., cancer testis antigens that do not play any role in tumorigenesis and survival) were mixed. One difference in our study was the use of 12 epitopes, all derived from different cancer pathways related proteins, whereas the majority of other studies have used 1 or a few epitopes from the same protein or utilized peptides derived from a small number of proteins. We believe that this large number of targets would reduce the chance of antigenic escape. Furthermore, because the peptides are derived from proteins which are relevant to the malignant phenotype, tumors may not as readily downregulate them. We chose single epitopes from multiple different peptides to reduce possible competition between epitopes from the same protein (34). Finally, we used a mixture of antigenic peptides with various HLA-binding affinities. In some cases, immune responses may be more effective when directed against epitopes with lower binding affinity that are poorly crosspresented and thus may be less susceptible to tolerance while still capable of activating tumor-specific immune responses (34).

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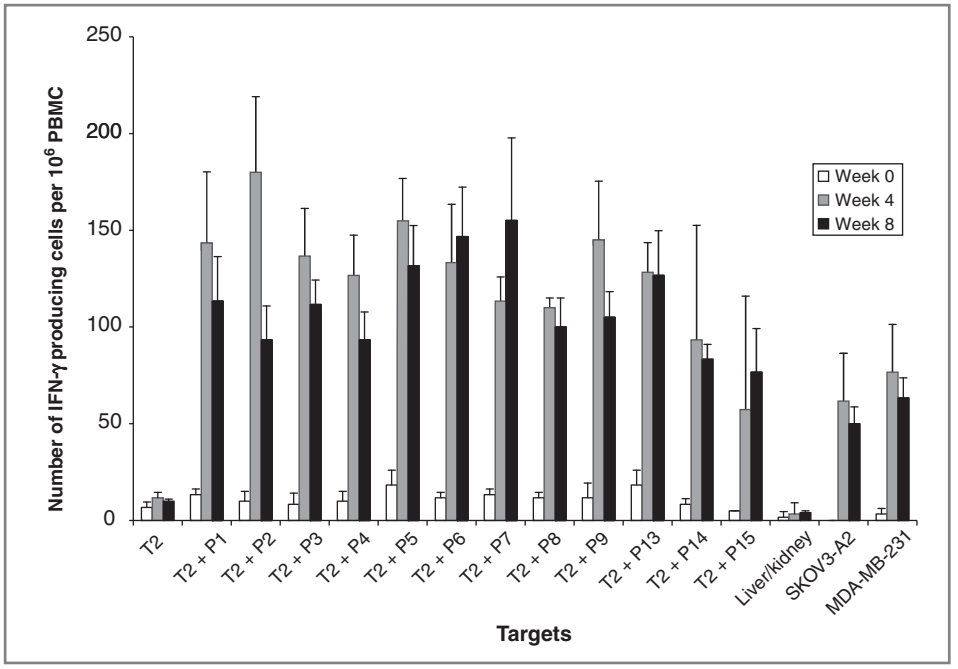

Figure 3. Patient PBMC peptide–specific response by ELISpot. Example of ELISPOT results for a breast cancer patient with immune response against peptides within the vaccine.

We acknowledge that there are also potential drawbacks of multiepitope vaccines. For example, there may be competition between peptides based on MHC affinity. In the low-dose cohort, we addressed this by choosing peptides to pool which would be less likely to result in 1 peptide outcompeting others for MHC binding (group 1 had low and medium MHC binders and group 2 had high binders). However, we found in our preclinical studies

Table 4. Peptide-specific T-cell responses

Patient ID	Peptides												
	P1	P2	P3	P6	P7	P8	P4	P5	P9	P13	P14	P15	Total[b]
Imm-01-001	+	+	+	−	+	+	−	+	+	+	+	−	9
Imm-01-002	+	+	+	+	+	+	+	+	+	−	+	+	11
Imm-01-003	−	−	−	−	−	−	−	−	−	−	−	−	0
Imm-01-004	+	+	−	−	+	+	−	−	+	+	+	+	8
Imm-01-005	−	−	−	−	−	−	−	−	−	−	−	−	0
Imm-01-006	+	+	+	+	+	+	+	+	+	+	+	+	12
Imm-01-007	−	−	−	−	−	−	−	−	−	−	−	−	0
Imm-01-008	+	+	−	+	+	+	+	+	+	+	+	−	10
Imm-02-001	−	−	+	−	−	−	−	+	−	−	+	+	4
Imm-02-002	−	−	−	−	−	−	−	−	−	−	−	−	0
Imm-02-003	−	−	−	−	−	−	−	−	−	−	−	−	0
Imm-02-004	+	+	+	+	−	+	+	+	+	+	−	−	9
Imm-02-005	+	+	+	+	+	+	+	+	+	+	+	+	12
Imm-02-006	+	+	+	+	+	+	+	+	+	+	+	+	12
Imm-02-007	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total[a]	8	8	7	6	7	8	6	8	8	7	8	6	

NOTE: Imm-01 (ovarian cancer); Imm-02 (breast cancer).
Positive response is defined as response to specific peptide greater than 40 spots (out of 10^6 PBMC) over prevaccination and/or negative control response by ELISpot.
ND: Pt Imm-02-007 received only 3 immunizations and did not return for follow-up testing.
[a]Total number of patients experiencing response to the particular peptide.
[b]Total number of peptides to which the patient responded.

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Table 5. ELISpot data with tumor cell line targets

Patient ID	Normal liver	SKOV3-A2	MDA-MB-231
IMM-01-001	−	+	+
IMM-01-002	−	+	+
IMM-01-003	−	−	−
IMM-01-004	−	+	+
IMM-01-005	−	−	−
IMM-01-006	−	+	−
IMM-01-007	−	−	−
IMM-01-008	−	−	−
IMM-02-001	−	−	+
IMM-02-002	−	−	−
IMM-02-003	−	−	−
IMM-02-004	−	−	+
IMM-02-005	−	+	+
IMM-02-006	+	−	−
IMM-02-007	ND	ND	ND

NOTE: Positive response is defined as response to specific peptide greater than 40 spots (out of 10^6 PBMC) over prevaccination and/or negative control response by ELISpot.

ND: Pt Imm-02-007 received only 3 immunizations and did not return for follow-up testing.

that even if all 12 were combined, there would not be competition (data not shown) and therefore in the high-dose clinical trial group, we combined all the peptides into 1 injection. Another potential drawback is the possibility that there would be multiple weaker responses rather than dominant robust responses to 1 or 2 of the peptides. In the high-dose cohorts of the clinical trials, we observed responses against all the peptides that were of a magnitude typical for peptide vaccine studies. Therefore, we do not believe that there was diminution or the dampening of immune response to a HLA-A2–binding immunogenic peptide, even when given in combination with other peptides.

A unique aspect of our study is the use of peptides identified by their association with the HLA-A2 molecules on tumor cells and not on nonmalignant cells. Peptide epitopes have previously been identified by utilizing cloned T cells specific for tumors and then by using genetic approaches to search for the epitopes recognized by the T-cell receptors. Others have been chosen by searching for proteins overexpressed by tumors and then by using motif algorithms for estimating what peptide fragments would fit within a particular HLA molecule. We believe that by utilizing epitopes naturally presented by tumor cells, we will be able to induce immune responses against the authentic T-cell targets.

We designed our vaccine to include GM-CSF and Montanide based on strategies previously shown to be safe and immunogenic. Numerous clinical trials testing tumor associated peptides have used adjuvants based on bacterial cell wall products [Incomplete Freund's adjuvant (IFA) or Montanide ISA-51] or with immune-activating agents such as GM-CSF (35–43). There is controversy as to whether GM-CSF improves immune responses or activates suppressive responses (44, 45). Parmiani and colleagues suggested GM-CSF may increase the vaccine-induced immune responses when administered repeatedly at relatively low doses (range 40–80 μg for 1–5 days), but an opposite effect could occur at dosages of 100 to 500 μg (46). We used GM-CSF at 100 μg per injection and believe the GM-CSF was likely immunostimulatory at this dose. Regarding our use of a weekly schedule (with a 1-week break in the middle), the best immunization schedule is unclear with some studies by using weekly or biweekly followed by monthly (28–37).

One negative aspect of our study was the substantial incidence of local reactions that persisted beyond the study participation. This has been observed with Montanide containing vaccines previously (32) and may have been exacerbated by giving a portion of the injection intradermally. We had hoped that this could lead to enhanced dendritic cell uptake of the peptides but possibly led to the injection reactions. Another potential criticism is that the immune responses remain modest. Low levels of immune responses may be because of the delivery of free peptides mixed with Montanide and GM-CSF, instead of a vaccine formulation that enhances immune activation and helper T-cell stimulation. Currently, we are carrying out a phase I/II study with DPX-0907 consisting of 7 of the 12 tumor-specific HLA-A2–restricted peptides from this study, a universal T-helper peptide, a polynucleotide adjuvant, in a liposome, and Montanide ISA-51 VG in ovarian, breast, and prostate cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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