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Circulating regulatory T cells ($CD4^+CD25^+FOXP3^+$) decrease in breast cancer patients after vaccination with a modified MHC class II HER2/*neu* (AE37) peptide☆,☆☆

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ABSTRACT

Regulatory T cells (T_{Reg}), $CD4^+CD25^+FOXP3^+$, are implicated in suppressing tumor immune responses. We analyzed peripheral blood lymphocytes (PBL) from breast cancer patients receiving a modified HLA class II HER2/*neu* peptide (AE37) vaccine for T_{Reg} cells and correlated their levels with vaccine-specific immune responses. The mean $CD4^+CD25^+FOXP3^+$ T_{Reg} cells decreased in patients with vaccination with no significant difference in serum TGF-β levels. IFN-γ ELISPOT and DTH increased after vaccination with a good correlation between T_{Reg} cell reduction and size of DTH to AE37. The T_{Reg} cell reduction and associated immune response suggest that AE37 may be clinically useful.

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1. Introduction

The interplay between neoplasia and the immune system is complex with multifactorial means by which a tumor is able to evade the immune system [1,2]. The phenotypic characterization of a subpopulation of $CD4^+$ T cells, regulatory T cells (T_{Reg}), which have the ability to down-regulate the immune response and provide a means of neoplastic escape from immunosurveillence, has now been established after having been a point of debate for nearly 40 years [3]. In light of these new findings, the comprehensive evaluation of peptide-based cancer vaccines, especially CD4-activating vaccines should include monitoring their effect on levels of T_{Reg} cells: $CD4^+CD25^+FOXP3^+$.

T_{Reg} cells are a subset of $CD4^+$ lymphocytes with a distinct phenotype which, unlike effector $CD4^+$ T cells, primarily have a suppressive role on the immune system. T_{Reg} cells constitutively express high levels of the IL-2 receptor α chain (CD25) along with increased amounts the FOXP3 protein (a member of the forkhead family of transcription factors), a pivotal molecule in T_{Reg} development and function. T_{Reg} cells originate in the thymus (naturally occurring T_{Reg}) or are induced in the periphery by antigens (adaptive T_{Reg}). Through cell–cell contact dependent mechanisms and TGF-β expression, T_{Reg} cells control the immune response to pathogens and prevent autoimmune processes [4]. The fact that most tumor-associated antigens are also self-antigens tolerated by the immune system, in part because of T_{Reg} cells, constitutes one of the chief obstacles to successful cancer immunotherapy.

HER2/neu is a proto-oncogene in the epidermal growth factor family of tyrosine kinases which encodes a transmembrane glycoprotein that is highly expressed in many epithelial derived cancers [5]. Breast cancer, one such epithelial cancer, is the most common cancer in women and responds best to multi-modal therapy, including surgery, chemotherapy, radiation therapy, hormone

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therapy, and immunotherapy, as indicated [6,7]. Despite patients being rendered disease-free, many women with high risk features, such as high expression of the HER2/*neu* protein (20–25% of breast cancer patients), will have recurrent disease after receiving primary treatments [8,9].

The HER2/*neu* protein is a tumor-associated antigen (TAA) containing several immunogenic peptides recognized by $CD8^+$ cytotoxic T lymphocytes (CTLs) [10–14]. The use of MHC class I peptides (E75 and GP2) from HER2/*neu* that target CTLs has been evaluated by our group [15,16]. These peptides have been shown to be safe as well as immunogenic. With accumulating evidence that $CD4^+$ T cells play a critical role in effective anti-tumor responses such as the induction of CTLs, our group has begun evaluating the induction and/or stimulation of $CD4^+$ T lymphocytes with the use of a MHC class II peptide (AE37) consisting of a HER2/*neu* peptide (776–790) hybridized to the Ii-Key moiety (a four amino-acid peptide) of the HLA class II-associated invariant chain, which enhances epitope interaction with the class II molecule [17–19]. In mice and with *in vitro* cultures using peripheral blood cells from cancer patients, these hybrid peptides have already been shown to potently stimulate antigen-specific $CD4^+$ T_H cells with therapeutic anti-tumor activity [20–23].

With the robust induction of $CD4^+$ T cells by AE37, there is a theoretical concern that T_{Reg} cells may proliferate and thereby lead to suppression of an effective immune response. Recently, the initial report of the phase Ib trial for AE37 documented the vaccine's efficient induction of immunity and minimal toxicity to escalating vaccine doses in node-negative breast cancer patients. The vaccine was shown to be highly immunogenic even without an immunoadjuvant, GM-CSF, in a subgroup of patients [24]. Therefore, in this study, we have assessed the levels of T_{Reg} cells prior to and after vaccination, as well as their relationship to vaccine-induced *in vitro* and *in vivo* immune responses.

2. Materials and methods

2.1. Patient characteristics and clinical protocol

The clinical trial testing the AE37 vaccine was Institutional Review Board-approved and conducted at Walter Reed Army Medical Center, Washington, DC under an Investigational New Drug Application (BB-IND#12229). All patients had histologically confirmed, node-negative (NN) breast cancer, and completed standard course of surgery, chemotherapy, and radiation (as required) before enrollment. Patients on hormonal therapy were continued on their regimen. After obtaining proper consent, breast cancer patients were enrolled into the study. Before vaccination, patients were skin tested with a panel of recall antigens (modified Mantoux test). Patients were considered immunocompetent if they reacted (>5 mm) to ≥2 antigens. All enrolled patients passed the Mantoux test.

2.2. Vaccine

The AE37 peptide (Ac-LRMKGVGSPYVSRLLGICL-NH_2) used in this study is a fusion of the four amino-acid Ii-Key peptide (LRMK) with the native HER2/*neu* peptide (aa776–790: GVGSPYVSRLLGICL). AE37 was commercially produced in accordance with federal guidelines for good manufacturing practices (GMP) by NeoMPS, Inc. (San Diego, CA). Peptide purity (>95%) was verified by high-performance liquid chromatography and mass spectrometry. Sterility and general safety testing was carried out by the manufacturer. Lyophilized peptide was reconstituted in 0.5 ml sterile saline at the following doses: 100, 500, and 1000 mcg. The peptide was mixed with different doses of GM-CSF in 0.5 ml (Berlex, Seattle, WA) resulting in a 1 ml inoculation. The 1.0 ml inoculation was split and given intradermally at two sites 5 cm apart in the same extremity.

2.3. Vaccination series

The study was performed as a dose escalation trial to define optimal dosing of vaccine and GM-CSF concentrations. Each dosing group consisted of three patients. The first three dose groups (patients A1–A9) were given escalating amounts of AE37 peptide and fixed initial GM-CSF dose. GM-CSF dose was chosen based on our previous E75 trials [15]. The local reactions at the sites of vaccine injection were monitored, and GM-CSF was reduced 50% in subsequent inoculations for local reactions ≥100 mm or grade 2 systemic toxicities. The cutoff of 100 mm was determined from previous experience; at ≥100 mm, the sites become confluent, local toxicity increases, and our goal is to minimize toxicity and prevent skin disruptions. All patients received 6 monthly inoculations, as previously described [24].

2.4. Toxicity

Patients were observed 1 h post-vaccination for immediate hypersensitivity and returned 48–72 h later to have injection sites measured and questioned in regards to local/systemic toxicities. Toxicities were graded using NCI Common Terminology Criteria for Adverse Events version 3.0 (reported on 0–5 scale). Progression from one dose group to the next occurred only if no significant toxicity occurred in the lower dose group.

2.5. Peripheral blood mononuclear cell (PBMC) isolation and cultures

Blood was drawn before each inoculation and at 1 (post-vaccine) and 6 months (long-term) after vaccine series completion. Forty milliliter of blood was drawn into Vacutainer CPT tubes (BD Biosciences) and PBMCs isolated. PBMCs were washed and re-suspended in culture medium (RPMI + 10%FCS + penicillin/streptomycin + L-glutamine) and used as a source of lymphocytes as previously described [25–27].

2.6. Immunofluorescent staining and flow cytometry analysis

PBMC were stained with anti-CD4–FITC and anti-CD25–APC (eBiosciences, USA) to determine their immunophenotype and measure the $CD4^+CD25^{+high}$ T cell populations. In addition, the PBMC samples were also subjected to intracellular staining for expression of FOXP3 using the PE-conjugated anti-FOXP3 antibodies PCH101 and 236A/E7 (eBiosciences, USA). Three-color flow cytometry was performed using FACSCalibur. Data was collected on the total cell population and subsequent analysis was performed on lymphocytes (gated by forward and side scatter properties) with eventual focus on the $CD4^+$ population of cells. The FACS datasets were analyzed using CellQuest software, version 3.3 (Becton Dickinson).

2.7. ELISPOT assay

Freshly isolated PBMCs were resuspended in culture medium supplemented with IL-7 (20 ng/ml) and added at 5×10^5 cells/200 μl/well into flat-bottom wells of anti-human interferon (IFN)-γ ELISPOT plates (BD PharMingen). Duplicate wells of the PBMC were stimulated in the absence or presence of AE37 at 25 μg/ml by placing the plate overnight in an incubator (37 °C +5% CO_2). After the overnight incubation, the plate was processed and developed as suggested by the manufacturer. The number of spots present in

each well was enumerated using the CTL ELISPOT analyzer (CTL Analyzers LLC, Cleveland, OH).

2.8. Serum TGF-β cytokine ELISA

Serum samples were prepared by drawing 10 ml of blood into a Vacutainer Gel & Clot Activator tube (Becton Dickinson, Franklin Lakes, NJ) that was then centrifuged and the serum aspirated. Serum was aliquoted in NUNC-Cryovial tubes and placed in −80° freezer. Serum samples were thawed and used for the measurement of TGF-β cytokine levels using ELISA kits (BioSource, Camarillo, CA). Total TGF-β was assayed by acidification (1N HCl) and neutralization (1N NaOH) of the serum as per manufacturer's instructions. The assay measured TGF-β1 which is the prevalent and most ubiquitously expressed form of the five isoforms. The procedures for collection, preparation, freezing, and thawing of all the serum samples used in this study were performed in a highly consistent manner and none of the serum samples had been previously thawed prior to thawing for this cytokine assay.

2.9. Delayed type hypersensitivity (DTH)

DTH reaction was performed prior to vaccination and at 1 month after completion of vaccine. Intradermal injections were performed on the back or extremity (opposite side from vaccination), using 100 mcg of AE37 (without GM-CSF) in 0.5 ml of saline and compared to an equal volume control inoculum of saline. DTH reactions were measured in two dimensions at 48–72 h using the sensitive ballpoint pen method and results reported as orthogonal mean [28].

2.10. Statistical analysis

p values for ELISPOT were calculated using χ^2 test. p values for comparing pre- and post-vaccine T cell levels and DTH were calculated using paired Student t-test. The Pearson's correlation coefficient (r) was obtained for comparison of the change in DTH with relation to reduction in T_{reg} levels. Statistical significance is defined as $p < 0.05$.

3. Results

3.1. Patients

Fifteen disease-free, NN breast cancer patients were enrolled after completion of standard adjuvant therapy and vaccinated. All patients expressed HER2/*neu* at varying levels (IHC 1–3+). No patients withdrew from this study [24].

3.2. Circulating CD4⁺ and CD4⁺CD25⁺ T cells

Peripheral blood samples obtained pre- and post-vaccination (1 month after completion of the series) were stained with anti-CD4/CD25 and anti-CD8/CD25. The mean $CD4^+$ and $CD4^+CD25^+$ T cell populations for all patients ($n = 15$) did not change from pre- to post-vaccination ($CD4^+ = 52.3 \pm 3.3\%$ vs. $50.5 \pm 3.9\%$, $p = 0.55$; $CD4^+CD25^+ = 1.9 \pm 0.2\%$ vs. $2.4 \pm 0.5\%$, $p = 0.19$). $CD8^+$ T cell data was available for 12 patients. The mean $CD8^+$ T cell populations did not change ($CD8^+ = 23.8 \pm 2.3\%$ vs. $23.4 \pm 2.9\%$, $p = 0.90$) and the mean $CD8^+CD25^+$ T cell populations trended toward an increase from pre- to post-vaccination ($CD8^+CD25^+ = 0.5 \pm 0.2\%$ vs. $1.5 \pm 0.4\%$, $p = 0.09$) (Fig. 1).

3.3. Circulating CD4⁺CD25⁺FOXP3⁺

The expression of the FOXP3 protein in $CD4^+CD25^+$ T cells has become widely accepted as a means of monitoring T_{Reg} cells. Therefore in our study, a more accurate identification of the T_{Reg} population was performed by staining for FOXP3 in the $CD4^+CD25^+$ T cell population in the latter 9 vaccinated patients (since the FOXP3 assay was not being performed during the vaccination period for the initial three patients). The intracellular staining assay was performed utilizing two anti-FOXP3 antibodies (PCH101 and 236A/E7) followed by analysis with flow cytometry (Fig. 2). T_{Reg} ($CD4^+CD25^+FOXP3^+$) cells were reduced in all 9 patients tested pre- to post-vaccination for both FOXP3 antibodies (Ab) (FOXP3 Ab1 = $2.1 \pm 0.2\%$ vs. $1.1 \pm 0.1\%$, $p = 0.002$; FOXP3 Ab2 = $2.0 \pm 0.2\%$ vs. $1.0 \pm 0.2\%$, $p = 0.0009$) (Fig. 3). Additionally, in two patients who received AE37 without GM-CSF immunoadjuvant the T_{Reg} population levels was observed to show a declining trend (FOXP3 Ab1 = $2.4 \pm 0.6\%$ vs. $0.9 \pm 0.4\%$, $p = 0.07$; FOXP3 Ab2 = $2.2 \pm 0.6\%$ vs.

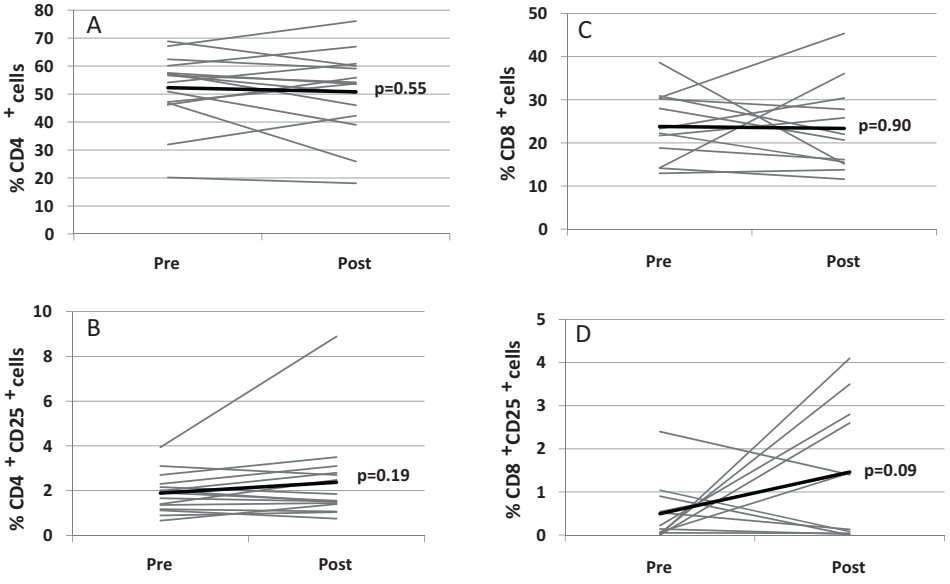

Fig. 1. There was not a significant change in percent of total lymphocyte populations from prior to vaccination (pre) to 1 month after completion of the vaccine series (post) with the dark line representing the mean. Lymphocytes are characterized as $CD4^+$, $n = 15$ (**A**); $CD4^+CD25^+$, $n = 15$ (**B**); CD8, $n = 12$ (**C**); and $CD8^+CD25^+$, $n = 12$ (**D**).

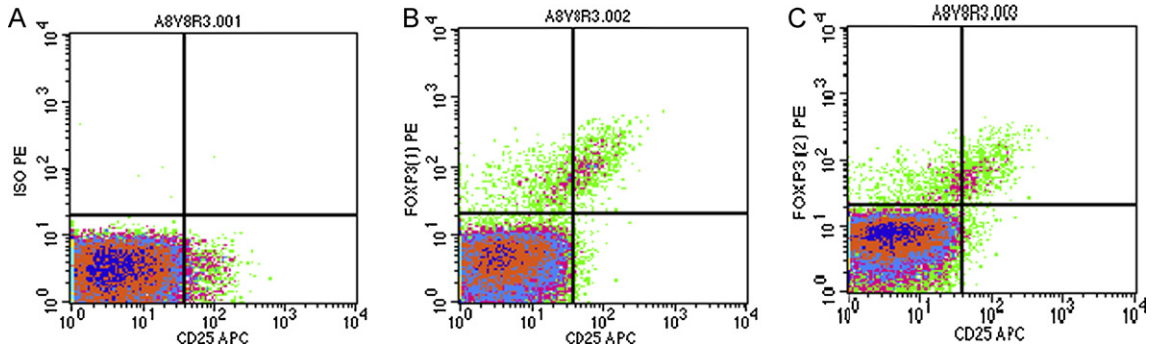

Fig. 2. Staining of patient PBMC with FOXP3 antibodies. PBMC were stained with FITC anti-human CD4 and APC anti-human CD25 and PE Rat IgG2a Isotype Control (**A**) or PE anti-human FOXP3–Clone PCH101 (**B**) or PE anti-human FOXP3–Clone 236A/E7 (**C**). Cells were gated to select the breakaway $CD25^{high}$ and FOXP3 positive cells.

$1.2 \pm 0.9\%$, $p = 0.18$).

3.4. Serum TGF-β levels

The cytokine TGF-β is a key mediator of immune suppression and is believed to play a pivotal role in the functional activity and/or generation of T_{Reg} cells. In order to monitor for changes in this cytokine as a result of vaccination, the serum obtained from pre- and post-vaccination blood samples was assessed for levels of TGF-β. The results obtained indicate that there was no difference in pre- to post-vaccination levels of TGF-β (2720 ± 582 pg/ml vs. 2957 ± 538 pg/ml; $p = 0.3$) (Fig. 4).

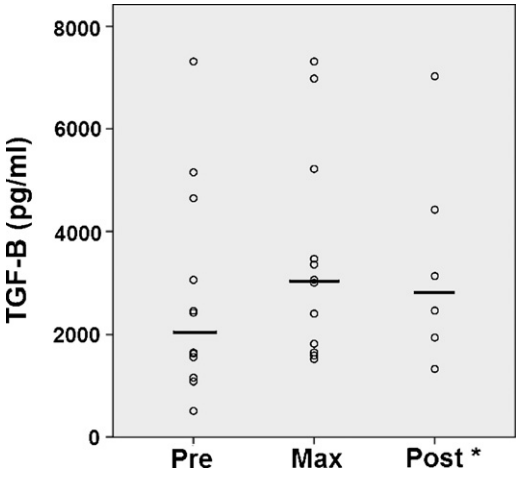

Fig. 4. There was no significant difference in the median amount of serum TGF-B (pg/ml) pre-vaccination (pre, 12 patients), maximum level during the vaccination series (max, 12 patients), and post-vaccination (post, 6 patients), $p = 0.3$.

3.5. Immune response

As a means of monitoring a functional immune response to the vaccine in the same blood samples that were being tested for T_{Reg} levels, PBMC were stimulated directly *ex vivo* with AE37 peptide to measure IFN-γ secretion by the ELISPOT assay. The results demonstrated an increased AE37 vaccine-specific response from pre- to max (median = 2 (range = 0–300) vs. 104 (range = 10–1202) spots/10^6 cells; $p < 0.0001$) during the primary vaccination series and long-term (6–12 months) post-vaccination (median = 2 (range = 0–300) vs. 34 (10–266) spots/10^6 cells; $p = 0.003$) (Fig. 5).

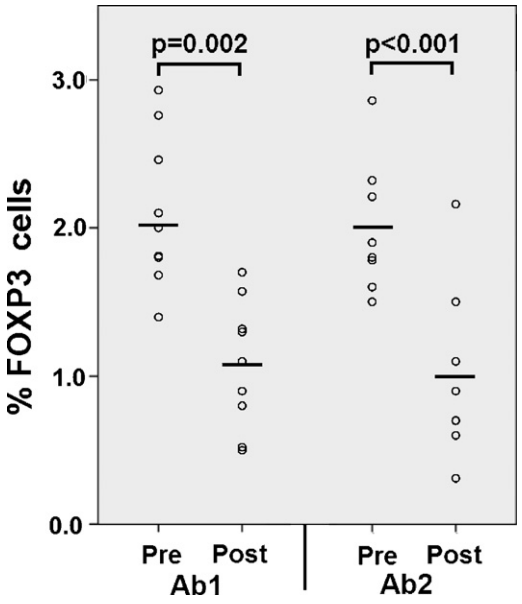

Fig. 3. Percent of total $CD4^+$ T cells which are $CD4^+CD25^{high}FOXP3^+$ T_{Reg} cells measured prior to vaccination (pre) and 1 month after completion of vaccine series (post) based upon PE anti-human FOXP3–Clone PCH101 (Ab1) or PE anti-human FOXP3–Clone 236A/E7 (Ab2) ($n = 9$).

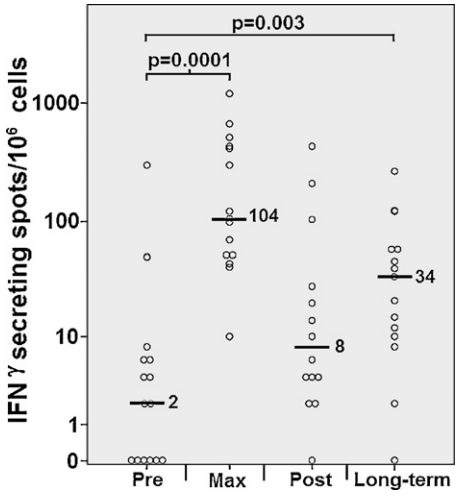

Fig. 5. Median IFN-γ ELISpot responses to AE37 for all patients ($n = 15$) prior to vaccination (pre), maximum response during the vaccine series (max), 1 month after completion of the vaccine series (post), and 6 months after completion of the series (long-term, $n = 14$).

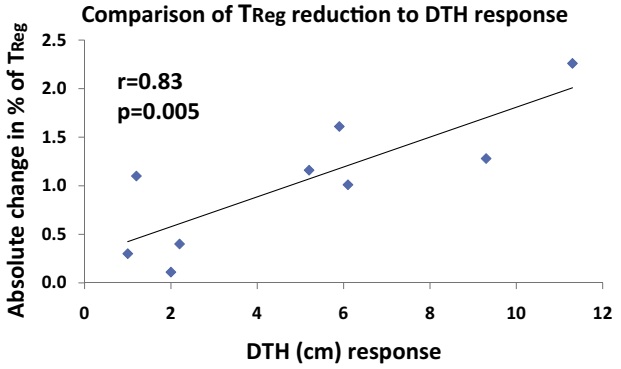

Fig. 6. Comparison of the change in DTH (cm) response to the absolute change in the percentage of T cells which are $CD4^+CD25^+FOXP3^+$ cells per patient pre- to post-vaccination (data shown for FOXP3 Ab1). Pearson's correlation coefficient reveals good correlation.

3.6. Correlation between T_{Reg} levels and immunologic response

In vivo clinical immune responses to the AE37 vaccine were detected by measuring DTH reactions to the vaccine peptide before the initial vaccination and 1 month after the last vaccine dose. DTH responses increased in all patients from pre- to post-vaccination (3.6 ± 1.4 mm vs. 56.0 ± 9.4 mm; $p < 0.0001$). Furthermore, with the exception of one outlier patient, there appeared to be a good correlation between the degree of T_{Reg} reduction and the size of DTH response to AE37 ($r = 0.83$, $p = 0.005$) (Fig. 6).

4. Discussion

We have recently reported the results of a phase 1b clinical trial investigating AE37, a novel HER2/*neu* peptide vaccine. The peptide is a highly immunogenic vaccine even in the absence of an immunoadjuvant [24]. Given the fact that AE37 is a MHC class II peptide, the potential for induction of T_{Reg} cells was concerning. In the current study, we have addressed this issue by assessing the levels of $CD4^+CD25^+FOXP3^+$ cells and TGF-β levels in the peripheral blood of patients before and after administration of the AE37 vaccine. We found no increase in the numbers of $CD4^+CD25^+FOXP3^+$ cells or levels of serum TGF-β in blood and serum samples, respectively. In fact, our findings demonstrate a reduction in $CD4^+CD25^+FOXP3^+$ cells after vaccination, and this reduction was inversely related to the clinical *in vivo* immune response as measured by DTH.

AE37 is a novel MHC class II hybrid vaccine that consists of a 15 amino-acid immunogenic epitope from HER2/*neu* linked to a four amino-acid peptide from the MHC invariant chain (Ii). AE37 takes advantage of the Ii-Key protein interaction with class II MHC molecules by directly charging MHC class II molecules with the antigenic epitope of HER2/*neu*. When compared to an unmodified epitope, these Ii-Key/antigenic epitope hybrids can display ≥250 times potency *in vitro* [29,30]. Preclinical studies demonstrated that AE37 was more potent than the native epitope-only peptide both in eliciting higher frequencies of Th cell responses in the PBMC of patients with HER2/*neu*-positive tumors and in potentiating CTL responses in a SCID mouse tumor model [31,32].

In the present study, we found that AE37 did not significantly change the composition of the circulating T cell populations; however, the percentage of activated $CD8^+$ T cells ($CD8^+CD25^+$) trended toward an increased number. This may represent activation of $CD8^+$ lymphocytes against MHC class I epitopes contained within AE37 by internalization and processing of AE37 antigen presenting cells or cross-activation by T-helper cells, This potential increase in activated $CD8^+$ lymphocyte response is encouraging given the previously published increases in AE36/AE37-specific $CD4^+$ T lymphocyte stimulation [24], which has been suggested to be important in generating an effective, long-term tumor-specific immune response [33]. Furthermore, the induction of an *in vivo* clinical immune response by the AE37 vaccine was also seen by an increased DTH response in the patients after vaccination. As previously stated, these results were observed corresponding with a decrease in $CD4^+CD25^+FOXP3^+$ cells.

The presence and effect of T_{Reg} cells in breast cancer is important and must be addressed in any cancer immunotherapy targeting this prevalent disease. As shown by Liyanage et al., T_{Reg} cells in breast cancer patients ($n = 35$) were found to be significantly higher than normal donors ($p < 0.01$) and were able to suppress proliferation and IFN-γ secretion from activated $CD8^+$ or $CD4^+CD25^-$ cells [34]. Our group has also previously reported similar findings of higher T_{Reg} in node-positive and node-negative breast cancer patients [35]. Furthermore, higher levels of T_{Reg} cells were found in invasive tumors compared to DCIS using immunohistochemical stained tissue microarrays, and more importantly, the number of T_{Reg} cells (≥15 cells/core) correlated with clinical outcome. In invasive breast cancer, high numbers of FOXP3+ cells were correlated with positive lymph node status, higher tumor grade and shorter overall and relapse-free survival. Shorter relapse-free survival was also demonstrated in DCIS patients with high numbers of T_{Reg} cells [36]. Similarly, FOXP3 expression has been demonstrated to be an independent prognostic factor in breast cancer with an inverse relationship between FOXP3 expression and overall survival [37].

The significant role that T_{Reg} cells play by suppressing antitumor immunity was elucidated within a decade of their discovery [38,39]. Since then, further phenotypic characterization of these cells as $CD4^+CD25^+FOXP3^+$ and the understanding that TGF-β is a predominant cytokine responsible for the suppressive activities associated with T_{Reg} cells has enabled more accurate monitoring in clinical studies [35,36,40–45]. The pre-vaccination level of TGF-β in the serum of the patients in the current study was found to be similar to our previously reported value for a separate group of breast cancer patients [35]. Three of six patients, for whom pre- and post-vaccination levels were available, did show a decrease in serum TGF-β levels but the trend did not prevail and the levels of TGF-β before and after completion of vaccination remained unchanged for the group as a whole. The utility of measuring serum TGF-β as a marker of T_{Reg} cells is unclear.

The potential for induction of T_{Reg} cells by cancer vaccines is a recognized issue. Both animal models and human trials have demonstrated increased T_{Reg} cells after therapeutic vaccination [46–49]. In contrast, others have reported decreased T_{Reg} cells with vaccination or decreases in the subset of patients with metastatic disease who clinically responded to vaccination [50–53]. This issue was previously addressed by our group regarding the effect of vaccination with a class I peptide from HER2/*neu*, E75, on T_{Reg} population and functionality [35]. Though E75 elicited $CD4^+$ recruitment, it was able to decrease circulating T_{Regs} and TGF-β levels in the majority of patients. To date, it is unclear what factors in the vaccines, individuals, or their pathology create the observed differences in T_{Reg} response.

Given the recognized immunosuppressive effects of T_{Reg} cells, efforts to modulate them in immunotherapy for cancer are ongoing. Researchers have demonstrated enhanced cancer vaccine effectiveness against neuroblastoma and adenocarcinoma after depletion of T_{Reg} cells with an anti-CD25 mAb in murine models [54,55]. Additionally, Nair et al. combined a melanoma antigen vaccine (TRP-2) with a truncated FOXP3 vaccine (directed at T_{Reg} cells) in mice and showed increased immunity and anti-cancer activity than either vaccine given individually [56]. Denileukin diftitox (Ontak), a CD25-targeted immunotoxin, is being utilized to deplete T_{Reg} cells and enhance cancer vaccine effectiveness [57,58]. Similarly, initial suc-

cess has been seen with efforts to combine cancer vaccines with inhibition of COX-2, which has been implicated in recruitment of T_{Reg} cells [59].

5. Conclusion

Based upon the results of our phase Ib trial using AE37 and the findings conveyed above, this CD4-eliciting, class II peptide vaccine is safe, immunogenic, and does not stimulate T_{Reg} cells. Furthermore, the reduced levels of T_{Reg} cells in vaccinated patients appear to be associated with robust *in vivo* DTH reactions suggesting that the AE37 vaccine may be clinically useful. Advancing our search for an effective adjuvant breast cancer vaccine, we have initiated a phase II trial assessing the impact of AE37 on clinical recurrence in disease-free breast cancer patients at high risk for recurrence. Additionally, we have initiated a phase I trial combining class I and II HER2/*neu* peptide vaccines (GP2 + AE37) in hopes that a multi-epitope vaccine will be even more effective in engendering robust and long-lived immune responses that can prevent recurrence of disease.

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