Expert Opinion

CD4+ T cells in antitumor immunity: utility of an Ii-Key HER_2/neu hybrid peptide vaccine (AE37)

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Background: Early clinical trials of HER2/neu-derived peptide vaccines indicate that they may be useful for preventing recurrence in breast cancer patients rendered disease-free after standard-of-care therapy. An effective vaccination strategy will probably require stimulation of T helper (Th) cells. AE37 is an HER2/neu-derived peptide that has been modified to enhance antigen-specific stimulation of Th cells by linkage of the Ii-Key moiety of the MHC class II-associated invariant chain (Ii protein). *Objective*: To review the literature regarding the role of a Th response in immunotherapy with a focus on this novel HER2/neu-derived AE37 peptide. *Results/conclusion*: Improved immuno-genicity of the AE37 Ii-key hybrid peptide has been demonstrated in animal models, *ex vivo* patient cells, and, most recently, in a Phase I clinical trial in breast cancer patients. Future clinical trials incorporating AE37 into a peptide vaccine strategy are warranted.

Keywords: AE37, breast cancer, Ii-Key, peptide, vaccine

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1. Background

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 [1-4]. Vaccines designed to target TAAs represent a therapeutic modality with great specificity and a low potential for toxicity. Numerous vaccination strategies have been described, including peptide vaccines, which are the focus of our group's laboratory and clinical trial efforts.

Peptide-based vaccines use antigenic epitopes derived from TAAs to induce antigen-specific immune regulators, including antibodies, T helper cells (CD4+ or Th cells), and cytotoxic T lymphocytes (CD8+ cells or CTLs). One TAA studied extensively in breast cancer is HER2/neu. Peptides have been identified that are derived from the HER2/neu protein and that can be presented by MHC class I or II molecules to stimulate CTLs or Th cells, respectively. The most studied of the HER2/neu-derived peptides in the laboratory and clinical trials is E75 (HER2/neu 369 – 377), an MHC class I peptide that stimulates CTLs [5-12].

Our group has used E75 mixed with the immunoadjuvant GM-CSF as a simple vaccine to prevent disease recurrence in high-risk women who have completed standard therapy, including surgery, chemotherapy and, when indicated, radiation. We recently reported the combined results of two clinical trials enrolling more than 185 patients; a dose escalation safety trial enrolling disease-free node-positive

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breast cancer patients and a dose optimization study enrolling node-negative patients [11]. In these trials, we demonstrated the vaccine to be safe and effective in raising HER2/neu-specific immunity. At a median follow-up of 20 months, the recurrence rate in the vaccinated group was 5.6%, compared with 14.2% in the observation group ($p = 0.04$), suggesting that vaccination has clinical efficacy [11]. In a more recent analysis performed at a median follow-up of 26 months, the recurrence rate was 8.3% in the vaccine group, compared with 14.8% in the observation group ($p = 0.17$). Although there continued to be a trend toward a benefit to vaccination, the statistical significance was lost [2]. Our finding that E75 immunity wanes over time was not unexpected, given that $CD8^+$ T 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 (APCs) [11].

In light of the findings from our clinical trials, our group is encouraged about the potential ability of peptide vaccines administered in the adjuvant setting to prevent disease recurrence. We recognize, however, that there are limitations to a single-peptide vaccine strategy. In order to be successful, a strategy will need to be identified that results not only in antigen-specific immunity but also in the generation of an immune response that persists after completion of the inoculation series [13]. Therefore, to enhance the efficacy of our CTL epitope peptide vaccine, we have explored additional strategies to enhance the immune response, maintain long-term immunity and increase protection against recurrence. Strategies that we are currently exploring include identification of the optimal biological dose, implementation of a program administering booster inoculations, and vaccination with HER2/neu-derived MHC class II epitope peptides that stimulate Th cells [2,14,15].

Although immunotherapeutic cancer vaccines have historically focused on eliciting a CTL response because of the ability of CTLs to directly kill tumor cells, more recent data have suggested the need for stimulation of a Th response in immunotherapy [16,17]. Recognizing this need, our group recently completed a Phase I clinical trial administering AE37, a modified HER2/neu class II epitope to disease-free, node negative breast cancer patients [14]. AE37 is a novel peptide in that it is a naturally occurring HER2/neu-derived peptide, AE36 (HER2/neu:776 – 790), linked to the Ii-Key moiety of the invariant chain. This technology has been demonstrated to dramatically increase antigen-specific stimulation of Th cells [18]. Whereas the non–Ii-Key–linked peptide was investigated clinically and shown to have immunological activity in patients [6], the Ii-Key–linked version (AE37) demonstrated significantly better activation of HER2/neu-specific T helper cells in peripheral blood mononuclear cells (PBMCs) from cancer patients and in animal models [19]. In this article, we review the potential role of class II epitopes in a peptide vaccine strategy. Particular emphasis is placed on the novel AE37 peptide and on the results of our clinical trial, which is, to our knowledge, the first human Phase I trial of an Ii-Key–linked class II epitope.

2. Role of $CD4^+$ T cells and antitumor immunity

Historically, Th cells appeared to be only minimally involved in direct tumor cell killing, functioning by activating or 'licensing' APCs to increase antigen presentation via MHC class I molecules and via cytokine secretion [20,21]. There is increasing evidence that generation of a Th cell response will be required for a peptide vaccine strategy to be effective in preventing disease recurrence. In one important experiment, mice were vaccinated with irradiated tumor cells prior to subcutaneous injection of tumor cells. Immediately before tumor challenge, some mice had their $CD4^+$ T cells eliminated by treatment with an anti-$CD4^+$ monoclonal antibody. Mice in this treatment group had a significantly slower rate of tumor rejection than that of mice that had not been treated with the antibodies [22]. A second group demonstrated that depletion of $CD4^+$ T cells resulted in an inability to reject MHC class I–negative tumors, because such tumors are not targets for CTLs [23]. Hung *et al.* [24] demonstrated that MHC class I–deficient tumor cells could elicit immune responses in mice similar to those elicited by tumors with functional MHC class I molecules. Subsequent depletion of $CD4^+$ cells, but not $CD8^+$ cells, resulted in the inability to reject tumors [24]. Clinically, the most effective, proven form of cancer immunotherapy has relied on the adoptive transfer of both $CD8^+$ and $CD4^+$ cells exogenously stimulated with tumor cells, which results in objective cancer regression in approximately 50% of patients [25]. The transfer of activated $CD8^+$ cells alone has not produced efficacy [26]. Taken together, these data suggest a central role for Th cells in anti-tumor immunity.

It is likely that activation of Th cells is an early step in the tumor-specific immune response. In patients with early-stage disease, dendritic cells (DCs), which are effective APCs, deliver antigen to lymph node T cells, where Th cells are activated [16,17]. There is evidence that in addition to DC-mediated activation of Th cells, tumor cells can also activate Th cells if they express MHC class II molecules [27,28]. Although many tumor cells do not express MHC class II molecules, melanoma, breast and lung cancers clearly do [29]. In addition to the expression of MHC class II molecules, additional costimulatory molecules are required to stimulate Th cells. Other cells, such as B cells, DCs and macrophages, all of which are present in the lymph nodes and tumor bed, express these costimulatory molecules, allowing for Th cell activation in the tumor cell environment [16,30].

There are two predominant Th cell subtypes: Th1 and Th2. Which subtype develops is influenced by cytokines present in the local environment, with Th1 differentiation relying on IL-12 and Th2 differentiation resulting from IL-4 in the absence of IL-12 [31]. Each of these subtypes plays a unique role in immunity. Th1 cells are responsible for

activating and regulating the development and persistence of cell-mediated immunity [20]. For example, Th1 cells can directly activate CTLs by secreting IL-2, which directly stimulates the growth of CTLs [32]. Th1 cells can also enhance CTL activity indirectly. Th1 cells secrete IFN-γ, which activates APCs to upregulate molecules that contribute to increased antigen presentation to CTLs, including large multifunctional peptidase (LMP)2, LMP7, multicatalytic endopeptidase complex (MECL) and HLA class I antigens [33]. In addition, Th1 cells induce the production of opsonizing antibodies that enhance the uptake of tumor cells into APCs. These APCs then promote the expansion of tumor-specific CTLs [16,24]. Th2 cells are associated with the humoral response. Th2 cells produce several different cytokines, including IL-10, IL-4 and IL-13, which play roles in B cell maturation, clonal expansion and class-switching [16]. Clearly, Th cells mediate an anti-tumor immune response via multiple mechanisms. Two comprehensive reviews on this subject have been published by Knutson and Disis [16,17].

3. HER2/neu-derived MHC class II peptides

Multiple HER2/neu-derived Th peptides have been described, the first of which was G89 (HER2/neu:777 – 789) [34]. In a study analyzing the ability of T cells in PBMCs from 18 breast cancer patients to recognize HER2/neu peptides, Tuttle *et al.* [34] found that the majority of patients responded by proliferation of T cells to at least one of the peptides tested, including G89. Most responding patients were HLA-DR_4^+, suggesting that this peptide is recognized preferentially in association with HLA-DR_4, which is present in 25% of humans. Analysis of the cytokine response to G89 by G89-stimulated T cells showed that the cells secreted high levels of IFN-γ, suggesting priming for a Th1 response [34].

Significant work on Th vaccines has been performed by Disis and colleagues at the University of Washington. Using a computer algorithm, these researchers identified 26 putative HER2/neu-derived MHC class II binding peptides. These 26 peptides were tested for T cell proliferation responses, and nine demonstrated the ability to elicit T cell responses *in vitro* [35]. These nine peptides were formulated into three different vaccine preparations: one targeting the extracellular domain of HER2/neu, a second targeting the intracellular domain, and a third that was unique in that each of the class II peptides contained an HLA-A2 class I binding peptide completely within its sequence [6,16]. In a Phase I trial evaluating these different preparations, they found that more than 90% of patients developed T cell immunity to their immunizing HER2/neu-derived MHC class II peptide and that 60% developed HER2/neu protein-specific immunity. Importantly, at a 1-year follow-up, immunity to the HER2/neu protein persisted in more than one-third of patients, suggesting that the immunity stimulated by vaccination with these MHC class II epitopes included immunological memory. Another important finding in their study was the generation of epitope spreading in the majority of patients. Epitope spreading suggests that the immune response stimulated by vaccination with the MHC class II peptide established a microenvironment at the tumor site conducive to promoting endogenous immunity to either other areas of the HER2/neu protein or other antigens present [6,16].

Vaccination with the third formulation, which had class II epitopes that encompassed the HLA-A2 class I epitopes, provided additional evidence of the important role Th cells may play in a peptide vaccine strategy. Included in this vaccine mixture were HER2/neu:369 – 386, HER2/neu:688 – 703, and HER2/neu:971 – 984. Concurrent with the study investigating the Th peptides, the same group conducted a study immunizing patients with the HLA-A2–binding E75 peptide (HER2/neu:369 – 377) [8]. Patients immunized with the Th epitopes generated T cell immunity to E75 that was of both greater magnitude and greater durability than immunity in patients immunized with only the E75 peptide. The higher E75 responses correlated with higher responses to the longer Th epitope that fully encompassed it. E75 peptide–specific T cells derived from patients who had more vigorous Th responses could lyse HER2-overexpressing HLA-matched cells after 1 year. In contrast, patients immunized with the E75 peptide alone developed low-level, short-lived E75-specific immunity [8,16,36]. Finally, the researchers performed binding assays and demonstrated that *in vitro* binding correlated with *in vivo* immunogenicity. Three peptides were identified as the most immunogenic: HER2/neu:98 – 114, HER2/neu: 369 – 386, and HER2/neu:776 – 790 [37]. Taken together, these data supported an important role of inducing T helper immunity and identified specific peptides that warranted further investigation.

4. Augmentation of $CD4^+$ T cell–mediated antitumor responses using the Ii-Key moiety of the invariant chain

Ii-Key/MHC class II hybrid peptides represent a novel method of enhancing the immunogenicity of MHC class II vaccine approaches [38,39]. A lengthy discussion of the Ii-Key technology is outside the scope of this article, but a comprehensive review has been published by Kallinteris *et al.* [40]. Briefly, the Ii protein normally binds to MHC class II molecules in the endoplasmic reticulum at synthesis and protects the epitope-binding site on MHC class II molecules from binding to endogenously derived epitopes in the endoplasmic reticulum [40,41]. A 16-amino-acid sequence of the Ii protein was found that enhances the presentation of antigenic peptides by APCs to T cell hybridomas [38,39]. Studies performed on homologs of that peptide, known as 'Ii-Key', demonstrated a core structure (LRMKLPK) that had significantly greater activity than that of the original 16-amino-acid peptide. Further studies identified that the shortest active sequence was four amino acids (LRMK), which is referred to as the 'Ii-Key peptide' [38,42]. Covalent linkage of the

Table 1. Dosing of peptide and immunoadjuvant in 15 patients.

Dose group*	AE37 dose (μg)	GM-CSF initial dose (μg)	Dose reduction of peptide required?	Dose reduction of GM-CSF required?
100:250:6	100	250	No	No
500:250:6	500	250	No	Yes (all 3 patients)
1000:0:6	1000	0	Yes (in 2 of 3 patients)	N/A
500:125:6	500	125	No	Yes (all 3 patients)
500:30:6	500	30	No	No

*Dose groups are given as peptide (μg):GM-CSF (μg):number of inoculations. There were three patients in each group.

Ii-Key segment to Th peptides facilitates direct antigenic epitope charging of MHC class II molecules at the cell surface [18,39]. This enhanced epitope charging results in increased antigen presentation that can increase potency to ≥ 250 times that of the unmodified class II epitope *in vitro* [43,44].

The HER2/neu:776 – 790 peptide sequence, also known as AE36, has been studied by several different groups. It is promiscuously presented by multiple alleles, including HLA-DR_4 [19,34,37], and is naturally processed and presented by numerous types of human tumor cells [19]. AE37 is an Ii-Key hybrid of HER2/neu:776 – 790 (AE36) generated by the addition of LRMK to the N terminal of the HER2/neu peptide. By taking advantage of the Ii-Key interaction with class II MHC molecules, AE37 can directly charge MHC class II molecules with the antigenic HER2/neu-derived epitope, bypassing the normal antigen processing pathway. The antigenic epitope can then be presented to the Th cells, stimulating a specific immune response [14]. In a recent study investigating several Ii-Key/HER2/neu hybrid peptides, including AE37, Voutsas *et al.* [45] showed that the hybrid peptides elicited higher frequencies of Th cell responses then did the native AE36 peptide in PBMCs from patients with HER2/neu-positive tumors. Furthermore, they showed that hybrid-peptide-induced Th cells stimulated higher HER2/neu peptide-specific CTL responses, which resulted in better tumor regression in SCID mice xenografted with a HER2/neu-overexpressing tumor cell line [45]. The efficiency of AE37 *in vitro* and in animal models thus warranted further investigation in clinical trials.

5. Phase I clinical trial of an Ii-Key/HER2/neu hybrid peptide vaccine (AE37)

Our group has recently published the results of the first human trial of a peptide vaccine developed using Ii-Key technology, a Phase Ib trial of AE37 in HER2/neu-positive (defined as immunohistochemistry (IHC) 1+-3+) breast cancer patients [14]. It is important to emphasize our definition of HER2-positivity and recognize that it is different then the definition currently used clinically to identify patients with HER2-overexpressing (defined as IHC 3+ or fluorescence *in situ* hybridization (FISH) positive) disease that may benefit from HER2-targeted therapeutics such as trastuzumab. Interestingly, in clinical trials using E75, another HER2-derived peptide, our group has found that patients with IHC 1+ or 2+ disease have a more robust immune response after vaccination then patients with IHC 3+ or FISH positive disease [46]. The Phase Ib trial of AE37 was conducted as a dose-escalation study to document safety and measure the immunologic response to escalating vaccine doses. Fifteen patients were enrolled, three in each of five dosage groups (**Table 1**). All patients had node-negative breast cancer and had been rendered disease free after a course of standard therapy that included surgery, chemotherapy and, if indicated, radiation. Toxicities were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events v3.0. *In vivo* immune responses were assessed using a standard delayed-type hypersensitivity (DTH) reaction and *in vitro* immune monitoring consisting of a standard radioactive [^{3}H]-thymidine incorporation assay. In order to ensure that measured immune responses were representative of reactivity against the native HER2-derived peptide, we measured immune responses against AE36 as well as AE37. AE37 was found to be safe and well tolerated. Among all patients, maximum local toxicities were grade 1 (40%) or grade 2 (60%) (**Figure 1**). Local toxicity was highest in the 500:250:6 group (dose groups are given as peptide [μg]:GM-CSF [μg]:number of inoculations), with all patients experiencing grade 2 toxicity in the form of a significant skin reaction. Maximum systemic toxicity among all patients was mild, including grade 0 (13%), grade 1 (73%), and grade 2 (13%) (**Figure 1**). Only two patients (one in dosing group 1000:0:6 and one in dosing group 500:125:6) experienced grade 2 symptoms, consisting of joint pain and stiffness. Forty-seven percent of patients had dose reductions, per protocol design, because of local reaction measurement of indurations 100 mm or greater in diameter or grade 2 systemic toxicity. Because each patient in the 500:250:6 dose group required multiple GM-CSF reductions, the next group (1000:0:6) was inoculated without GM-CSF. One patient in this group failed to respond with a local reaction so GM-CSF was reinstated with that patient's fourth inoculation. Importantly, two patients in this group did not receive GM-CSF, and they still mounted significant reactions. Historically, peptides alone have been demonstrated to be only minimally immunogenic and to require the administration of an immunoadjuvant to induce

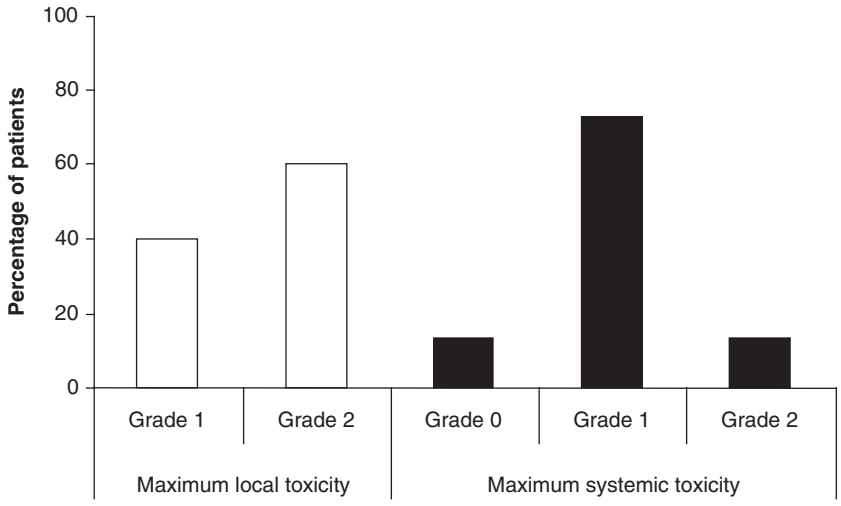

Figure 1. Combined toxicity response. Maximum local and systemic toxicities are given for 15 patients. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events v3.0.

detectable T cell responses [47]. To our knowledge, this is the first peptide vaccine to show potency in the absence of an immunoadjuvant.

The AE37 peptide vaccine was also found to be highly immunogenic. All dosage groups had statistically significant increases in their pre- to postvaccine DTH responses (**Figure 2A**), and reaction measurements were enhanced as peptide dose increased. When GM-CSF was reduced from 250 to 125 µg, there was no decrease in DTH reactions. When GM-CSF was decreased to 30 µg, there was a significant decrease in DTH reactions. *In vitro* assays also demonstrated a significant response, with mean proliferative responses to AE37 increasing significantly from before to after vaccination in all dose groups (**Figure 2B** and **C**).

This trial demonstrated the hybrid AE37 vaccine to be safe and well tolerated with minimal toxicity at proper doses. Given the positive results of this trial, our group is currently conducting a multicenter, Phase II trial further investigating the AE37 hybrid vaccine. We have also begun a Phase I trial of a multi-epitope vaccine strategy administering to patients the combination of AE37 with GP2, a class I HER2/neu-derived peptide that we have demonstrated to be more immunogenic then E75 [48]. A multi-epitope vaccine should work to stimulate antigen-specific CTL responses both directly and indirectly. We propose therefore that there will be an enhanced immune response to the doublet GP2 and AE37 + GM-CSF vaccine as compared with either the GP2 + GM-CSF or AE37 + GM-CSF vaccines alone.

The purpose of this Phase I multi-epitope trial is to determine the safety of and assess the immune response to doublet vaccination with GP2 and AE37 in disease-free, conventionally treated HLA-A2+ or HLA-A3+ patients with intermediate- to high-risk breast cancer. The study design is a standard 3 × 3 cohort design: three patients will be assigned to each successive cohort and receive six sets of inoculations (four inoculations, two per peptide, per visit for a total of 24 inoculations) every 3 – 4 weeks. Each vaccine will be administered intradermally, in two 0.5 ml inoculums ≤ 5 cm apart. The GP2 and AE37 vaccines will be injected at two different sites ≥ 10 cm from each other in the same extremity. In Phase I trials investigating AE37 and GP2 as single agents, both peptides were demonstrated to be safe and well tolerated with minimal toxicity at doses as high as 1000 µg per injection. For this Phase I multi-epitope vaccine trial, dose escalation with the combination of AE37 and GP2 will be initiated at ¼ the dose of each, such that the initiating combined dose will be 50% less than previously demonstrated 'no toxic effect' dose level. Given the robust local reactions we saw in response to the AE37 vaccine, it is possible that we will see significant local toxicity in this multi-epitope trial. The trial is designed to address such a finding with a well-defined dose reduction strategy to be implemented in the case of grade 3 local toxicity.

6. Conclusions

There is increasing evidence that generation of a Th cell response will be required for a cancer vaccine to have adequate antitumor activity. AE37 is a modified MHC class II epitope peptide that employs the novel Ii-Key technology to enhance antigen-specific immunogenicity. *In vitro* and *in vivo* work, including the recently published results of a Phase I trial, has suggested that this peptide is effective in stimulating HER2/neu-specific immunity. Ongoing clinical trials will determine the utility of incorporating AE37 into a multi-epitope vaccine.

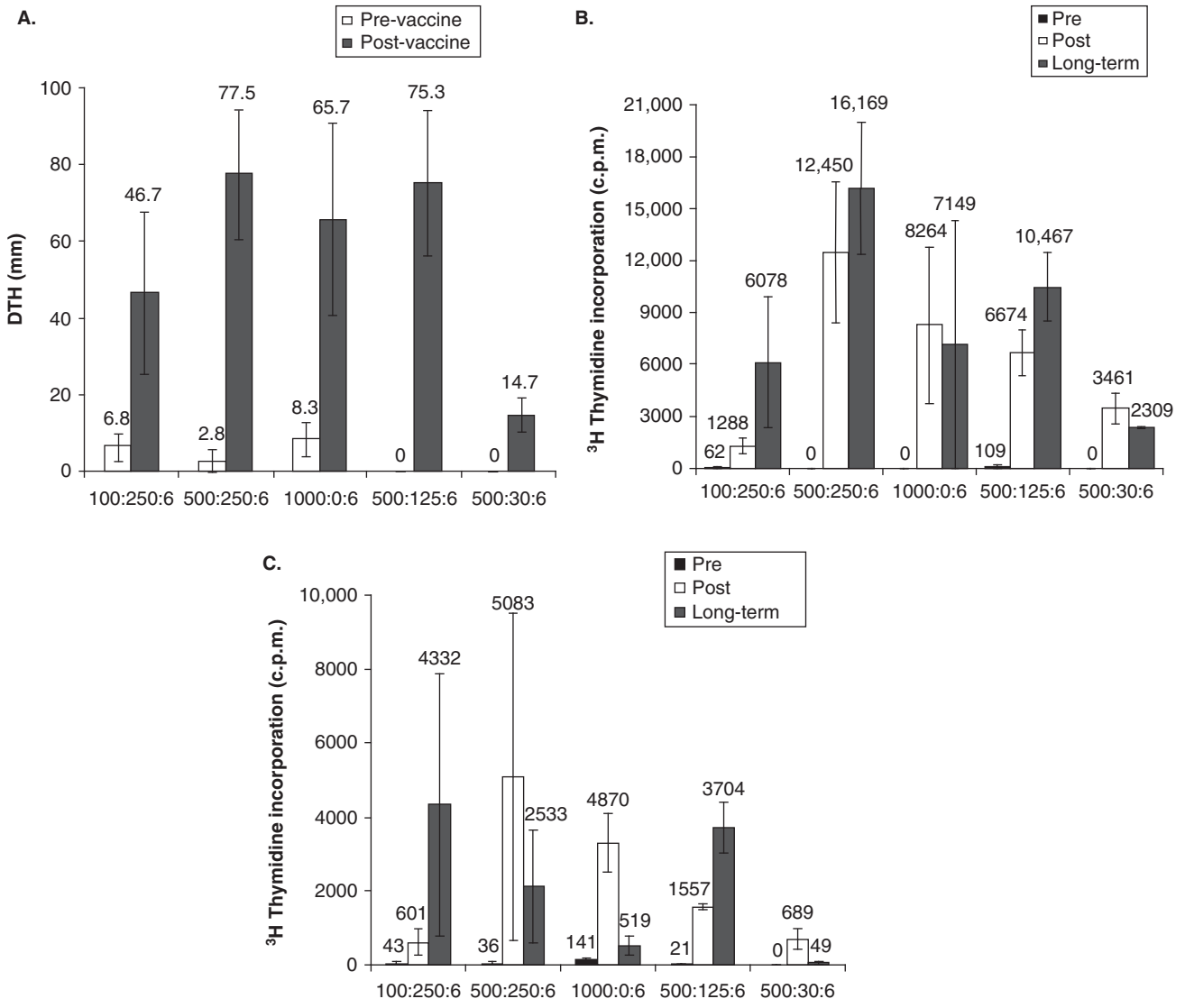

Figure 2. Immunological responses. (**A**) Delayed-type hypersensitivity (DTH; mean ± standard error) pre- and postvaccine per dosing groups. (**B**) Proliferative responses (mean ± standard) to AE37 increased prevaccine (pre) to postvaccine (post) and long-term (6 months after completion of the inoculation series) responses. (**C**) Proliferative responses (mean ± standard error) to AE36 increased prevaccine (pre) to postvaccine (post) and long-term. Dose groups are given as peptide (μg):GM-CSF (μg):number of inoculations (three patients per group).

7. Expert opinion

Multiple clinical trials investigating cancer vaccines have failed to demonstrate clinical efficacy. The majority of these trials enrolled patients with late-stage disease and were utilizing vaccines as a therapeutic modality. Our group believes that vaccines will have the greatest utility if they are administered to prevent disease recurrence in breast cancer patients rendered disease free after the completion of standard-of-care therapy. Results of our early clinical trials are encouraging and suggest that peptide vaccines will have clinical efficacy in this setting. We recognize, however, a need to enhance the efficacy of CTL epitope peptide vaccines used in our initial trials by employing strategies to specifically stimulate Th cells such as the administration of MHC-class II epitope peptides. Based on early observations of patients enrolled in our Phase II AE37 trial, we are confident that this Ii-Key hybrid peptide will effectively stimulate an HER2/neu-specific immune response and will be an important component as we move forward with our plans to develop a multiepitope vaccine. Because it is nontoxic and has great specificity, a cancer vaccine would have important potential advantages over other available therapies for breast cancer [49]. We therefore anticipate that interest in immunotherapy will continue to grow.

Declaration of interest

Murray has received $100,000 in grant funds from Antigen Express.

von Hofe is president of Antigen Express.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army, Department of the Navy, or Department of Defense.

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