Clinical Cancer Research

Results from a Phase I Clinical Study of the Novel Ii-Key/HER-2/*neu*(776–790) Hybrid Peptide Vaccine in Patients with Prostate Cancer

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Abstract

Purpose: Active immunotherapy is emerging as a potential therapeutic approach for prostate cancer. We conducted the first phase I trial of an Ii-Key/HER-2/*neu*(776–790) hybrid peptide vaccine (AE37) with recombinant granulocyte macrophage colony-stimulating factor as adjuvant in patients with HER-2/*neu*$^+$ prostate cancer. The primary end points of the study were to evaluate toxicity and monitor patients' immune responses to the vaccine.

Experimental Design: Thirty-two HER-2/*neu*$^+$, castrate-sensitive, and castrate-resistant prostate cancer patients were enrolled. Of these, 29 patients completed all six vaccination cycles with AE37. Immunologic responses in the total patient population were monitored by delayed-type hypersensitivity and IFN-γ ELISPOT and intracellular staining. Regulatory T-cell (Treg) frequency and plasma HER-2/*neu* and transforming growth factor-β levels were also determined. Immunologic responses were also analyzed among groups of patients with different clinical characteristics. Local/systemic toxicities were monitored throughout the study.

Results: Toxicities beyond grade 2 were not observed. Seventy-five percent of patients developed augmented immunity to the AE37 vaccine and 65% to the unmodified AE36 peptide as detected in the IFN-γ–based ELISPOT assay. Intracellular IFN-γ analyses revealed that AE37 elicited both CD4$^+$ and CD8$^+$ T-cell responses. Eighty percent of the patients developed a positive delayed-type hypersensitivity reaction to AE36. Additionally, significant decreases could be detected in circulating Treg frequencies, plasma HER-2/*neu*, and serum transforming growth factor-β levels. Patients with less extensive disease developed better immunologic responses on vaccination.

Conclusion: AE37 vaccine is safe and can induce HER-2/*neu*–specific cellular immune responses in patients with castrate-sensitive and castrate-resistant prostate cancer, thus emphasizing the potential of AE37 to target HER-2/*neu* for the immunotherapy of prostate cancer. *Clin Cancer Res; 16(13); 3495–506.*

Despite conventional treatments for prostate cancer, such as surgical resection and radiotherapy, many patients will finally develop metastases. Hormone deprivation is initially effective in the majority of patients with metastatic disease (1). Eventually, however, prostate cancer patients will relapse due to progression of prostate cancer toward hormone-independent growth of the tumor. Unfortunately, advanced prostate cancer responds poorly to chemotherapeutic agents of which taxane-based treatments are nowadays considered as the common treatment of castrate-resistant prostate cancer (2). Therefore, novel approaches are required for the prevention and/or effective management of patients with advanced prostate cancer.

Prostate glands are frequently diffusely infiltrated with both CD4$^+$ and CD8$^+$ T cells (3, 4), suggesting that prostate cancer may represent an attractive target for immunotherapy. Indeed, several prostate-specific gene products have been reported that may function as tumor/tissue antigens (5–7). Active immunotherapies in prostate cancer have used a variety of methods to augment immune responses to prostate cancer–associated antigens. Of these, the most promising agents include PROSTVAC and APC8015 (Provenge). PROSTVAC-VF comprises two recombinant viral vectors, each encoding transgenes for prostate-specific antigen (PSA). In two phase II studies, PROSTVAC-VF immunotherapy was well tolerated and

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Translational Relevance

Tolerance mechanisms against (self) tumor proteins hamper the generation of robust antitumor responses and thus represent a major limitation for tumor immunotherapy. One method to overcome such tolerance mechanisms is to increase the immunogenicity of synthetic peptide vaccines containing tumor protein epitopes. In a phase I clinical trial, we provided immune activation signals through an Ii-Key/HER-2/*neu*(776–790) (AE37) hybrid peptide/granulocyte macrophage colony-stimulating factor vaccine in patients with prostate cancer. We describe the safety and vaccine-induced immunity of this combination in these patients. Our results show that the AE37 hybrid peptide/granulocyte macrophage colony-stimulating factor vaccine strategy is safe and effective in eliciting an immune response against the HER-2/*neu* protein.

These observations suggest that further investigation of AE37 in a randomized phase II clinical trial, in which clinical benefit in terms of evaluating time to disease progression can be assessed, is warranted.

associated with a significant reduction in the death rate and an improvement in median overall survival in men with metastatic castrate-resistant prostate cancer (8, 9). APC8015 is a novel immunotherapeutic, which consists of autologous dendritic cells pulsed *ex vivo* with PA2024, a recombinant fusion protein consisting of granulocyte macrophage colony-stimulating factor (GM-CSF) and prostatic acid phosphatase, as an immunogenic agent. Two phase III randomized clinical trials have shown a survival benefit in patients with metastatic hormone-refractory prostate cancer (10, 11). A novel method to augment potency of MHC class II epitope peptide presentation has now become available in the form of Ii-Key/MHC class II hybrid peptides (12). Such Ii-Key hybrid peptides contain an immunoregulatory segment of the Ii protein that catalyzes direct charging of MHC class II epitopes to the peptide-binding groove, circumventing the need for intracellular epitope processing (13). The shortest active sequence of the Ii immunoregulatory region consists of four amino acids [LRMK (Ii-Key peptide); ref. 12]. Covalent linkage of this Ii-Key segment to MHC class II epitopes significantly augments antigen presentation (13–16). Work from our laboratory has shown that the HER-2/*neu*(776–790) epitope serves as a compelling tumor antigen and that $CD4^+$ T cells primed with the synthetic HER-2/*neu*(776–790) peptide help autologous CTL for increased antitumor activity (17–19). In a series of studies (14, 17–20), we have shown that the Ii-Key/HER-2/*neu*(776–790) hybrid (AE37) induces more potent immunologic responses both *in vitro* and *in vivo* compared with the nonmodified HER-2/*neu*(776–790) peptide (AE36). Recently, Holmes et al. (21) conducted a phase I study of the AE37 hybrid peptide in HER-2/*neu*–positive breast cancer patients and showed strong immunologic responses to the vaccine.

In prostate cancer, several studies have reported increased HER-2/*neu* expression in patients with clinically localized and more advanced hormone-refractory disease (22–24). In addition to stimulating cell division, HER-2/*neu* also confers an increased malignant potential to prostate cancer cells by activating the androgen receptor pathway in the absence of androgen (25), thus contributing to the development of castrate-resistant prostate cancer. Indeed, clinical studies have shown that progression of prostate cancer toward androgen independence is characterized by a gradual increase in HER-2/*neu* expression (26, 27). Therefore, HER-2/*neu* targeting represents a promising therapeutic intervention for prostate cancer patients. Active immunotherapy provides the added benefit in that specifically activated immune cells can recognize and kill tumor cells expressing lower levels of HER-2/*neu* than are required for recognition by Herceptin. We report here the results of the first trial with AE37-immunized prostate cancer patients.

Materials and Methods

Patient population, clinical protocol, and study design

The phase I clinical trial protocol was approved by the Hellenic National Organization for Medicines (EOF) under investigational number IS107/2006, EudraCT 2006-003299-37. Patients' primary tumors were evaluated by immunohistochemistry (IHC) for HER-2/*neu* expression (27). After proper counseling and written informed consent, castrate-sensitive and castrate-resistant prostate cancer patients with HER-2/neu^+ (IHC score 1^+ to 3^+), nonmetastatic and metastatic disease, and Eastern Cooperative Oncology Group (ECOG) 0 or 1 were considered eligible. Before vaccination, patients were skin tested with *Candida albicans* (Lofarma) and considered immunocompetent if they reacted to the antigen. Exclusion criteria included patients with ECOG ≥2; active infection; severe cardiovascular comorbidity; acute/chronic HBV, HCV, and HIV; HIV seropositivity; diagnosis of other primary solid/hematologic malignancy and any pathologic comorbidity affecting patients' compliance in the proposed clinical protocol (e.g., mental disorder); and immunologic inactivity (negative skin test). Patients were assigned to receive 500 μg of the AE37 vaccine mixed with 125 μg of GM-CSF at each vaccination cycle. The doses for both AE37 and GM-CSF used were found to be optimal in a phase I study with disease-free, node-negative breast cancer patients (21) where a similar vaccination schedule was followed.

Vaccine

The Ii-Key/HER-2/*neu*(776–790) hybrid peptide, AE37 (Ac-LRMKGVGSPYVSRLLGICL-NH2), was produced in accordance with current federal guidelines for good manufacturing practices by NeoMPS, Inc. Peptide purity (>95%) was verified by high-performance liquid chromatography and mass spectrometry. Sterility testing was

carried out by the manufacturer. The vaccine was prepared as previously described (21). Briefly, 500 μg of lyophilized peptide were reconstituted in 0.5 mL of sterile saline and mixed with 125 μg GM-CSF (Berlex) in 0.5 mL. The 1.0 mL inoculum was split and administered intradermally at two sites 5 cm apart in the same extremity. All patients received 6 monthly vaccinations with the AE37 vaccine. GM-CSF was reduced by half serially in subsequent inoculations when local reactions of ≥100 mm in diameter (21) were observed. If the latter patients continued with robust local reactions without GM-CSF, then the peptide amount was also reduced by half serially.

Toxicity

Patients were observed 1 hour postvaccination for immediate hypersensitivity and returned 48 hours later to have the injection sites examined. Both local toxicity at the injection sites and systemic toxicity were evaluated in all patients for all inoculations. Toxicities were graded using the National Cancer Institute Common Terminology Criteria for Adverse events v3.0 and were reported on a 0 to 5 scale.

Peptides and proteins

The AE37 peptide (Ac-LRMKGVGSPYVSRLLGICL-NH2) is a fusion of the Ii-Key peptide (LRMK) with the native HER-2/*neu* peptide AE36 (aa776–790: GVGSPYVSRLLGICL; ref. 19). To assess whether immune responses elicited *in vivo* reflected reactivity against the native HER-2/*neu* peptide, we evaluated immune responses against both AE36 and AE37. *C. albicans* and pokeweed mitogen (Sigma) were used as positive controls for *in vitro* immunogenicity assays. Peripheral blood mononuclear cells (PBMC) in culture medium alone were used as a negative control.

ELISPOT assay

Blood was drawn from patients before treatment to establish a baseline (pre vac), then monthly before each inoculation, 1 month after the final (6th) vaccination (post vac), and 6 months after completion of vaccinations (long term). PBMCs were isolated by Ficoll gradient separation. Freshly isolated PBMCs were cultured in X-VIVO 15 medium (BioWhittaker, Cambrex) supplemented with 2% AB human serum (Sigma), with the individual peptides (10 μg/mL of AE36 or AE37) or controls (*C. albicans*, 1:50; pokeweed mitogen, 2 μg/mL) in precoated IFN-γ ELISPOT plates (MABTECH AB), in quadruplicate at 2.5×10^5 cells per well. The plates were incubated at 37°C in a humidified 5% CO_2 incubator for 40 hours and developed as described by the manufacturer. Spots were enumerated using an ELISPOT analyzer (A.EL.VIS GmbH). Data were calculated as previously described (28). Briefly, data are presented as specific spots (experimental spots minus background spots; i.e., PBMCs in medium alone) per 10^6 PBMCs, or as a calculated 1/frequency of specific spots in 10^6 PBMCs. Patients were considered to have preexisting immunity if, at baseline, the mean antigen-specific spots per well were statistically different ($P < 0.05$) from background. Patients were considered to have increased response if the mean number of specific spots per well (i.e., experimental number of spots per well minus the mean number of spots of the background wells at the corresponding cycle of vaccination) at a given vaccination round was greater than 2 SD above the pre vac–specific spots, remained the same if the mean number of specific spots per well was within 2 SD of the pre vac–specific spots, and decreased if the mean number of specific spots per well was greater than 2 SD below the pre vac–specific spots.

Intracellular staining

Cultures set up as above were harvested, and recovered cells were stained with allophycocyanin-labeled anti-CD4 and peridinin chlorophyll protein–labeled anti-CD3 antibodies (BD Biosciences) at room temperature for 15 minutes. Cells were then washed, fixed, permeabilized, and stained with fluorescein isothiocyanate (FITC)–labeled anti–IFN-γ (BD Biosciences) using BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) according to the manufacturer's protocol. Stained cells were analyzed by FACSCalibur and CellQuest software (BD Biosciences). The percentage of IFN-γ^+ cells are presented corrected for background.

Phenotypic characterization of Tregs

Peripheral blood from patients was collected pre vac, post vac, and at long term. Treg cells were detected in whole blood by a lyse-no-wash method. In brief, 50 μL of whole blood (with heparin or EDTA) were stained for

Table 1. Patient characteristics

Age, median (range)	66 y (46–84 y)	
	%	No. patients
Race		
Caucasian	100	32
Gleason score		
Low (3–6)	31	10
Intermediate (7)	38	12
High (8–10)	31	10
Stage*		
Stage I	0	0
Stage II	9	3
Stage III	47	15
Stage IV	44	14
Biochemical castration		
Castrate sensitive	66	21
Castrate resistant	34	11
PSA (ng/mL)		
<0.2	41	13
0.2–10	22	7
>10	37	9
All median (range)	0.86 (0.01–367)	

*Staging according to American Joint Committee on Cancer.

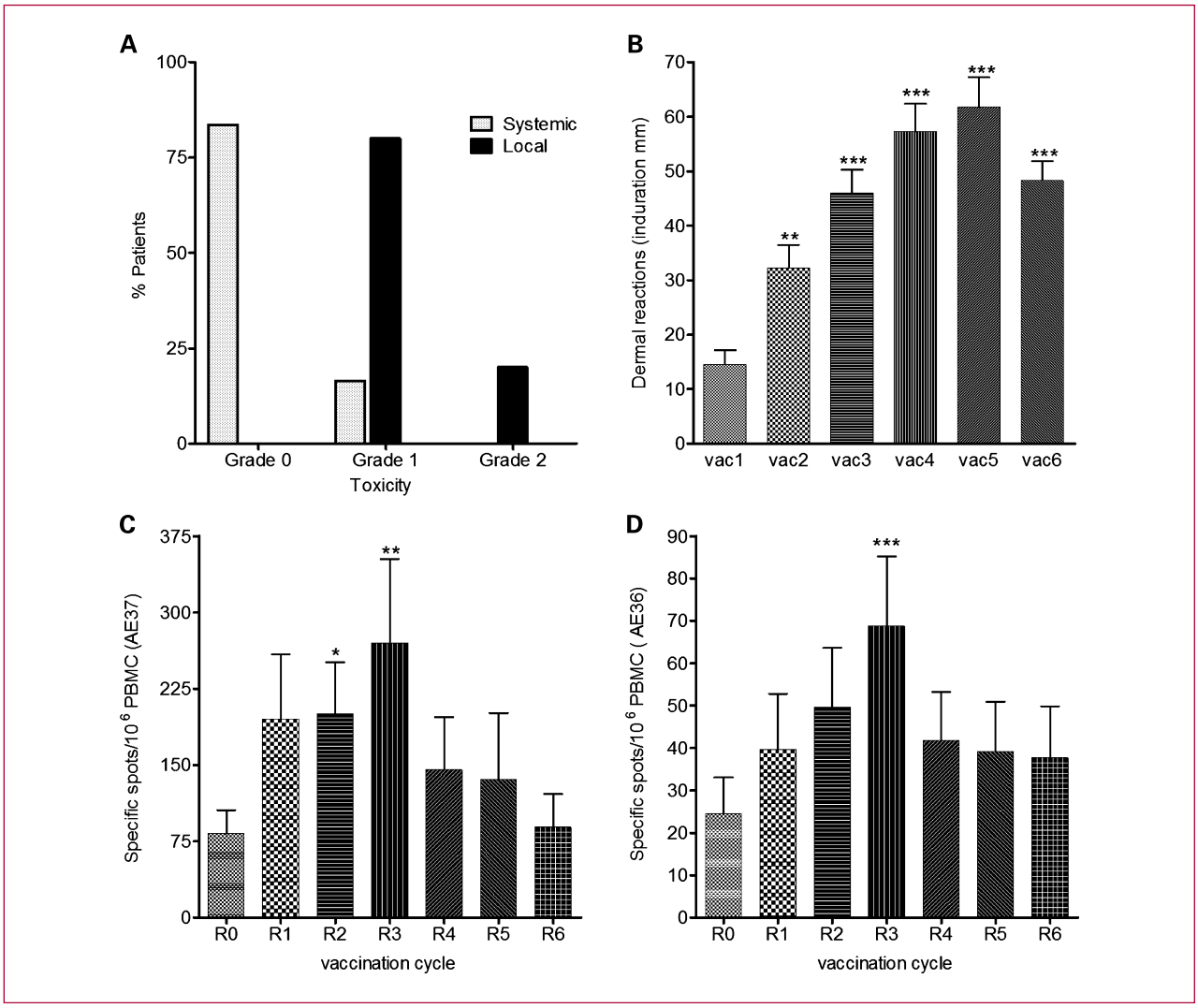

Fig. 1. Toxicity, dermal reactions, and immunity to AE37. A, maximum local and systemic toxicities observed during vaccinations (graded according to common terminology criteria). B, dermal reactions (orthogonal mean ± SEM) were recorded 48 hours postvaccination throughout the vaccination series. **, $P = 0.0013$; ***, $P < 0.0001$. IFN-γ ELISPOT responses to AE37 (C) *, $P < 0.05$; **, $P < 0.01$ or to AE36 (D) ***, $P < 0.001$ during vaccinations with AE37. Specific spots (mean ± SEM) are shown at baseline (R0) and 1 month after each vaccination (R1–R6).

15 minutes at room temperature with pretitrated combinations of anti–CD45-peridinin chlorophyll protein, CD4-allophycocyanin, CD25-fluorescein isothiocyanate, and CD127-phycoerythrin (all purchased from BD Biosciences). Red blood cells were lysed with 450 μL of ammonium chloride lysing solution and analyzed within 1 hour on a FACSCalibur. Tregs were defined by gating on low SSC $CD45^+$ $CD4^+$ lymphocytes expressing high CD25 (higher than the non $CD4^+$ lymphocytes) and negative/low for CD127 (29).

Plasma human epidermal growth factor receptor extracellular domain and transforming growth factor-β determination

Plasma samples were collected pre vac, post vac, and at long term. Commercially available immunoassay kits were used for measurements of transforming growth factor-β (human TGF-β1 instant enzyme-linked immunosorbent assay, Bender MedSystems GmbH), and HER-2/*neu* extracellular domain (HER-ECD; Immuno 1, Bayer Diagnostics).

Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) reaction was done before the vaccination regimen (pre vac DTH), post vac, and at long term. The DTH reaction was assessed with 100 μg AE36, which were injected intradermally at a site on the extremity opposite the one used for vaccination. DTH reactions, as well as dermal reactions to the vaccine, were measured in two dimensions at 48 hours using the sensitive ballpoint pen method, and results are reported as an orthogonal mean (21). Patients must have an induration of >5 mm post vac to be considered as having developed a positive DTH reaction (21).

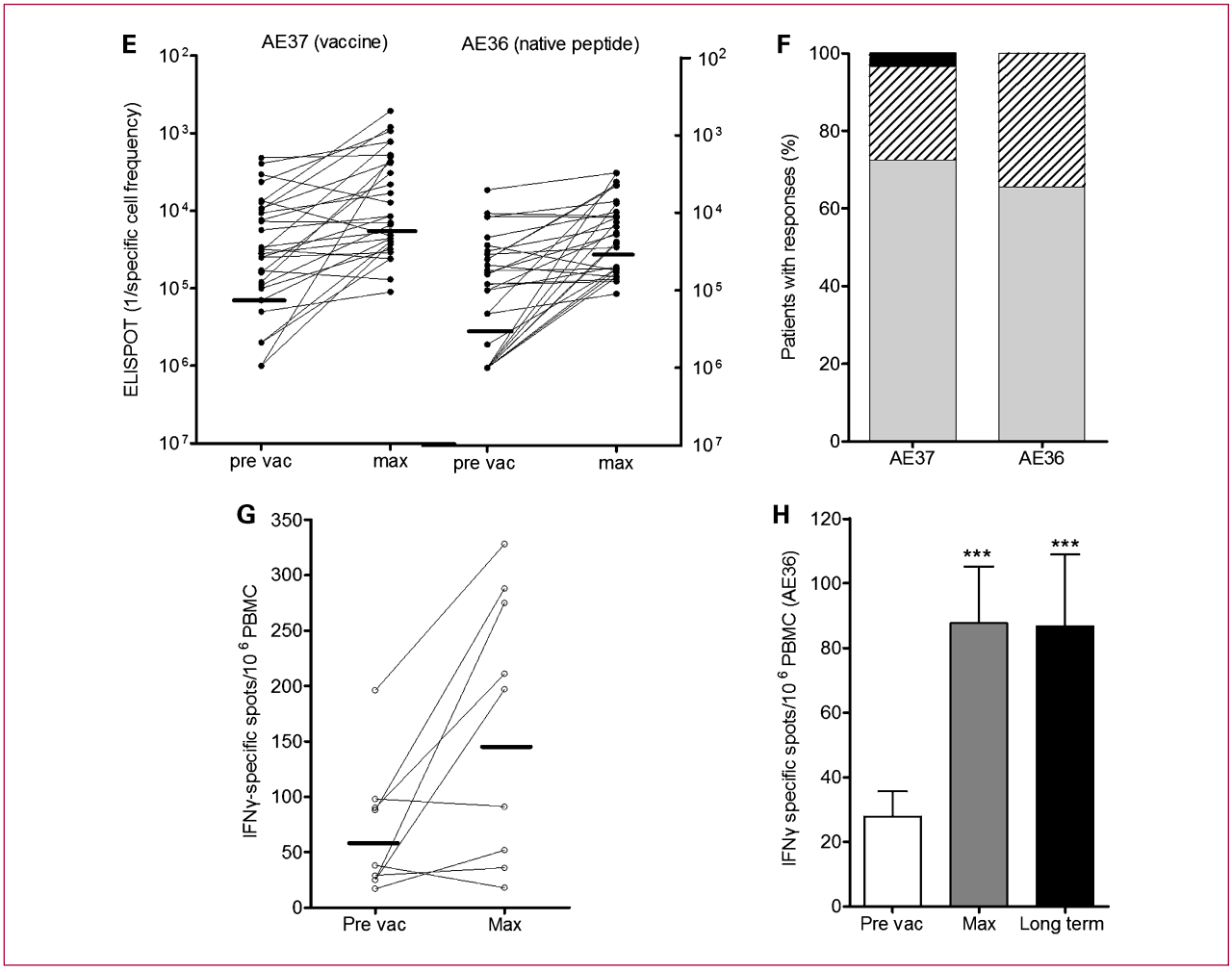

Fig. 1. ***Continued.*** E, individual pre vac and maximal (max) ELISPOT IFN-γ responses obtained during vaccination. Horizontal bars, median values. F, percent AE37 and AE36 specific immunity after vaccination. Gray, increased; hatched, unchanged; black, decreased. G, pre vac and maximal ELISPOT IFN-γ responses in patients with preexistent immunity to AE36. H, durable immunity to AE36 induced by the AE37 vaccine: mean ± SEM. IFN-γ ELISPOT responses to AE36 at long term did not differ compared with maximal responses. ***, $P < 0.0001$.

Statistical analysis

GraphPad Prism version 4 software was used for the statistical analysis of data. Two-tailed Wilcoxon matched pair test and Spearman correlation test at 95% confidence interval were used for statistical evaluation. Statistically significant differences were considered when the *P* value was ≤0.05.

Results

Patients

Thirty-two patients with HER-2/*neu*$^+$ prostate cancer were enrolled. Patient characteristics are given in Table 1. Twelve of the stage IV patients were metastatic with bone metastases. Three of the enrolled metastatic patients discontinued vaccinations (one patient at the second cycle and two at the third cycle) at time of severe disease progression. Thus, 29 of the patients completed all vaccinations and could be evaluated immunologically. Treatment before vaccinations included surgery, radiotherapy, chemotherapy (docetaxel), and androgen ablation (bicalutamide and luteinizing hormone-releasing hormone (LHRH) agonist). During vaccinations, 19 patients did not receive any other treatment; 5 patients who had progressive disease received additional chemotherapy; the remainder received hormonal therapy alone or combined with radiotherapy.

Toxicity

Maximum local and systemic toxicities for all 32 patients are shown in Fig. 1A. We observed no toxicities beyond grade 2. Predominant systemic symptoms were grade 0, with only three patients experiencing grade 1 fever during the first night, which was easily manageable with paracetamol. Local toxicity was mild with most of the patients having grade 1 symptoms (pain at the injection site accompanied with mild swelling and itching), whereas grade 2 symptoms (large swelling with blisters at the

injection site) were scored in six patients. Two patients required GM-CSF reduction, one after the first vaccination and one after the fourth vaccination. One additional patient required, initially (after the first vaccine), GM-CSF reduction; after the second vaccine, GM-CSF omission; and finally at the fourth vaccine, peptide reduction.

Dermal reactions

Dermal reactions at each vaccination cycle (AE37 with GM-CSF) were determined 48 hours after immunization by measurement of induration. Already after the first vaccination, the vast majority of patients (25 of 29) developed dermal reactions that progressively increased after the second vaccination (27 of 29 patients), with all patients responding after the third vaccination cycle (Fig. 1B).

The AE37 vaccine induces IFN-γ–producing circulating T cells

To study the effect of the AE37 vaccine on the immune system, PBMCs were isolated pre vac and during vaccinations, and then analyzed for the presence of AE37-specific T cells by IFN-γ–based ELISPOT. The overall PBMC-derived IFN-γ response to AE37 and native AE36 peptide pre vac and during each round of vaccination is shown in Fig. 1C and D. The number of IFN-γ–secreting cells in response to *in vitro* stimulation with AE37 increased already after the first round of vaccination (R1) and peaked 2 months later (i.e., after the third vaccine, R3; Fig. 1C). Patients' PBMCs were also assessed *in vitro* for IFN-γ response to the nonmodified AE36 HER-2/*neu*(776–790) peptide, which is naturally processed and expressed on both tumor cell lines and primary tumors from various types of cancers, including prostate adenocarcinomas (18, 19). The maximum AE36 peptide–specific immune responses were also observed after the third vaccination (Fig. 1D). Individual patient responses are shown in Fig. 1E. The median AE37-specific T-cell frequency pre vac was 1 in 33,333 (Fig. 1E). The maximal response was a median frequency of 1 in 11,765 PBMCs ($P < 0.0001$ compared with pre vac). Seventy-two percent of the patients (21 of 29) developed augmented immunity to AE37 peptide, seven patients (24%) did not increase, and one patient had a significant decrease on immunization (Fig. 1F). The median T-cell response to AE36 peptide pre vac was 1 in 83,333 (Fig. 1E). During vaccinations, the median response to this peptide was increased up to a frequency of 1 in 19,231 ($P < 0.0001$ compared with pre vac; Fig. 1E). Nineteen of 29 patients (65.5%) developed augmented immunity, 10 patients (34.5%) did not augment, and none had a significant decrease to AE36 with immunization (Fig. 1F). Of note, 9 (31%) of 29 patients had preexistent immunity to the native peptide, and five of those significantly increased their responses during vaccinations ($P = 0.0017$; Fig. 1F). The average IFN-γ response to AE36 at long-term assessment was comparable with average maximal response (Fig. 1H) suggesting that the AE37 vaccine induces long-lasting systemic immunity.

Both vaccine-specific and AE36-specific $CD4^+$ and $CD8^+$ T cells are induced by vaccination

Development of immunity to either the AE37 vaccine peptide or to the native AE36 peptide was shown by estimating the percentage of patients' circulating T cells that positively stained for intracellular IFN-γ after exposure to the respective peptide. As shown in Fig. 2, vaccination caused the successful induction of patients' AE37-specific $CD4^+$ and $CD8^+$ T-cell subsets (Fig. 2A and D). We also observed a parallel increase of both T-cell subsets responding to the native AE36 peptide (Fig. 2B and E). The median maximal percentage of $CD4^+$ T cells specific for AE37 was 0.025 (Fig. 2A), and 0.017 for AE36 (Fig. 2B). Median preimmunization values were less than 0.0001% for both AE37 and AE36. We also observed remarkable $CD8^+$ T cell–mediated responses to AE37 (median maximal percent 0.074%; $P < 0.0005$ compared with pre vac) and to AE36 (median maximal percent 0.085%; $P < 0.0001$ compared with pre vac; Fig. 2D and E, respectively). Average maximal responses to AE36 for both subsets remained unchanged at long-term assessment (Fig. 2C and F).

Regulatory T cells in patients

Tregs were defined as $CD4^+CD25^{high}CD127^{low/-}$ cells (29). Immunization with the AE37 peptide and GM-CSF was associated with a statistically significant decrease of Treg frequency at long term (median 4.89%; range 1.30–9.00% in vaccinated versus 5.76%; range 1.46–10.75% pre vac; Fig. 3A). Post vac Treg frequency was also reduced compared with pre vac (median 5.18%; range 2.12–10.46%), but without reaching statistical significance ($P = 0.4363$).

TGF-β and HER-2/*neu* extracellular domain levels in plasma

TGF-β, a potent immunosuppressant of T-cell immunity in cancer patients (30), has been shown to be elevated in the serum of patients with prostate cancer (31, 32). The median level of plasma TGF-β pre vac was 9.34 ng/mL (range 4.11–31.84 ng/mL). Post vac TGF-β levels, although reduced, did not reach statistical significance (median 8.46 ng/mL; range 4.42–31.99 ng/mL; $P = 0.4043$). However, at long-term assessment, a statistically significant reduction was achieved (7.49 ng/mL; range 2.90–24.23 ng/mL; Fig. 3B) Importantly, percent changes in long-term, compared with pre vac, TGF-β plasma levels statistically correlated with the corresponding percent changes in circulating Tregs (Fig. 3C).

HER-2/*neu* extracellular domain (HER-ECD) levels in serum have been reported to be elevated in prostate and breast cancer patients (33, 34). We show herein a significant decrease in plasma HER-ECD levels post vac (median 5.9 ng/mL; range 4.0–12.5) compared with pre vac (median 9.2 ng/mL; range 6.3–15.3 ng/mL) in our prostate cancer patients (Fig. 3D).

***In vivo* immunologic responses**

To more closely determine the effectiveness of the AE37 vaccine at inducing *in vivo* immunologic responses, we

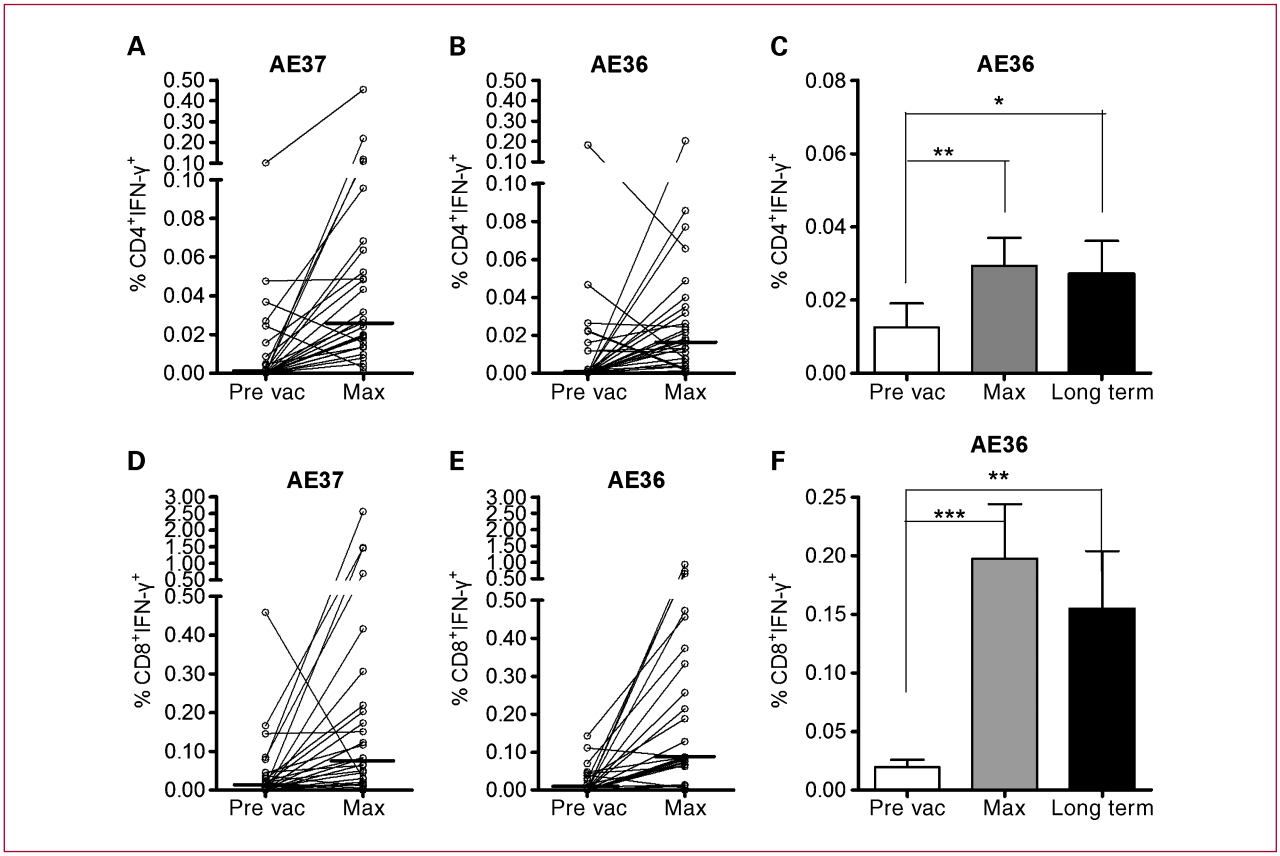

Fig. 2. Increased numbers of both $CD4^+$ and $CD8^+$ IFN-γ–producing T cells after vaccination. Individual responses to AE37 (A and D) and AE36 (B and E). Vaccine-induced AE36-specific $CD4^+IFN\text{-}\gamma^+$ (C) or $CD8^+IFN\text{-}\gamma^+$ (F) subsets during vaccinations (maximal responses) and at long term. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

measured and compared DTH responses to the nonmodified AE36 peptide pre vac, post vac, and at long term. Of the 29 patients who completed the vaccination series, 23 (79%) had significant post vac DTH reactions (>5 mm) with a median post vac induration diameter of 11.5 mm (range 0.5–38.75 mm) compared with a median of 0.0 mm (range 0.0–8.0 mm) at baseline (Fig. 4). We did not observe any significant changes in long-term DTH reactions (median 10.0 mm; range 0.5–45.0 mm) compared with post vac DTH (Fig. 4).

Comparison of AE37-induced immunity among different patient groups

Patients were retrospectively stratified for data analysis, which might be useful for providing insight for patient selection preceding a phase II clinical trial. Retrospective stratification was done by staging [i.e., stage II-III (nonmetastatic patients) versus stage IV (patients with advanced disease)], by response to testosterone lowering (i.e., castrate-sensitive versus castrate-resistant), and by HER-2/*neu* expression (i.e., HER-2/*neu* low expressors with IHC 1^+ and 2^+ versus *HER-2/neu* overexpressors with IHC 3^+). As can be seen from Fig. 5, there was a tendency for better *in vitro* and *in vivo* immune responses to vaccination in the groups of patients with less-progressed disease. The maximum increase of specific IFN-γ–producing T cells in response to vaccination with AE37, detected by ELISPOT and expressed as mean fold increase from baseline levels, was higher in stage II-III (Fig. 5A) and castrate-sensitive (Fig. 5B) patients compared with stage IV and castrate-resistant patients, respectively, although this difference was not statistically significant. Similarly, DTH responses to the vaccine, determined as the difference of post vac minus baseline DTH, were statistically significantly lower in stage IV patients (Fig. 5D) and also lower (although not statistically significant) in castrate-resistant patients (Fig. 5E). Interestingly, IFN-γ responses in patients with HER-2/*neu*–overexpressing tumor at first diagnosis were highly reduced (with statistical significance) compared with patients exhibiting low HER-2/*neu* expression in their biopsies (Fig. 5C). However, no statistically significant difference was observed in the DTH responses with regard to HER-2/*neu* expression (Fig. 5F).

Taking into consideration that such *in vitro* (IFN-γ) or *in vivo* (DTH) immunologic responses might have been influenced by the tumor-induced suppressive milieu, we also evaluated the plasma TGF-β levels and the Treg frequencies before the initiation of vaccinations (baseline values) in the same groups of patients. Increased baseline

TGF-β plasma concentrations were recorded in patients with progressed disease, i.e., stage IV (Fig. 5G) and castrate-resistant (Fig. 5H), compared with stage II-III and castrate-sensitive patients, although not reaching statistical significance. No differences in pre vac Treg frequencies could be documented in any patient group (Fig. 5J–L).

Discussion

We report here the results of a phase I trial evaluating the safety and immunologic activity of a HER-2/*neu* hybrid peptide vaccine (AE37) in patients with castrate-sensitive and castrate-resistant, metastatic and nonmetastatic prostate cancer. This treatment was not associated with significant adverse events; as anticipated, patients experienced local injection site reactions, and a few experienced low-grade constitutional symptoms such as fever during the first night. The majority of patients developed T-cell ($CD4^+$ and $CD8^+$) immune responses specific for AE37 as well as for the native HER-2/*neu*(776–790) (AE36) peptide.

The Ii-Key technology has been designed to enhance presentation of antigenic peptides by promoting exchange of peptides in the MHC class II molecules at the cell surface (13). Accordingly, the AE37 hybrid peptide [Ii-Key/HER-2/*neu*(776–790)] is capable of directly charging the HLA-DR alleles with the epitope(s) present in HER-2/*neu* (776–790), thus inducing stronger immunologic responses *in vitro* and antitumor immunity *in vivo* than the native AE36 peptide. Indeed, we have shown that AE37 elicits higher frequencies of IFN-γ^+ $CD4^+$ responder cells among cancer patients' PBMC compared with AE36 and provided significantly stronger help to autologous CTL in lysing tumor cells both *in vitro* and *in vivo* (18, 19). Further, the AE37 hybrid vaccine was reported to elicit strong

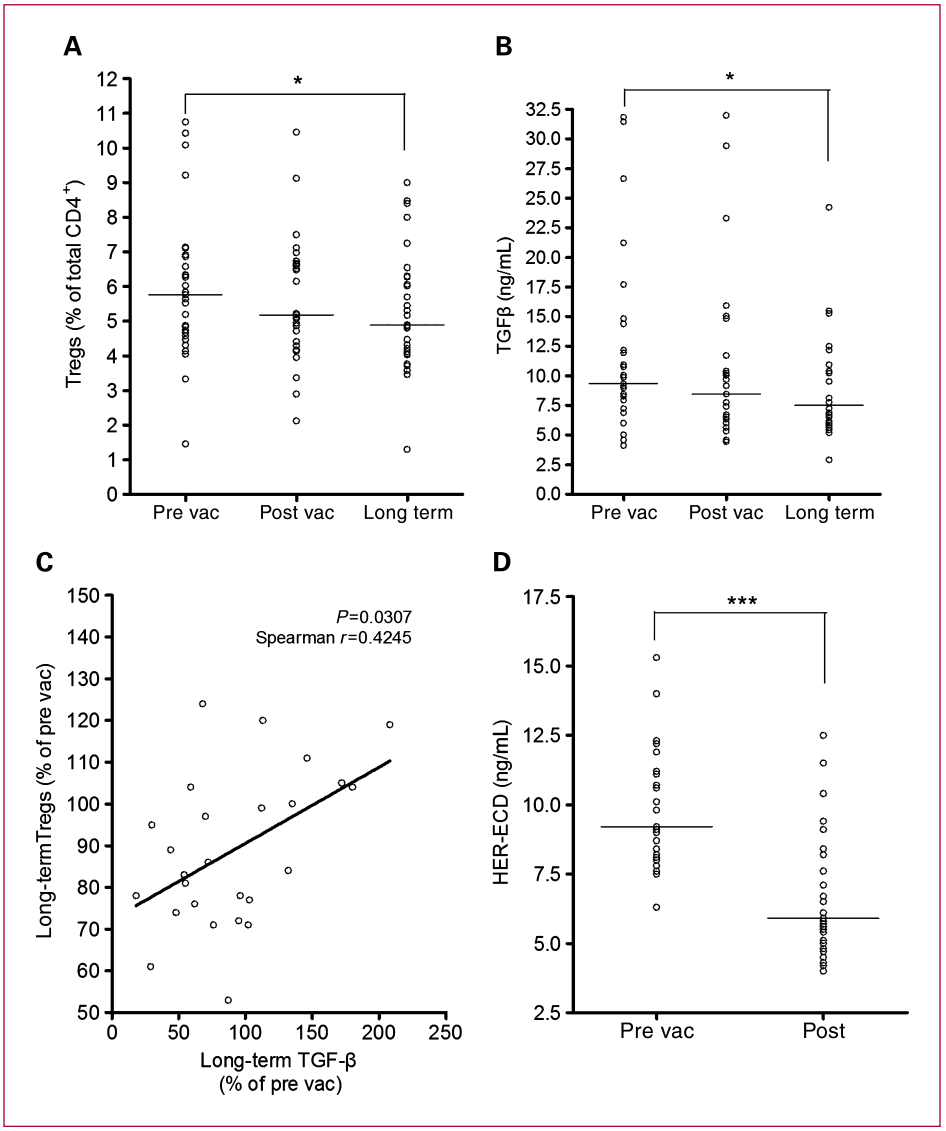

Fig. 3. Biological and immunologic parameters pre vac, post vac, and at long term. A, circulating Tregs. Serum levels of TGF-β (B) and HER-ECD (D). C, correlation between percent change in TGF-β and percent change in Treg frequency at long term compared with pre vac values. Horizontal bars, median values. *, $P < 0.05$; ***, $P < 0.001$.

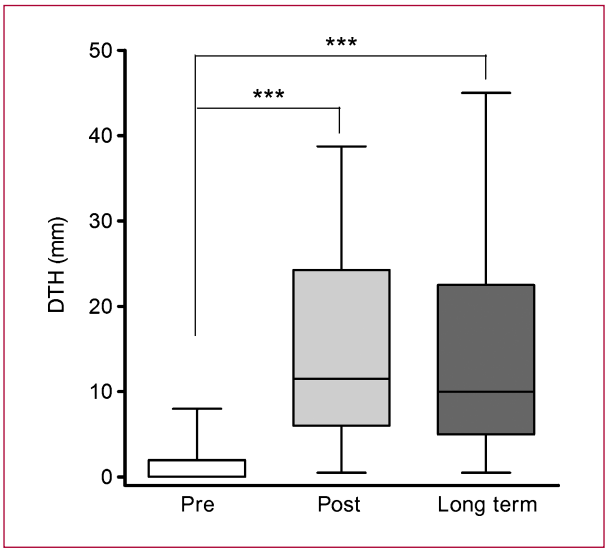

Fig. 4. DTH pre vac, post vac, and at long term. Box and whiskers (5–95% percentile) plot. Horizontal bars, median values; vertical bars, range. ***, $P < 0.0001$.

HER-2/*neu*–specific immune responses in a trial with breast cancer patients even in the absence of GM-CSF (21).

Peptide vaccine trials have focused predominately on stimulating CTLs, through the administration of 8-10mers binding on HLA class I alleles. Such vaccine-induced CTLs can directly kill tumor cells but lack long-lasting immunity (35). This AE37 peptide vaccine focuses on stimulating $CD4^+$ T-helper cells with the goals of inducing immunologic memory and persistent stimulation of CTLs. Clinical studies using class II peptide vaccines have been reported by Disis et al. (36) in breast cancer. The Ii-Key modification of HER-2(776–790) has been used to vaccinate disease-free, node-negative breast cancer patients (21). In melanoma, there are also class II peptide vaccines in clinical trials (37, 38). This is the first clinical trial using the Ii-Key modification in prostate cancer patients.

The HER-2/*neu*(776–790) peptide is promiscuously presented by many HLA-DR alleles (39). Such presentation theoretically could reflect one epitope that binds to many HLA-DR alleles and/or multiple closely overlapping, but slightly offset, HLA-DR epitopes. In either case, the AE37 vaccine seems to increase presentation and potency of T-cell activation regardless of HLA allele. Whether the Ii-Key (LRMK) moiety actually confers additional MHC class II promiscuity to AE36 requires further investigation. Interestingly, our data from intracellular IFN-γ analyses detected potent responses mediated by patients' $CD8^+$ T cells, suggesting that the AE37 vaccine, in addition to $CD4^+$ T cells, also primes $CD8^+$ T cells. Such responses were also detected in response to the native AE36 peptide, suggesting that the Ii-Key moiety in AE37 enhances immunity directed against sequences with MHC class I binding motifs encompassed within HER-2/*neu*(776–790) (suggested by an algorithm-prediction software). Moreover, IFN-γ production by $CD8^+$ T cells in response to AE37 may also be triggered by MHC "neo-determinants" generated by the linkage of Ii-Key to AE36. Although this has to be confirmed, our data suggest that this might be the case given the higher frequencies of responders' IFN-γ^+ cells with AE37 compared with AE36. The efficacy of our vaccine formulation to induce a specific T-cell response against AE37 and/or AE36 has been confirmed by the high number of patients with augmented immune responses. Such responses usually peaked by the third round of vaccination and then declined. One possibility for this could be that after a number of vaccinations, subsets of the vaccine-induced T cells acquire different migratory or homing characteristics and they selectively accumulate to various body compartments, including lymph nodes, bone marrow, and/or tumor tissue (40).

Stimulating $CD4^+$ T-helper cells is essential for inducing long-term immunity. In most of our vaccinated patients, we could detect specific immunity to AE36 both *in vitro* (IFN-γ) and *in vivo* (DTH) even 6 months after completion of the vaccination series. However, vaccinating with MHC class II–restricted peptides might additionally activate Tregs, which are already elevated in prostate cancer patients (41–43). By monitoring circulating Tregs in our AE37 trial, we detected a reduction in the frequency of Tregs postvaccination, which was even more pronounced at long-term assessment. Furthermore, Treg frequency reduction correlated with a decrease in TGF-β plasma levels, thus indicating that vaccination with AE37 may effectively modulate the immunosuppressive milieu induced by the tumor. The fact that Tregs, as well as TGF-β, were found to be more reduced 6 months after completion of vaccinations (long-term values) compared with postvaccination is indicative of ongoing immune responses triggered by the vaccine, probably not solely against the targeted epitope (AE36) but against broad tumor-associated specificities (epitope spreading). This has also been reported when vaccinating metastatic breast cancer patients with MHC class II–restricted HER-2/*neu* peptides (28).

HER-ECD serum levels have been shown to be elevated in prostate cancer patients and shown to be an independent prognostic factor associated with a high risk of biochemical recurrence (33). For this reason, it was of interest to determine plasma HER-ECD levels at enrollment and after completion of vaccinations. Overall, a significant vaccine-induced decrease in HER-ECD plasma levels was observed. Because of the limited number of patients, however, we could not detect any statistical difference among patients with different clinical characteristics. Further clinical studies will be required to clarify the reason for this decrease.

By retrospectively analyzing our patients according to their pre-enrollment characteristics, that is, stage, sensitivity to biochemical castration, or HER-2/neu expression, we have detected a correlation between the extent of the disease and the *in vitro* and *in vivo* vaccine-induced immune responses against AE36. Patients with advanced disease, stage IV and/or castrate-resistant, exhibited decreased circulating IFN-γ–producing cells and lower post vac DTH responses. It has been previously documented (44)

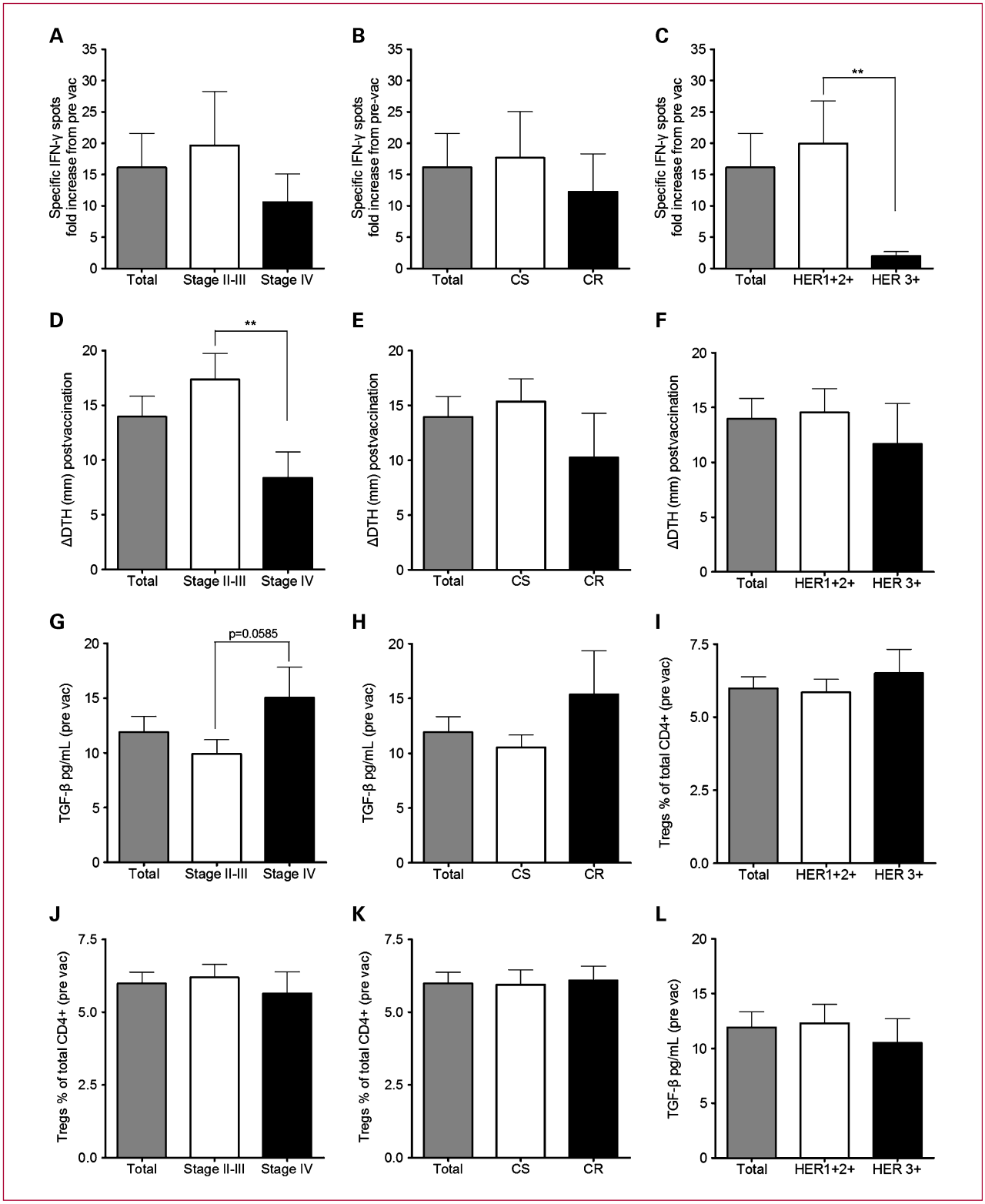

Fig. 5. Comparison of immune parameters in vaccinated patients classified according to staging (stage II-III versus stage IV), sensitivity, or resistance to testosterone lowering (castrate-sensitive versus castrate-resistant, respectively) and the levels of their tumors' HER-2/*neu* expression (HER1^{+}2^{+} versus HER3^{+}). A to C, results represent maximal-specific spots divided by pre vac–specific spots. D to F, results represent DTH post vac minus DTH pre vac. G to I, plasma TGF-β concentrations. J to L, % circulating Tregs. Where not indicated, intragroup comparisons were not statistically significant. Total, all patients. Bars, mean values ± SEM. **, $P < 0.01$.

that cancer progression leads to tumor-mediated local and systemic immunosuppression resulting in compromised antitumor immunity. Indeed, we found in the present study that prevaccination baseline plasma TGF-β levels were increased in patients with advanced disease (stage IV and/or castrate-resistant), at least partially accounting for a hostile milieu in these groups leading to decreased AE37 vaccine-induced immune responses. In contrast, similar percentages of peripheral blood Tregs were detected among the various groups. Our data are in line with those of Yokokawa et al. (42) who found no significant differences in Treg populations in peripheral blood of prostate cancer patients with localized or advanced disease, yet showed that Tregs from metastatic patients were more suppressive.

It has been previously reported that breast cancer patients with low HER-2/*neu* (IHC 1^+)–expressing tumors responded better to the E75 vaccine than those with HER-2/*neu* IHC 2^+ and 3^+, suggesting an element of immunologic tolerance in the high HER-2/*neu* expressors (45). In the present study, we also found significantly reduced INF-γ–producing cells and lower (although not statistically significant) DTH responses in patients with high (IHC 3^+) HER-2/*neu* expression compared with HER-2/*neu* IHC 1^+ and 2^+.

Given the small number of vaccinated patients, their heterogeneity, and the various types of treatments, it was difficult to determine whether an association exists between the development of an immune response and clinical response. Thus, we could not detect a significant association between vaccine-specific immunologic responses and PSA levels or overall survival/progression-free survival (data not shown). Furthermore, most patients (13 of 29, of which 12 did not receive any therapy during vaccinations), when entering the study, had negligible PSA values that remained low (below 0.2 ng/mL) throughout the relatively short vaccination period. Obviously, this could be a result of (*a*) the previous therapies, (*b*) the vaccine, or (*c*) a combination thereof.

In conclusion, the use of the AE37 immunotherapeutic vaccine is a novel strategy for managing patients with prostate cancer. This clinical trial shows that the vaccine is safe and immunologically active. However, proof of clinical benefit will require a phase II trial in a homogeneous group of patients with less extensive disease, including disease-free patients at high risk of recurrence.

Disclosure of Potential Conflicts of Interest

N.L. Kallinteris and E. von Hofe: ownership interest, Generex Biotechnology.

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