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SYMPOSIUM PAPER

The E75 HER2/*neu* peptide vaccine

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Abstract E75 (HER2/*neu* 369–377) is an immunogenic peptide from the HER2/*neu* protein which is overexpressed in many breast cancer patients. A large amount of preclinical work and a small number of Phase I trials have been completed evaluating the vaccine potential of the E75 peptide mixed with an immunoadjuvant. Our group has performed two concurrent E75 + GM-CSF Phase II trials in node-positive and node-negative disease-free breast cancer patients. These trials, totaling 186 patients, were designed to assess the ability of the E75 vaccine to prevent disease recurrence in these high risk patients. In this review article, we discuss the safety of the vaccine, the immunologic response to the peptide, and most importantly, the potential clinical benefit of the vaccine. The recurrence rate, mortality associated with recurrence, and the distribution of recurrences are presented and discussed. Additionally, the lessons learned from these trials to include optimal dosing and the need for booster inoculations are addressed. We also present data exploring possible explanations and mechanisms behind the potential clinical utility of this simple single epitope vaccine. Finally, we present some of the future directions for our Cancer Vaccine Development Program assessing multi-epitope peptide vaccines and combination immunotherapies.

Keywords Breast cancer · E75 · Peptide · Vaccine · Clinical trials

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This work represents original research that has not been submitted elsewhere for publication.

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Introduction

Interest in the development of cancer vaccines increased after advances in the molecular characterization of human tumors led to the identification of tumor-associated antigens (TAAs) that can be recognized by human T lymphocytes [1–3]. TAAs expressed by tumors are capable of eliciting a very specific immune response; therefore, a vaccine is appealing in that it represents a nontoxic therapeutic modality with great specificity. To date, researchers have investigated numerous vaccination strategies including

whole tumor cell-based vaccines, anti-carbohydrate-antigen (Ag) vaccines, anti-idiotype vaccines, DNA vaccines and tumor Ag protein-based or peptide-based vaccines. Novel vaccination strategies to include fusing a tumor cell with an antigen presenting cell such as a dendritic cell (DC), have also been described. These strategies are nicely reviewed in manuscripts by Ko et al. [4] and more recently by Mittendorf et al. [5].

Our group has focused on developing a peptide-based vaccine. Peptide-based vaccines use antigenic epitopes derived from TAAs for the induction of peptide-specific immune regulators including antibodies, helper T-cells and cytotoxic T lymphocytes (CTLs) that recognize and lyse tumor cells expressing the immunogenic peptide on their surface. When combined with an immunoadjuvant, peptides can be injected without a delivery system. Therefore, they represent a simple and pure way to stimulate an immune response to TAA. Such a vaccine can be easily produced and efficiently exported to the community [6].

One source of TAA that has been extensively studied in breast cancer is HER2/*neu*. Several peptides capable of inducing CTLs have been described from the HER2/*neu* protein including E75 (HER2/neu 369-377) which is the most studied in laboratory and clinical studies [7–15]. We have used E75, mixed with the immunoadjuvant granulocyte macrophage-colony stimulating factor (GM-CSF) as a preventive vaccine. In this article, we review preclinical work investigating the E75 peptide, results of early clinical trials utilizing the peptide, as well as the results of our large, Phase II clinical trial investigating the use of E75 as a preventive vaccine in high-risk breast cancer patients. We also address areas of ongoing investigation regarding HER2/*neu* peptide vaccines and future directions for our vaccine program.

E75 HER2/*neu*-derived peptide: preclinical data and early clinical trials

HER2/neu as a tumor-associated antigen

HER2/*neu* is a member of the epidermal growth factor receptor family of transmembrane tyrosine kinases [16, 17]. The HER2/*neu* gene is amplified and the encoded protein is overexpressed in a variety of malignancies, including up to 30% of breast and ovarian cancers [18, 19]. This overexpression can result in a 100–200-fold increase in concentration of the HER2/*neu* protein in tumor versus normal tissue [10]. Recognizing this differential expression, Fisk and colleagues proposed that HER2/*neu* may serve as a TAA since processing of overexpressed HER2/*neu* protein would theoretically result in an increased peptide supply which could occupy a significant number of MHC molecules in competition with other peptides. This in turn could activate or reactivate an immune response against a HER2/*neu*-expressing tumor [10, 20]. In an early study investigating this hypothesis, Fisk et al. [10] identified common immunogenic epitopes of HER2 that were recognized by $CD3^+CD4^-CD8^+$ ovarian-specific CTL lines that were isolated from tumor-associated lymphocytes from HLA-$A2^+$ ovarian cancer patients. All peptides were selected from the HER2/*neu* sequence based on HLA-A2 anchor motifs. HLA-A2-presented peptides were chosen because the HLA-A2 allele is expressed in 40–50% of the general population [21] and had been demonstrated to play an important role in tumor antigen presentation from a variety of tumors [22, 23]. The E75 epitope, derived from the protein's extracellular domain, was dominantly recognized by all CTL lines tested [10]. Finding a specific peptide derived from the HER2/*neu* sequence that was recognized by tumor-specific CTLs confirmed this oncogene as a TAA. Subsequent to the identification of E75, this peptide was studied by other investigators both in vitro and in an in vivo animal model. This additional work confirmed that E75 was capable of inducing a peptide-specific, CTL-mediated immune response [24, 25].

E75 as a therapeutic vaccine: early clinical trials

After description of the peptide, three clinical trials used E75 in combination with an immunoadjuvant [incomplete Freund's adjuvant (IFA) or GM-CSF] as a cancer vaccine. The trials were small and all targeted patients had metastatic disease. All three trials demonstrated E75 to be safe and capable of inducing a peptide-specific immune response in vivo [11, 13, 15].

Other investigators have utilized E75 in different vaccination strategies. One such strategy loaded the peptide onto autologous DCs which were then re-infused into the patient [7, 12]. This strategy has the potential benefit of loading the immunogen directly onto an antigen presenting cell however we believe this modality is limited by its complexity. A second strategy utilized longer peptides capable of binding HLA Class II molecules including some with sequestered CTL epitopes (i.e., E75). The purported benefit of this strategy is potential stimulation of $CD4^+$ helper T-cells, however, it requires the host to process a longer peptide fragment in order to generate the E75 peptide for CTL stimulation [8, 9]. These additional approaches were proven to be safe and effective at stimulating E75-specific immunity. However, because these early trials were not designed to compare the efficacy of these strategies in eliciting an immune response, it is unclear whether either approach can produce better E75-specific responses than E75 alone mixed with an immunoadjuvant.

HER2/*neu* (E75) vaccine for the prevention of recurrence in high-risk breast cancer patients: Phase II clinical trial

A preventive strategy for cancer vaccines

Though early clinical trials using E75 as a cancer vaccine were effective in stimulating an E75-specific response, little is known about the clinical efficacy of this peptide. This has been attributed both to the design of the trials which were initiated to assess safety and feasibility as well as to the fact that the majority of patients enrolled in initial trials had stage IV disease with large tumor burdens [26, 27]. Recognizing the potential difficulty in treating late-stage disease with a vaccine, our group has pursued a different strategy in our clinical trials shifting focus on preventing disease recurrence in those patients at high risk for relapse. We have enrolled breast cancer patients considered to be at high risk for recurrence that have been rendered disease-free through conventional treatment with surgery, chemotherapy, and radiation therapy when appropriate.

Prior to instituting a preventive vaccine trial, important preclinical work was done to determine the feasibility of priming cytolytic activity in healthy donors. The ability to induce tumor-reactive CTLs in healthy donors at risk, or in patients rendered disease-free is important for a preventive strategy dependent upon the hypothesis that CTLs recognizing tumors early may be more effective in containing tumor progression than CTLs that only expand upon disease progression [24]. In a study reported by Anderson et al., the ability of the E75 peptide to prime E75-specific cytolytic activity in healthy donors when presented on autologous DCs was investigated. Of ten healthy donors, two responded at priming with E75 presented on autologous DCs by induction of E75-specific CTL activity. Three other responders were identified after two additional restimulations suggesting that cytolytic activity could be elicited in 50% of healthy donors. Induction of cytolytic activity at priming was enhanced in responders but not in nonresponders by tumor necrosis factor-α and interleukin 12. Also, αB7.1 monoclonal antibody added at priming enhanced induction of lytic activity in only one of the nonresponding donors tested, suggesting, in the opinion of the authors, that in the majority of donors E75-precursor CTLs were not tolerized [24]. This study had important implications for the ability of an E75-peptide vaccine to be used in a preventive strategy administered to patients already treated for breast cancer and thought to be at high risk for disease recurrence.

Node-positive breast cancer trial

Our initial E75 breast cancer vaccine trial enrolled patients with node-positive (NP) disease that expressed HER2/*neu* by standard immunohistochemistry (IHC). Importantly, patients with any HER2 expression to include 1+, 2+or 3+ by IHC were eligible for participation. All patients completed a standard course of surgery, chemotherapy, and radiation therapy as clinically indicated and any patient on chemoprevention was continued on their prescribed regimen. After enrollment in the study, patients were HLA typed. Because E75 binds the HLA-A2 allele, HLA-A2$^+$ patients were vaccinated and HLA-A2$^-$ patients were observed prospectively as matched controls for clinical recurrence. All patients were tested for immunocompetence prior to vaccination by performing skin tests with a panel of recall antigens. The vaccine itself consisted of the E75 peptide mixed with the immunoadjuvant GM-CSF. The inoculation was given intradermally at two sites within 5 cm of each other on the same extremity.

The initial trial was performed under an Investigational New Drug application (IND #9187) that was approved by the Food and Drug Administration [26]. Multiple dosing groups were assessed using E75 peptide doses ranging from 100–1,000 μg with 250 μg of GM-CSF. Local and systemic toxicity were determined and immunologic response was assessed in vivo and in vitro.

Delayed-type hypersensitivity (DTH) reactions were used to assess in vivo immunologic response. Briefly, DTH was measured with 100 μg of E75 injected intradermally with a saline volume control. In vitro monitoring involved quantification of E75-specific CD8$^+$ T-cells using the HLA-A2:immunoglobulin dimer assay. This assay measures the ability of the HLA-A2 dimer to stain CD8 cells whose T-cell receptors are capable of interacting with and recognizing the peptide being presented by the MHC molecule. Our group had previously demonstrated comparable results when comparing the dimer assay to an E75-specific HLA-A2-tetramer assay [28]. The use of tetramer assays has previously been reported in clinical vaccine trials but tetramer technology is limited by the extensive biochemical process associated with the synthesis of the molecules and the inability to associate certain antigenic peptides within these structures. In contrast, peptide-specific HLA-A2 dimers can be readily prepared merely by incubating the dimer molecules with the peptide of interest [28]. We have previously shown that results of the dimer assay used to detect the presence of vaccine-specific CTL correlated well with the results observed in standard functional immunologic assays for immune responses including cell proliferation, cytokine secretion and cytotoxicity [28].

The initial results of this NP trial were reported after 53 patients were enrolled; 24 HLA-A2$^+$ patients were vaccinated and 29 HLA-A2$^-$ patients served as controls [26]. The vaccine was demonstrated to be safe with no reported grade 4 or 5 local or systemic toxicities. There was one patient with a grade 3 (severe bone pain) systemic toxicity,

and three patients with grade 2 systemic toxicities. All patients had either a grade 1 or 2 local reaction which is a desired effect of the vaccine. DTH reactions were observed in vaccinated patients with a mean diameter of induration of 33 mm (range, 17–53 mm) to E75 versus 7 mm (range, 0–17 mm) for controls. The vaccine was also demonstrated to be effective in stimulating clonal expansion of E75-specific $CD8^+$ T-cells in all patients. We observed a pattern of clonal expansion that was similar in most patients with an increasing percentage of $CD8^+$ E75-specific cells with successive vaccinations that peaked during the series and then contracted and reached a plateau by completion of the series. It did not appear that the peaking and contracting pattern was a result of overvaccination and clonal exhaustion because the same pattern was observed in patients in all dose groups. Importantly, the induced HER2/*neu* immunity appeared to reduce the recurrence rate in patients with NP breast cancer as the disease-free survival at a 22-month median follow-up was 85.7% in the vaccinated group compared to 59.8% in the controls ($P < 0.19$). Although this difference did not reach statistical significance, it suggests that vaccinated NP breast cancer patients did achieve some benefit from the inoculation series.

Node-negative breast cancer trial

After documentation of vaccine safety in the NP population, a second protocol enrolling patients with node-negative (NN) disease was begun. The NN trial was designed to further delineate optimal biological dosing by varying the dose of GM-CSF and altering the inoculation schedule. Importantly, patients with non-HER2/*neu*-expressing tumors were allowed to participate in this trial to determine the feasibility of vaccinating a presumably antigen-naïve host. In addition, shortly after the NN trial began enrolling, we determined that E75 could be used in HLA-$A3^+$ patients based on binding affinity data obtained from two commonly used HLA-peptide binding algorithms [29, 30]. Preclinical work also demonstrated that E75-stimulated HLA-$A3^+$ CTL could lyse HLA-$A3^+$ HER2/*neu*-expressing cancer cells (unpublished data). During this trial, we determined that the toxicity profile, immunologic response, and recurrence rates were similar between HLA-$A2^+$ and HLA-$A3^+$ patients, confirming the appropriateness of expanding the use of the E75 vaccine to HLA-$A3^+$ patients. Because approximately 15% of the population is HLA-$A3^+$ [21] by vaccinating both HLA-$A2^+$ and HLA-$A3^+$ patients, this single E75 peptide may address two-thirds of the general population [27].

Combined clinical trial results

We have recently reported the combined results of our NP and NN vaccine trials [27]. The decision was made to analyze these trials together since they were run simultaneously, they utilized the same peptide and immunoadjuvant administered the same way (intradermally) and both enrolled disease-free breast cancer patients. After combining the data, we assessed the vaccinated and control groups (Table 1) to ensure that no bias had been introduced. This comparison found the two groups to be comparable with respect to the number of NP patients as well as with all other prognostic factors except hormone receptor (HR) status. There were more HR negative patients in the vaccinated group (32 versus 17% HR negative in the observation group), therefore fewer patients in the vaccine group were on adjuvant hormonal therapy (66 versus 79%, $P = 0.05$). This difference would suggest that vaccinated patients were actually at higher risk for disease relapse as adjuvant hormonal therapy has been demonstrated to reduce the risk of recurrence [31]. The remainder of the adjuvant treatment profiles to include chemotherapy, radiation therapy and/or trastuzumab were the same for the vaccinated patients

Table 1 Demographic and prognostic factors for vaccinated and observation patients

	Vaccinated, HLA-$A2^+$, $A3^a$ ($n = 96$)[a]	Observed, HLA-$A2^-$, $A3^-$ ($n = 81$)[b]	*P*
Median age, years	58.9	55.1	
Range, years	32–80	34–87	0.33
Race			
White %	89.6	81.5	
Other %	10.4	18.5	0.12
Tumor size			
T1 %	69.8	60.5	0.20
T2–T4 %	30.2	39.5	0.20
Histological grade			
I–II %	64.5	59.5	0.50
III %	35.5	40.5	0.50
Node-positive %	46.9	56.8	0.19
Median + nodes (NP only)	2.0	2.5	
Range	1–25	1–15	0.17
HER2/*neu* IHC 3 + or FISH + %	25.8	28.4	0.32
Hormone receptor negative %	31.6	17.3	0.03
XRT %	71.9	80.2	0.20
Chemoprevention %	65.6	78.8	0.05
Adjuvant Herceptin %	5.2	3.7	0.60

[a] 101 patients enrolled to vaccine arm, 2 switched to observation, 1 withdrew for adjuvant trastuzumab, 1 due to an extended unrelated illness, and 1 patient for personal reasons

[b] 85 patients enrolled to observation arm, 2 lost to follow-up and 4 withdrew to another vaccine trial. Two patients were gained from the vaccine arm [27]

versus the control patients. At the time of this combined analysis, 186 patients had been enrolled (NP, 95; NN, 91). 101 patients were vaccinated with the remaining 85 being assigned to observation. Nine patients withdrew from the study leaving 177 patients available for analysis making this the largest HER2/*neu*-based peptide vaccine trial as well as the largest trial of a preventive vaccine strategy in breast cancer to date.

Local and systemic toxicities were again mild, and all patients completed the vaccine series. Grade 1 (81%) or grade 2 (19%) local toxicity, a desired effect suggesting in vivo immunogenicity, was seen in all patients. To formally measure the vaccine's in vivo effectiveness, a post-vaccine DTH was measured. Among all vaccinated patients, 74% had a positive post-vaccine DTH with an average induration to E75 of 14.0 ± 1.4 mm compared to control of 2.1 ± 0.5 mm ($P < 0.0001$) (Fig. 1a). The in vitro immune response, assessed using the dimer assay to quantify E75-specific CTL demonstrated a pattern of increasing E75-specific $CD8^+$ T-cells seen during the series with peak levels (occurring after the third or fourth dose in 65% of patients) followed by recession to a plateau by series completion (Fig. 1b). There was a statistically significant increase in the median percentage of E75-specific $CD8^+$ cells from pre-vaccine to maximum levels and post-vaccination. The long-term levels of E75-specific $CD8^+$ cells were not significantly different from pre-vaccination levels with only 43% of the patients maintaining significant residual immunity defined as dimer >0.5 6 months after vaccination.

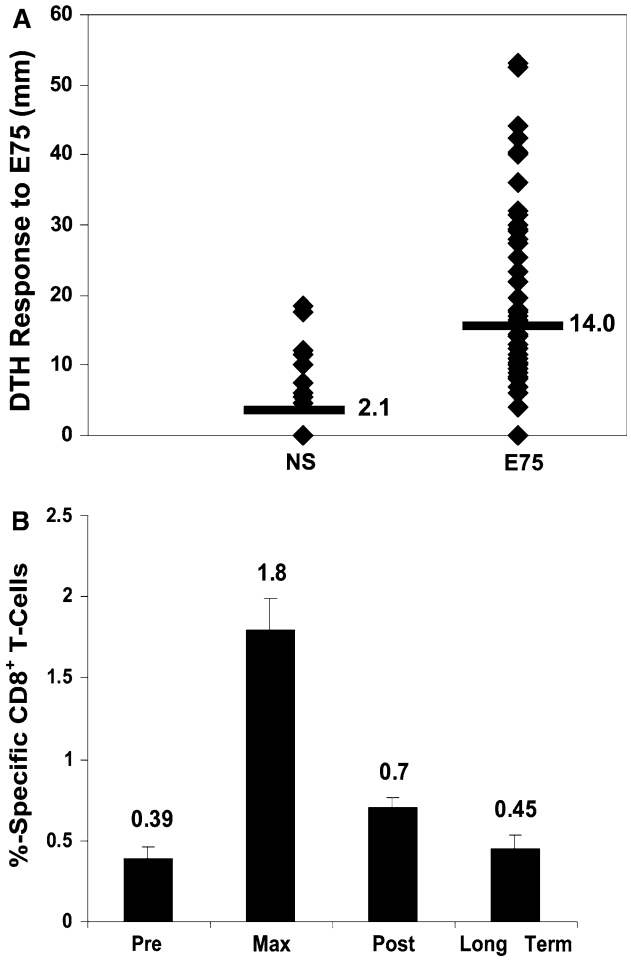

Fig. 1 Immune responses after vaccination with E75-peptide. **a** Delayed type hypersensitivity (DTH) reactions to the E75 peptide were used to assess in vivo immunologic response. Post-vaccination DTH to the E75 peptide was significantly larger (14.0 ± 1.4 mm) then to normal saline control (2.1 ± 0.5 mm); $P < 0.0001$. **b** A dimer assay detecting vaccine-induced E75-specific $CD8^+$ cytotoxic T lymphocytes (CTL) was used to assess in vitro immune response. The median levels of E75-specific CTL were significantly increased from pre-vaccination levels (0.39%; range, 0–3.28%) to a maximum level (1.8%; range, 0.4–12.2%; $P < 0.0001$) and post-vaccination level (0.70%; range, 0.06–2.91%; $P = 0.002$). There was no difference between pre-vaccine levels and long-term (6 month) levels of E75-specific CTL [27]

Clinical benefit of vaccination

In accordance with the original protocol design, primary analysis of the clinical response to vaccination was initiated at 18-months median follow-up. At this analysis, the recurrence rate was 5.6% in the vaccinated group compared to 14.2% in the observation group ($P = 0.04$). The disease-free survival rates were 92.5 and 77% in the vaccinated and control groups, respectively. There was one death in the vaccinated group for an overall survival rate of 99% compared to four deaths in the observation group for an overall survival rate of 95% ($P = 0.1$). Given these initial clinical results, follow-up of both trials was extended to 5 years. At the time of our most recent analysis, at a median follow-up of 26 months, the recurrence rate is 8.3% in the vaccine group compared to 14.8% in the observation group ($P = 0.17$); mortality rates were 1.0 and 6.2%, respectively ($P = 0.1$). For patients who recurred, the mortality rate for the control and vaccinated groups were 41.7 and 12.5%, respectively (Fig. 2) [32].

Despite the fact that there continues to be a trend towards a benefit to vaccination, the statistical significance has been lost. This finding was not entirely unexpected in that we had determined that the E75 immunity wanes over time consistent with what is known about the biology of $CD8^+$ T-cells, such as those stimulated by our MHC Class I vaccine. These cells are frequently not capable of sustaining a prolonged memory immune response in the absence of continued antigen exposure and stimulation by antigen-presenting cells [27]. Therefore, one must question how to maintain long-term immunity and protection against recurrence with a CTL epitope peptide vaccine. One consideration is to give booster inoculations. We have recently begun administering such booster doses of E75 to patients

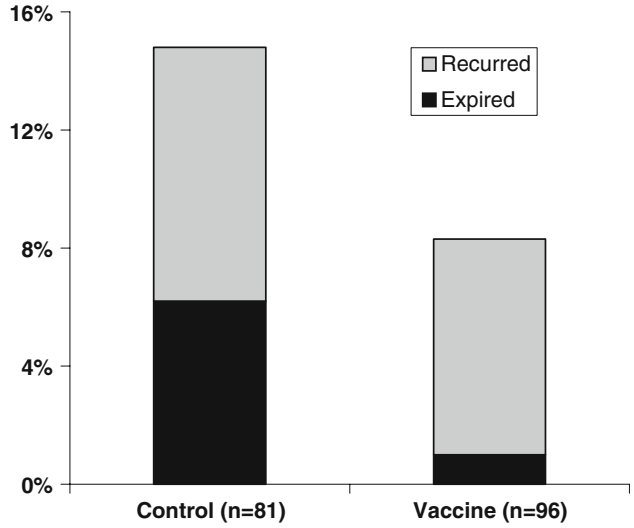

Fig. 2 Recurrence and survival rates for patients completing a HER2/*neu* (E75) vaccine trial for the prevention of recurrence in high-risk breast cancer patients ($n = 177$). The control and vaccinated groups had recurrence rates of 14.8 and 8.3%, respectively ($P = 0.17$), and mortality rates of 6.2 and 1.0%, respectively ($P = 0.1$). In the recurrent patients, the mortality rate for the control and vaccinated groups were 41.7 and 12.5%, respectively ($P = 0.3$) [32]

enrolled in our clinical trials, and the preliminary results are promising for a sustained E75-specific immune response.

Optimal biologic dose

The above clinical trials analysis was performed on patients receiving seven different dose/schedule groups of the E75 vaccine. To determine the optimal biologic dose (OBD), we analyzed the toxicity and immune responses from our trials. Importantly, all doses produced an immunologic response. The greatest response was seen in the highest dose group which received 1,000 μg E75 + 250 μg GM-CSF. There was no increase in toxicity with increasing doses of E75.

To validate the OBD, we have compared patients receiving the OBD to all others enrolled in our trials [33]. There was no difference between the two groups with respect to local or systemic toxicity (Fig. 3a). When we compared immunogenic responses between the two groups, the DTH response was significantly larger in those receiving the OBD (Fig. 3b). In addition, there was a trend towards an increase in the average post-vaccine dimer levels; however, there was no difference in the average long-term dimer levels at 6 months (Fig. 3c). At a median of 30 months, the recurrence rate in the group of optimally vaccinated patients was 3.4 versus 12.9% in all others ($P = 0.3$) (Fig. 3d) despite the fact that the OBD group was younger, had larger tumors, had a greater proportion of NP patients, and a trend towards higher grade tumors. However, the median follow-up for the optimally dosed patients is significantly shorter than for all others; therefore, longer follow-up will be needed to confirm these results and to determine if the difference reaches statistical significance.

Distribution of recurrence after vaccination

In order to determine if the vaccine had any impact in the patients who recurred, we compared vaccinated recurrent patients with those who recurred in the control group. There was a trend towards higher grade tumors and hormone receptor negativity in the vaccinated patients suggesting that the vaccine may be limiting recurrences among patients with less aggressive disease. Given this finding, we next analyzed the sites of disease recurrence. For breast cancer patients, bone is the most common site of metastasis with 35–50% of initial recurrences being bone only [34]. Consistent with this, in our trials, bone only recurrences accounted for 50% of the initial recurrences in the control group. In our vaccinated patients, none of the recurrences were bone only ($P = 0.04$) (Fig. 4) [32]. This further suggests that vaccination alters the immune process leading to fewer recurrences in more indolent disease.

It has been demonstrated that patients with bone as the sole initial site of metastasis have a better prognosis than patients with visceral recurrences with median survival from diagnosis of up to 35 months compared to 11–26 months [35]. Given this, and recognizing that the number of patients with recurrent disease in our trials is small, it is interesting to note that there is an 88% survival rate for vaccinated patients who recurred versus 58% for control patients that recurred.

Taken together, these data suggest that vaccination with the E75 peptide vaccine may limit recurrences among patients with less aggressive disease and may alter the distribution of disease. A multi-center, randomized, prospective Phase III clinical trial is currently being designed. Results from this trial will validate the true clinical benefit of the E75 peptide vaccine.

Immunologic response to vaccination

In order to elucidate the mechanism by which vaccination with the E75 peptide may confer clinical benefit, we have performed extensive studies to assess different aspects of the immune system which may be affected. As discussed, in vivo response has been demonstrated using DTH reactions. In vitro response has been demonstrated by quantifying E75-specific CTL using the dimer assay. This phenotypic data has correlated with functional data to include standard chromium release assays that measure CTL-mediated tumor cell killing (Fig. 5a, b) and ELISPOT assays that measure cytokine production [26, 28].

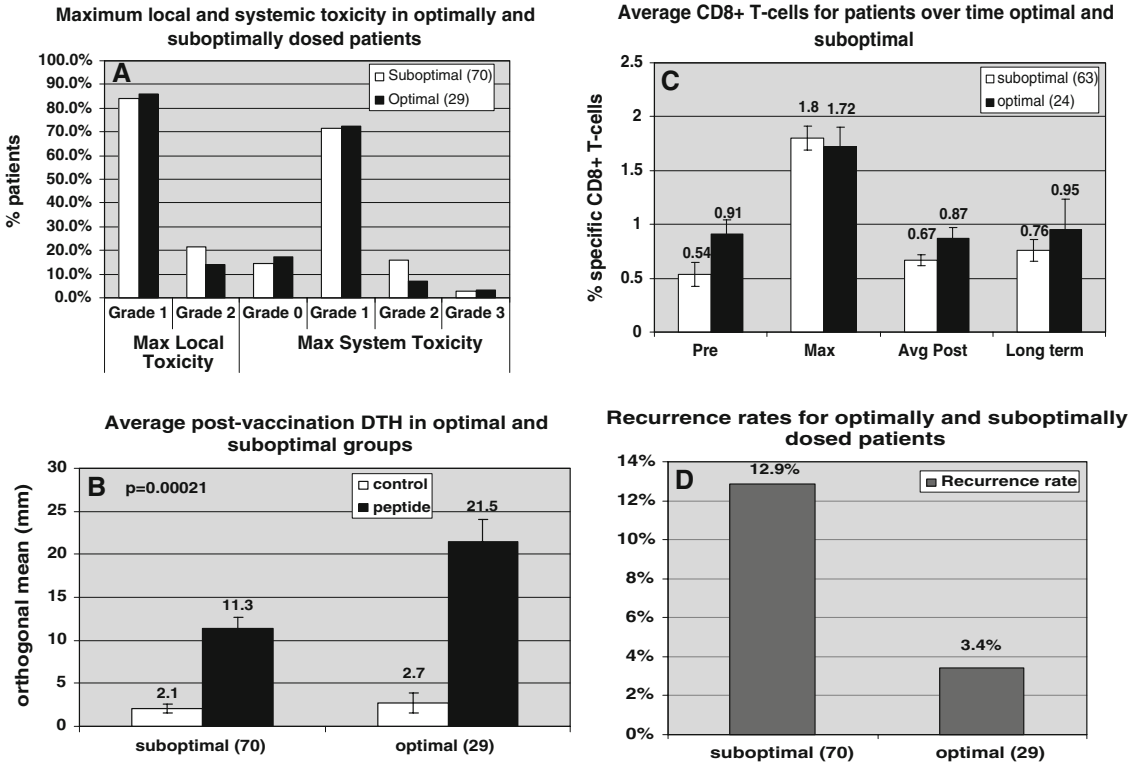

Fig. 3 Clinical and immunologic data in patients receiving the optimal biologic dose (OBD) versus all others. The optimal dose group (ODG; $n = 29$) received 1,000 μg E75 + 250 μg for 6 inoculations. The suboptimal dose group (SDG; $n = 70$) consists of the combined 6 remaining dose groups. **a** There was no significant difference identified in local (grade 1 and 2, $P = 0.58$) or systemic toxicity (grade 0, $P = 1$; grade 1, $P = 0.64$; grade 2, $P = 0.72$; grade 3, $P = 1$). **b** Orthogonal mean DTH response (mm) between the ODG versus SDG showed no difference to the normal saline control inoculum (3.0 ± 1.1 vs. 2.0 ± 0.5 mm). DTH to the peptide was significantly elevated in the ODG vs. the SDG (21.5 ± 2.5 vs. 11.3 ± 1.3 mm, $P = 0.00021$). **c** Dimer assay was used to detect E75-specific $CD8^+$ T-cells. There was a significant difference in the ODG vs. SDG in the average pre-vaccine $CD8^+$ E75-specific T-cell levels (0.91 ± 0.13 vs. $0.54 \pm 0.11\%$, $P = 0.03$). No significant difference was seen between the average maximum $CD8^+$ E75-specific T-cell levels. The optimal dose showed a trend toward an increase in the average of monthly post-vaccination percent of $CD8^+$ E75-specific T-cells (0.87 ± 0.10 vs. $0.67 \pm 0.05\%$, $P = 0.07$). No difference was seen in the average long-term $CD8^+$ E75-specific T-cell levels between groups at 6 months. **d** Compared to the SDG, the ODG demonstrated a trend toward lower recurrence rates ($P = 0.27$). The ODG consisted of younger patients with more aggressive disease, however, they have significantly shorter median follow-up [33]

Other investigators have used a murine model to show that in vivo generation of a specific immune response to a tumor epitope requires cross-priming of tumor antigens by antigen-presenting cells and is strictly $CD4^+$ T-cell dependent [36]. This suggests that activation of $CD4^+$ T helper cells is required to sustain a CTL response. We therefore investigated the number of circulating $CD4^+$ T-cells in patients enrolled in our trials and demonstrated a generalized increase after vaccination as well as alterations in specific subpopulations of memory/naïve and effector cells [37, 38]. The importance of $CD4^+$ T-cells in regulating an immune response has been enhanced by the identification and description of a regulatory $CD4^+CD25^+$T-cell population (T_{reg}). T_{reg} cells have been appreciated for their role in preventing autoimmunity, controlling activated autoreactive effector cells, and regulating inflammatory reactions [39]. It has also been suggested that T_{reg} may be associated with certain malignancies and that they have the potential to prevent elicitation of immune responses against tumor tissues [40]. We therefore analyzed samples from a subgroup of our vaccinated patients in order to determine T_{reg} levels. Importantly, although levels of circulating, activated $CD4^+$ T-cells increased post-vaccination, T_{reg} levels were significantly reduced [38].

To further study the immune response stimulated by E75 vaccination, we have used the Luminex® assay to determine serum cytokine profiles in vaccinated patients. We found significant differences in cytokine levels in sera of breast cancer patients compared to healthy controls and in vaccinated patients compared to unvaccinated patients [41]. The most significant difference was in the levels of MCP-1, a chemokine that is active in the tumor microenvironment influencing tumor-associated macrophages, angiogenesis, and metastasis [42–44]. In a subsequent study, high serum

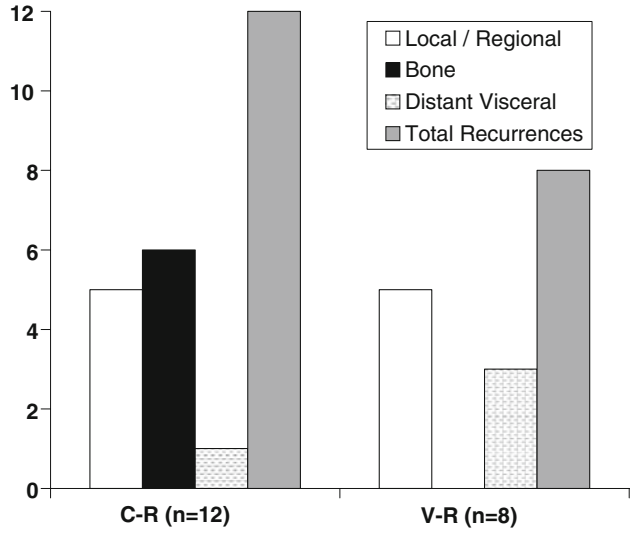

Fig. 4 Location of recurrences in control (*C-R*) ($n = 12$) and vaccinated groups (*V-R*) ($n = 8$) enrolled in a HER2/*neu* (E75) breast cancer vaccine trial. No bone-only recurrences were observed in the *V-R* patients. *Asterisks* denote statistical significance [32]

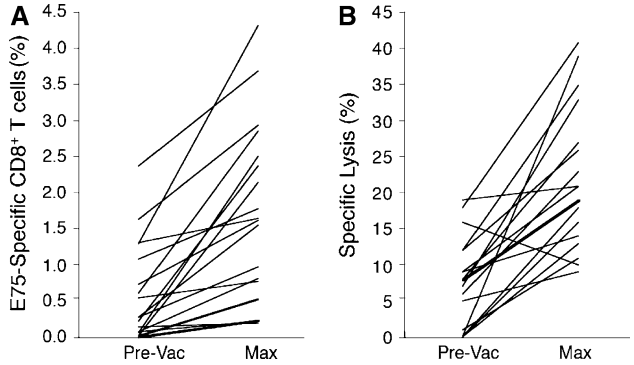

Fig. 5 Correlation between phenotypic (dimer) and functional (cytotoxicity) immunologic responses in node positive breast cancer patients receiving the E75-peptide vaccine. Results depicted are for the first 53 patients enrolled in a clinical trial of the E75 vaccine in node-positive breast cancer patients. **a** Prevaccination (Pre-Vac) and maximum (Max) responses are given for clonal expansion of E75-specific cytotoxic T lymphocytes. **b** Functional assessment of short-term cultured, peptide-stimulated peripheral blood mononuclear cells in standard chromium-release assays against a panel of HLA-A2$^+$, HER2/*neu*$^+$ tumor targets with nonspecific lysis of HLA-A2$^-$, HER2/*neu*$^+$ tumor targets subtracted [26]

MCP-1 levels in breast cancer patients were found to correlate with favorable prognostic variables and increased preexisting HER2/*neu* immunity. Vaccination with E75 induced the largest MCP-1 response in patients with low serum MCP-1 levels and unfavorable clinicopathologic variables. The increase in serum MCP-1 levels correlated with vaccine-induced, peptide-specific immunity suggesting that low serum MCP-1 levels may identify patients with worse prognosis and those most likely to benefit from vaccination [45].

Epitope spreading

The above data suggests that the E75 vaccine is successful in eliciting a broad immunologic reaction. We were therefore interested in investigating whether vaccination with this single peptide was effective in generating epitope spreading. Epitope spreading refers to the generation of an immune response to a particular portion of an immunogenic protein, and the natural spread of that immunity to other areas of the protein [8]. The mechanism by which this occurs has not been fully elucidated although it has been postulated that it represents enhanced processing of endogenous antigens at sites of inflammation [46, 47]. Given our findings that vaccination with the E75 peptide stimulates a broad immunologic reaction, we hypothesized that an environment is established with enhanced stimulation of immune effector cells and that these cells respond more effectively to tumor antigen that is present in the body.

In a subset of 44 patients enrolled in our vaccine trials, we have investigated the phenomenon of epitope spreading [48]. We observed epitope spreading to GP2, a subdominant epitope of the HER2/*neu* protein in all patients with NP disease and 85% of patients with NN disease. The finding of epitope spreading in such a high percentage of NN patients was interesting because it suggests that these patients, with lower tumor antigen burden, likely have occult disease [48].

Circulating tumor cells

In an attempt to better quantify the presence of occult disease after completion of standard therapy in breast cancer patients, we have recently investigated the ability of the CellSearch System (Veridex-LLC, Warren, NJ) to quantify and phenotype circulating tumor cells (CTC). The presence of CTC has been shown in metastatic breast cancer patients to have clinical significance. Using this system, we were able to isolate, enumerate and phenotype CTC in patients without clinical evidence of disease but who were at high risk of relapse [49]. These data confirm that despite administration of chemotherapy, patients do have occult disease suggesting that they may benefit from vaccination. Vaccination in these patients would be expected to elicit a CTL response to both the peptide with which they were immunized as well as to other epitopes that may not be included in their vaccination mix, due to epitope spreading. We found that E75 vaccination significantly reduced the number of CTC in our patients suggesting a potential

clinically relevant means to monitor our ongoing vaccine trials.

Future directions

Multi-epitope vaccines

Although our initial trial results are encouraging, we recognize that there are limitations to a single peptide vaccine strategy. Because a single peptide vaccine targets one epitope from a TAA, lack of antigen diversity is one such concern. This limitation may be partially overcome by epitope spreading. Another strategy would be to administer a multi-epitope vaccine.

In addition to suggesting the presence of occult disease, epitope spreading to GP2 confirms GP2 as an in vivo immunogenic peptide [48]. GP2 is a 9-amino acid peptide (HER2/*neu* 654-662) from the HER2/*neu* protein's transmembrane portion that was first described by Peoples et al. [14]. One concern regarding the use of GP2 as a peptide vaccine is that it has relatively low binding affinity for the HLA-A2 molecule [14]. Theoretically, this could result in a limited number of GP2-MHC Class I complexes being expressed on the cell surface resulting in limited CTL induction in vivo. However, in vitro studies have shown that, despite its lower binding affinity, GP2 has a similar capacity for CTL induction as E75 suggesting that it may be more immunogenic [50]. We have enrolled patients in a Phase I trial that uses GP2 mixed with GM-CSF. The goal of the trial was to document the safety of the peptide such that GP2 could be incorporated into a multi-epitope vaccine with E75. Such an approach is likely to be necessary in NN patients. After vaccination with E75, NN patients did demonstrate epitope spreading to GP2; however, this response was less robust than in NP patients [48]. We hypothesize that a multi-epitope vaccine may be able to elicit a response in such patients with earlier stage disease or against tumors expressing lower levels of HER2/*neu* [6].

We are also interested in a multi-epitope vaccine strategy incorporating Class II epitopes in order to stimulate the $CD4^+$ T-cell population. We have recently completed a Phase I clinical trial administering AE37, a HER2/*neu* Class II epitope to disease-free, NN breast cancer patients [51]. The vaccine was safe, well tolerated, and capable of eliciting HER2/*neu*-specific immune responses. The AE37 peptide was highly immunogenic, and at large doses did not require the use of an immunoadjuvant. A clinical trial investigating a multi-epitope vaccine designed to stimulate both the cell-mediated and humoral immune systems is currently under design.

Combination immunotherapy

We anticipate that peptide-based vaccines will be incorporated into adjuvant treatment algorithms using already determined standard therapeutics to include trastuzumab for women with HER2-overexpressing breast cancer. We therefore have become interested in combination immunotherapy. Preclinical data generated in our lab demonstrate that pretreatment of breast cancer cells with trastuzumab results in increased specific cytotoxicity by E75- and GP2-stimulated CTL [52]. Our data suggest that the enhanced lytic activity is attributable to increased HER2/*neu* receptor internalization and turnover. Theoretically, this would lead to enhanced proteolytic processing and degradation of the protein resulting in an increased number of HER2/*neu*-derived peptide available to be complexed with MHC Class I molecules on the cell surface [52]. This increased susceptibility of trastuzumab-treated cells to lysis by HER2/*neu* peptide-stimulated PBMC occurred even in MCF-7 breast cancer cells which have low HER2/*neu* expression, a finding which could potentially expand the clinical indications for trastuzumab use as a "vaccine sensitizing" agent in patients with HER2/*neu* immunohistochemistry 1+ and 2+ tumors [52].

Importantly, PBMC sample from breast cancer patients receiving the E75 vaccine in our NP clinical trial showed increased recognition and lysis of breast cancer cells treated with trastuzumab. This finding represents the first clinical evidence supporting potential benefits that may be derived from combination immunotherapy [52]. We have recently designed a Phase I clinical trial investigating a combination immunotherapy strategy.

Conclusion

HER2/*neu* peptide vaccines, to include E75, show significant promise, particularly when administered in the adjuvant setting to prevent disease recurrence. Confirmation of the clinical utility suggested by our initial trial results will require a large, multi-center Phase III randomized trial testing the optimally dosed vaccine with booster inoculations in HLA-A2 and A3 patients. It is likely that an effective peptide-vaccine strategy will require a multi-epitope vaccine, combination immunotherapy, or both, and Phase I trials have been designed to address these issues. Positive trial results will suggest a role for peptide-based vaccines in the treatment and prevention of breast cancer.

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