

Tumor Antigen – Specific T-Cell Expansion Is Greatly Facilitated by *In vivo* Priming

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Abstract Purpose: Adoptive T-cell therapy is a promising strategy for the treatment of patients with established tumors but is often limited to specific cancers where tumor-infiltrating lymphocytes, the source of T cells for *ex vivo* culture, can be obtained. In this study, we evaluated the feasibility of expanding HER-2/*neu* – specific T cells derived from peripheral blood *ex vivo* following *in vivo* priming with a HER-2/*neu* peptide vaccine.

Experimental Design: Peripheral blood mononuclear cells from cytomegalovirus (CMV) – seronegative and CMV-seropositive donors as well as HER-2/*neu* – positive cancer patients who had or had not been vaccinated with a HER-2/*neu* peptide – based vaccine was used as a source of T lymphocytes. Antigen-specific T-cell lines were generated by *in vitro* stimulation with antigen followed by nonspecific expansion on CD3/CD28 beads. The ability to expand antigen-specific T cells was assessed using IFN- γ and granzyme B enzyme-linked immunosorbent spot. The phenotype of the resultant T-cell lines was evaluated by flow cytometry, including the presence of FOXP3-expressing CD4⁺ T cells.

Results: The frequencies of CMV-specific T cells generated from CMV⁺ donors were >11-fold higher than the frequencies from CMV⁻ donors ($P = 0.001$), with 22-fold increase of total number of CD3⁺ T cells. The frequencies of HER-2/*neu* – specific T cells generated from the primed patients were >25-fold higher than the frequencies from unvaccinated patients ($P = 0.006$), with an average of a 19-fold increase of total number of CD3⁺ T cells. Using peripheral blood as the source of T cells did not result in concurrent expansion of FOXP3⁺CD4⁺ regulatory T cells despite the use of interleukin-2 in *in vitro* culture. Both CD4⁺ and CD8⁺ HER-2/*neu* – specific T cells could be expanded. The extent of *ex vivo* expansion correlated with the magnitude of immunity achieved during immunization ($P = 0.008$).

Conclusion: Tumor-specific T cells can be efficiently expanded from the peripheral blood *ex vivo* following *in vivo* priming with a vaccine. This approach provides an effective method to generate tumor-specific polyclonal T cells for therapeutic use that could be applied to cancer patients with any tumor type.

The adoptive transfer of tumor-specific T cells into animals with established cancers has effectively resulted in tumor eradication in a variety of malignancies (1–5). In human clinical trials, infusion of tumor-specific T cells derived from

tumor-infiltrating lymphocytes or draining lymph nodes has shown limited but encouraging clinical responses in specific settings (6–10). Unfortunately, the ability to expand tumor antigen-specific T cells *ex vivo* from cancer patients is technically difficult due to numerous obstacles, including initiating cultures with low numbers of tumor-specific T cells and the physical inability to obtain tumor-infiltrating lymphocytes from patients with the most common malignancies (11, 12).

Studies by a number of investigators have shown that significant T-cell immunity can be generated via active immunization (13–16). Our group has previously vaccinated patients with HER-2/*neu* – overexpressing tumors with HER-2/*neu* T helper peptide vaccines (17, 18). HER-2/*neu* is a well-defined tumor antigen expressed in several solid tumors such as breast, ovarian, and non-small cell lung cancer. We have shown that significant CD4⁺ and CD8⁺ T-cell immunity can be elicited against HER-2/*neu* peptides as well as HER-2/*neu* protein with active immunization (17, 18). Early clinical studies have indicated that HER-2/*neu* vaccines may have benefit in preventing disease relapse (19). However, based on vaccine trials in other solid tumors, there is a limited

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probability that active immunization will affect established and progressive cancers, inducing a clinical response (20).

In this study, we evaluate the feasibility of expanding tumor-specific T cells *ex vivo* following *in vivo* priming with a HER-2/*neu* vaccine as the prelude to developing a combination immunotherapy approach for the treatment of advanced stage refractory HER-2/*neu*-expressing tumors. Data presented here show that *in vivo* priming greatly facilitates tumor antigen-specific T-cell expansion, that the extent of immunity elicited with active immunization predicts the magnitude of T-cell growth *ex vivo*, and that generating tumor-specific T cells from the peripheral blood may limit the proliferation of T regulatory cells despite the use of IL-2 to expand T-cell cultures. Rapid *ex vivo* T-cell expansion after immunization, resulting in a large number of tumor-specific T cells to be derived from the peripheral blood, will allow the clinical application of adoptive T-cell therapy to the most common human tumors.

Materials and Methods

Peripheral blood mononuclear cells derived from volunteer donors and cancer patients. Volunteer donors were leukapheresed after informed consent ($n = 15$). The purification and cryopreservation of peripheral blood mononuclear cells (PBMC) were done as previously described (21). Briefly, PBMC were purified by density gradient centrifugation and cryopreserved in liquid nitrogen in freezing media.

PBMC samples from HER-2/*neu*-positive cancer patients, stage III or IV breast or ovarian cancer, were taken after informed consent ($n = 18$). Among these patients, 11 completed a vaccine regimen with HER-2/*neu* peptides, and 7 had not been vaccinated. HLA-A2 typing was done as part of the original study, and 3 of the 11 subjects were HLA-A2⁺. The vaccinated patients were immunized monthly with three 14-18 amino acid HER-2/*neu*-derived T helper peptides for 6 months (17, 18). Three vaccine formulations were given: one vaccine was derived from the extracellular domain of HER-2/*neu* protein (p42-56, p98-114, and p328-345), and another was derived from the intracellular domain (p776-790, p927-941, and p1166-1180; ref. 17). The final vaccine consisted of helper epitopes that encompassed HLA-A2 binding peptides within the 14-18 amino acid sequence (p369-384, p688-703, and p971-984; ref. 18). HER-2/*neu*-specific T-cell immunity was evaluated by antigen-specific proliferation via tritiated thymidine incorporation, peptide-specific delayed-type hypersensitivity response, and IFN γ enzyme-linked immunosorbent spot (ELISPOT) in some patients as previously reported (17, 18, 22). The majority of patients developed HER-2/*neu*-specific T-cell immunity after the vaccination. The median of stimulation indices specific for HER-2/*neu* peptides from the 11 peptide-vaccinated patients was 6.5 (range, 2-25). The median of stimulation indices specific for HER-2/*neu* peptides from the seven unvaccinated patients was 0.9 (range, 0.8-1.2).

ELISA for the detection of cytomegalovirus-specific IgG antibodies. Ninety-six-well microtiter plates (Dynex Technologies, Inc., Chantilly, VA) were coated with 2.5 $\mu\text{g}/\text{mL}$ cytomegalovirus (CMV) lysate (East Coast Biologics, Inc., North Berwick, ME) diluted with carbonate buffer and added at 50 μL per well. Control wells were coated with carbonate buffer alone. A standard curve was generated by evaluating serial dilutions of purified human IgG (Sigma, St. Louis, MO) ranging from 2.5 to 0.0097 $\mu\text{g}/\text{mL}$. After an overnight incubation at 4°C, all wells were blocked with 200 μL of 1% bovine serum albumin/PBS at room temperature on a rocker for 2 h. Plates were washed four times with 1 \times PBS/0.5% Tween 20, and serum was diluted with 1% bovine serum albumin/PBS to 1:400, 1:800, 1:1,600, and 1:3,200 dilutions, and 50 μL per well was added to the plates. After 2 h of incubation at room temperature, plates were washed four times with PBS/0.5% Tween 20 and then incubated with 50 μL per well IgG-horseradish peroxidase conjugate (Zymed, San Francisco, CA) diluted 1:5,000 in 1%

bovine serum albumin/PBS for 45 min at room temperature. After a final four washes with PBS/0.5% Tween 20, TMB reagent at 75 μL per well (KMI Diagnostics, Inc., Minneapolis, MN) was added, and color reaction was monitored at 640 nm until the well containing 0.3125 $\mu\text{g}/\text{mL}$ of IgG standard reached an absorbance of 0.3. Reaction was stopped with 75 μL per well of 1 N HCl and analyzed at 450 nm. The absorbance of each serum dilution was calculated from a four-variable equation of the line for the standard curve on each plate.

Reagents. Media used for T-cell expansions (T-cell media) consisted of X-Vivo-15 (Bio Whittaker, Walkersville, MD) supplemented with 10% human AB serum (Valley Biomedical, Winchester, VA), 10 mmol/L acetylcysteine (Faulding, Paramus, NJ), 20 mmol/L HEPES, 2 mmol/L L-glutamine, 100 $\mu\text{g}/\text{mL}$ penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen, Grand Island, NY). HER-2/*neu* peptides were synthesized by Genemed Synthesis, Inc. (South San Francisco, CA), purified by high-performance liquid chromatography, and characterized by mass spectrometry. Recombinant HER-2/*neu* extracellular domain protein was provided by Corixa Corp. (Seattle, WA). Human lyophilized recombinant interleukin-2 (IL-2) was manufactured by Hoffmann-La Roche (Nutley, NJ), reconstituted with sterile water, and stored at 4°C. A portion of the stock IL-2 was diluted with T-cell media before use. Human lyophilized recombinant IL-12, purchased from R&D Systems (Minneapolis, MN), was reconstituted and aliquoted into PBS/1% human serum albumin (Bayer, ZLB Bioplasma AG, Berne, Switzerland), and stored at -20°C before use. Anti-CD3/CD28 beads were kindly provided by Xcyte Therapies Corp. (Seattle, WA).

Ex vivo expansion of CMV-specific and HER-2/*neu*-specific T cells. The *ex vivo* expansion consisted of two phases: antigen-specific enrichment (days 0-12) and nonspecific expansion (days 12-24). On day 0, cryopreserved PBMC were thawed, washed, and resuspended at a concentration of 3×10^6 cells/mL in T-cell media. The cells from volunteer donors were stimulated with 2.5 $\mu\text{g}/\text{mL}$ of CMV lysate (EastCocast Bio, North Berwick, ME). The cells from cancer patients were stimulated with a pool of three T helper peptides, each at a concentration of 10 $\mu\text{g}/\text{mL}$. For the vaccine primed patients, the peptides used were those that the patients had developed immune responses to after the course of vaccination. For the unvaccinated patients, p98-114, p369-384, and p776-790 were used, as these peptides were associated with the greatest number of responses in vaccinated patients (23). The cells, either stimulated with CMV lysate or HER-2/*neu* peptides, were incubated at 37°C in 5% CO₂ at the initiation of the culture. On days 4 and 8, 10 units/mL of recombinant human IL-2 and 10 ng/mL of recombinant human IL-12 were added to the stimulated cells. On day 12, the stimulated cells were harvested from the culture flasks and resuspended at a concentration of 1×10^6 /mL in fresh media containing 1×10^6 /mL of Xcyte CD3/CD28 beads. The cells and the beads were mixed gently and cocultured at 37°C in 5% CO₂. From days 14 to 23, the number of the cells was evaluated every 2 to 3 days, and the cells were diluted to a concentration of 0.5 to 1×10^6 /mL with fresh media. IL-2 was added at a final concentration of 30 units/mL into the cell culture.

Phenotypic analysis of T-cell lines. PBMC or the expanded T cells were collected, washed, and incubated with 10% of normal mouse serum in PBS for 15 min at 4°C to block nonspecific binding. The cells were aliquoted and stained with appropriate FITC-, PE-, PE-Cy5-, or PE-Cy7-conjugated monoclonal antibodies at 4°C in the dark for 25 to 30 min. These antibodies included human CD3, CD4, CD8, CD14, CD19, CD25, CD28, CD56, CD45RA, CD45RO, and isotype controls (all antibodies were purchased from BD PharMingen, San Diego, CA). After washing twice with PBS, the cells were resuspended in 400 μL of PBS containing 1% paraformaldehyde. Human FOXP3 PE antibody and FOXP3 Fix/Perm buffer set (obtained from Biolegend, San Diego, CA) were also used to stain T regulatory cells according to the manufacturer's instructions. Stained cells were analyzed with FC500 flow cytometer using CXP cell quest software (Beckman Coulter, Miami Lakes, FL).

IFN- γ ELISPOT. A 3-day IFN- γ ELISPOT assay was used to determine the frequencies of CMV or HER-2/*neu*-specific T cells

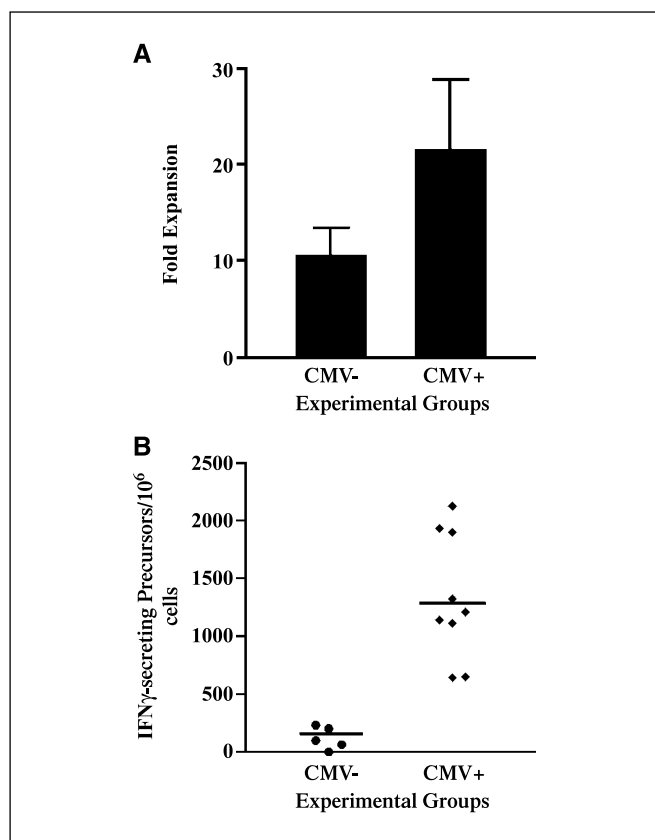


Fig. 1. CMV-specific T cells are readily expanded from CMV-seropositive donors. *A*, T-cell growth (fold expansion) from the initiation of the culture to the end of the culture period (day 24). Columns, mean of 5 CMV⁻ and 10 CMV⁺ donors; bars, SE. *B*, precursor frequencies of CMV-specific T cells in expanded cells from CMV⁻ (●) and 10 CMV⁺ (◆) donors. Points, mean measurement of IFN- γ ELISPOT from a single donor calculated from six replicates. The solid lines indicate the mean frequencies for the groups.

(24, 25). Some ELISPOT assays were done using HER-2/*neu* class I epitopes (described below) as stimulating antigen (Fig. 4). On day 1, the expanded T cells were resuspended at a concentration of 1×10^6 /mL in fresh media, and 100 μ L was placed in 96-well tissue culture plate. The cells were mixed with 100 μ L of 1×10^6 /mL of irradiated (3,000 rad) autologous PBMC with or without varying concentrations of antigen (six replicates per condition). The plates were cultured at 37°C in 5% CO₂ overnight. On day 2, the stimulated cells were transferred into anti-human IFN- γ monoclonal antibody 1-D1K (Mabtech, Nacka, Sweden)-coated 96-well nitrocellulose plate (Millipore Corp, Bedford, MA) and further incubated for 20 h. On day 3, the cells were washed with 0.05% Tween 20 in PBS. A solution of 50 μ L of anti-human IFN γ biotinylated monoclonal antibody 7-B6-1 (Mabtech) at 1 μ g/mL in PBS was added into each well. After incubation for 2 h at 37°C, plates were washed and developed for 1 h at room temperature with 50 μ L of streptavidin-alkaline phosphatase (diluted 1:1,000 in PBS; Bio-Rad Lab, Hercules, CA). After washing, substrate (Bio-Rad Lab) was added and incubated for 10 to 20 min. The resultant spots were then counted using an ELISPOT reader (Cell Technology, Columbia, MD). Data are presented as the mean frequency/million cells calculated from six-well replicates subtracted from no-antigen or irrelevant peptide wells.

Granzyme B ELISPOT. The granzyme B ELISPOT assay was carried out as a measure of HER-2/*neu*-specific CTL cytotoxicity as previously described with modification (26, 27). Briefly, 96 polyvinylidene difluoride-bottomed well plates (Millipore) were coated with granzyme B capture antibody (Cell Sciences, Canton, MA) overnight at 4°C.

The wells were washed and blocked with 2% dry skimmed milk in PBS for 2 h. *Ex vivo* expanded HER-2/*neu*-specific T cells derived from patients with an HLA-A2 phenotype were added to the wells at a concentration of 1×10^5 per well. HLA-A2⁺ T2 cells (American Type Culture Collection, Manassas, VA) were incubated with 9-amino-acid HER-2 class I peptides (p369-377, p689-p697, and p971-979) or irrelevant peptide as control. The peptide-loaded T2 cells were irradiated (3,500 rad) and added to each well, respectively, with an effector/target ratio of 2:1. The cells were incubated at 37°C, 5% CO₂ for 4 h in a total volume of 200 μ L per well. After washing, the plates were incubated with biotinylated detection antibody (Cell Sciences) at room temperature for 90 min. The resulted granzyme B spots were detected with streptavidin to alkaline phosphatases and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Cell Sciences) according to the manufacturer's protocol. The spots were counted, and the HER-2/*neu* peptide-specific CTL frequency was calculated as it was for IFN- γ ELISPOT.

Statistical analysis. Differences in CMV-specific T-cell expansion between CMV⁻ and CMV⁺ donors and differences in HER-2/*neu*-specific T-cell expansion between HER-2/*neu*-vaccinated and unvaccinated patients were analyzed by the Mann-Whitney *U* test. Differences

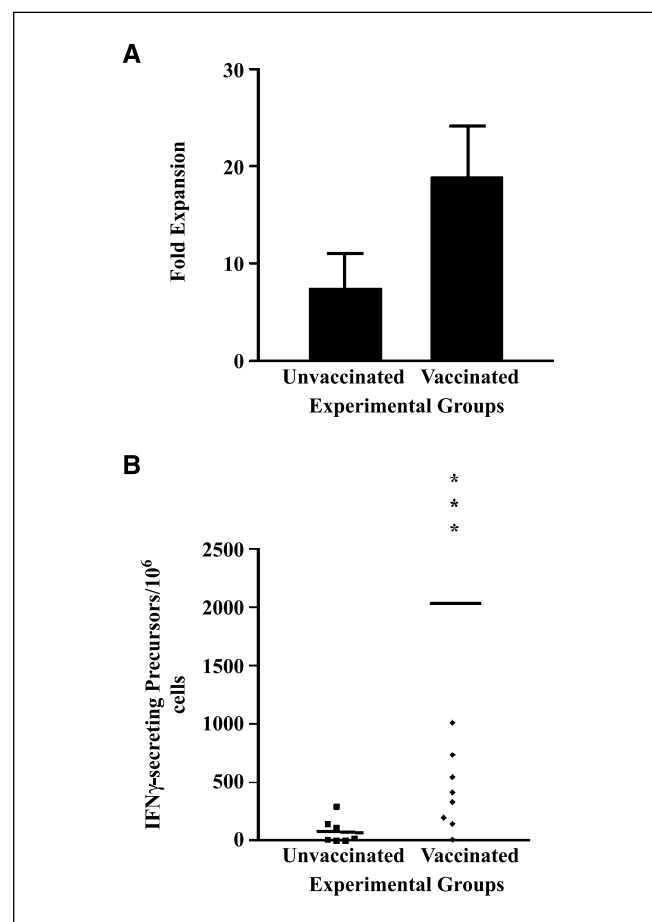


Fig. 2. Tumor antigen-specific T cells can be readily expanded from HER-2/*neu* vaccine-primed patients. *A*, T-cell growth (fold expansion) from the initiation of the culture to the end of the culture period (day 24). Columns, mean of 7 naive HER-2/*neu*-positive breast cancer patients and 11 HER-2/*neu*-positive vaccinated patients; bars, SE. *B*, precursor frequencies of HER-2/*neu*-specific T cells in expanded cells from vaccine-primed patients (◆) compared with nonvaccinated patients (■). Points, combined measurement of three individual HER-2/*neu* peptide-induced IFN- γ ELISPOT from each patient. ***, three values off the graph (8,036, 6,520, and 5,190 precursors per 10^6 cells). The solid lines indicate the mean precursor frequencies for the groups.

in the magnitude of CMV-specific precursor frequency between CMV⁻ and CMV⁺ donors and differences in magnitude of HER-2/*neu*-specific precursor frequency between HER-2/*neu*-vaccinated and unvaccinated patients were also analyzed by the Mann-Whitney *U* test. The strength of the relationship between precursor frequencies of HER-2/*neu*-specific T cells post-expansion and HER-2/*neu*-specific response (stimulation index) post-vaccination was analyzed by Pearson's product moment correlation.

Results

CMV-specific T cells are readily expanded from CMV-seropositive donors. The CMV serostatus of the donors was determined using a CMV IgG ELISA described above. Among the 15 donors, 5 were CMV seronegative (CMV⁻), and 10 were CMV seropositive (CMV⁺). The CMV⁻ donors did not show a T-cell response to CMV lysate as evaluated by antigen-specific proliferation via tritiated thymidine incorporation with a median stimulation index of 0.9 (range, 0.6-1.0). The CMV⁺ donors did show a T-cell response to CMV lysate with a median stimulation index of 23 (range, 8.7-128.2). In addition, ELISPOT and cytokine flow cytometric assays for IFN- γ secretion were strongly positive (28).

To evaluate the feasibility of *ex vivo* expansion of tumor-specific T cells from vaccine primed patients, we first used the well-characterized CMV model as an example of an endogenously primed antigen. PBMC from CMV⁻ and CMV⁺ donors were stimulated with CMV lysate to enrich CMV-specific T cells at the initiation of the culture. At the end of the enrichment phase (day 12), the total number of the cultured cells was largely decreased in both CMV⁻ and CMV⁺ donors. However, the number of cells from CMV⁺ donors was 2-fold higher than that from CMV⁻ donors (mean \pm SE of fold expansion: CMV⁻, 0.13 \pm 0.02, *n* = 5; CMV⁺, 0.26 \pm 0.06, *n* = 10). At the end of the second-phase, nonspecific expansion (day 24), the growth of total CD3⁺ T cells from CMV⁺ donors was double of that

from CMV⁻ donors, although the difference was not significant (mean \pm SE: CMV⁻, 10.5 \pm 3.5; CMV⁺, 21.5 \pm 8.0; *P* > 0.05), as illustrated in Fig. 1A.

We next examined the cell phenotype pre- and post-expansion. Pre-expansion, the percentage of CD3⁺ T cells was 65 \pm 3% (*n* = 10), with 38 \pm 3% of CD4⁺ cells and 15 \pm 1% of CD8⁺ cells in CMV⁺ donors. B lymphocytes (CD19), monocytes/macrophages (CD14), and natural killer cells (CD56) accounted for 5 \pm 1%, 15 \pm 2%, and 10 \pm 2% of total cells, respectively. Post-expansion, the average of CD3⁺ T cells reached to 95 \pm 1%, with 3 \pm 1% CD56⁺ natural killer cells. Among the CD3⁺ cells, 67 \pm 5% were CD4⁺ cells, and 26 \pm 5% were CD8⁺ cells. The cell composition pre- and post-expansion from CMV⁻ donors was not significantly different with that from CMV⁺ donors for any variable measured (*P* > 0.05).

However, the precursor frequencies of CMV-specific IFN- γ secreting cells in 10⁶ cells from CMV⁺ donors (1,336 \pm 180) were significantly higher than that from CMV⁻ donors (118 \pm 43; *P* = 0.001) after *in vitro* expansion (Fig. 1B).

Tumor antigen-specific T cells are readily expanded from HER-2/*neu* vaccine-primed patients. Based on the data generated with CMV, we evaluated the ability to expand T cells from patients who had been previously vaccinated with a HER-2/*neu* peptide-based vaccine. Similar to the cells stimulated with CMV lysate, the total number of the cultured cells from unvaccinated and vaccinated patients was largely decreased at the end of the antigen enrichment phase. However, the fold expansion of vaccine-primed cells was 3-fold higher than that of unvaccinated cells (unvaccinated, 0.14 \pm 0.05, *n* = 7; vaccinated, 0.42 \pm 0.06, *n* = 11; *P* = 0.003). As shown in Fig. 2A, at the end of expansion, the total number of CD3⁺ cells was 2.6 times higher in vaccinated than unvaccinated cells (unvaccinated, 7.4 \pm 3.8; vaccinated, 18.9 \pm 5.1; *P* = 0.006).

We quantitated antigen-specific precursor frequency in the expanded cultures by ELISPOT. Each of the three HER-2/*neu*

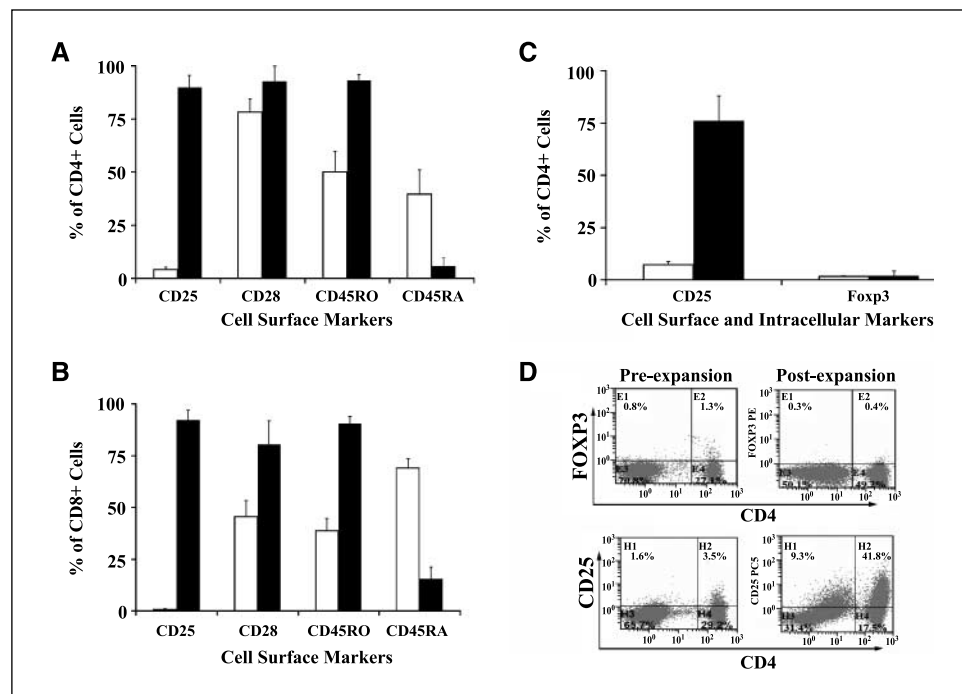
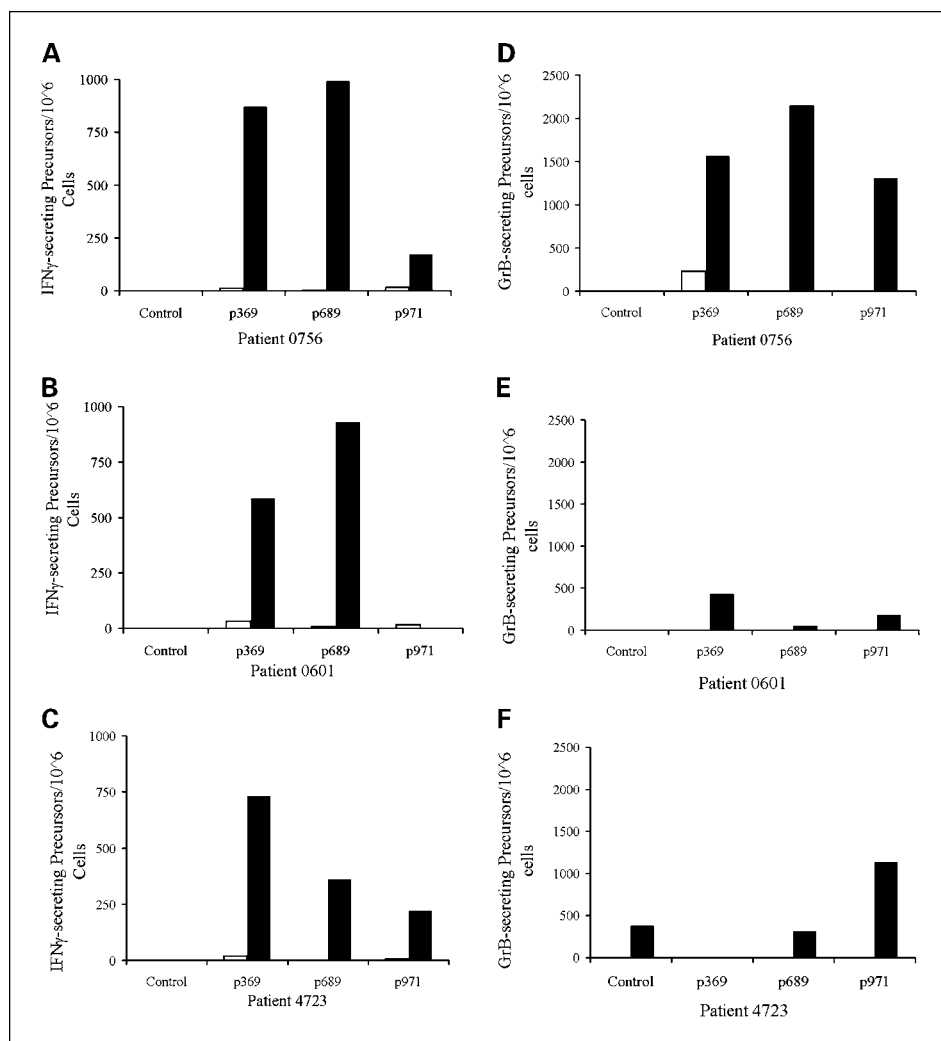


Fig. 3. *Ex vivo* expanded HER-2/*neu*-specific T-cell lines exhibit activated and memory phenotype. **A**, expression of CD25 and CD28 (*n* = 11) and CD45RO and CD45RA (*n* = 4) on pre-expanded (□) and post-expanded (■) CD4⁺ T cells analyzed by flow cytometry. Columns, mean; bars, SE. **B**, expression of CD25 and CD28 (*n* = 11) and CD45RO and CD45RA (*n* = 4) on pre-expanded (□) and post-expanded (■) CD8⁺ T cells analyzed by flow cytometry. Columns, mean; bars, SE. **C**, expression of CD25 and FOXP3 (*n* = 4) on pre-expanded (□) and post-expanded (■) CD4⁺ T cells analyzed by flow cytometry. Columns, mean; bars, SE. **D**, representative dot plots. Left, pre-expanded cells; right, post-expanded cells.

Fig. 4. Functional HER-2/*neu* – specific CD8⁺ T cells are also expanded after *in vitro* culture with T helper peptides. The PBMC from three vaccinated HLA-A2 patients were expanded with three T helper peptides. The expanded T cell lines were tested against class I HER-2/*neu* peptide – loaded autologous PBMC in an IFN- γ ELISPOT assay (A-C), or class I peptide – loaded T2 cells in granzyme B (*GrB*) ELISPOT assay (D-F). Columns, mean (six replicates) of pre-expanded (□) and post-expanded (■) cells.



T helper peptides used to expand the cells was used to stimulate the expanded T-cell lines. The precursor frequencies in 10^6 expanded cells from vaccinated patients ($2,102 \pm 892$) were significantly higher than that from unvaccinated patients (82 ± 41 ; $P = 0.006$), as shown in Fig. 2B.

Ex vivo expanded HER-2/*neu* – specific T-cell lines exhibit both an activated and memory phenotype. We analyzed the cell phenotype pre- and post-expansion in the vaccinated patients. Pre-expansion, the average percentage of CD3⁺ T cells was $49 \pm 5\%$, with $30 \pm 5\%$ CD4⁺ cells and $19 \pm 3\%$ CD8⁺ cells. B lymphocytes (CD19), monocytes/macrophages (CD14), and natural killer cells (CD56) accounted for $12 \pm 2\%$, $21 \pm 5\%$, and $13 \pm 2\%$ of total cells, respectively. Post-expansion, the average CD3⁺ T cells reached to $93 \pm 4\%$, with $5 \pm 4\%$ CD56⁺ cells. Among the CD3⁺ cells, $45 \pm 6\%$ were CD4⁺ cells, and $48 \pm 4\%$ were CD8⁺ cells. The cell composition pre- and post-expansion from unvaccinated patients was similar to that from vaccinated patients ($P > 0.05$).

We further characterized the expanded T cells derived from vaccinated patients for changes in activation markers and memory phenotype. We found that the expression of IL-2 receptor α chain (CD25) was up-regulated from $4 \pm 1\%$ in pre-expansion CD4⁺ cells to $90 \pm 6\%$ post-expansion (Fig. 3A) and

$1 \pm 1\%$ in pre-expansion CD8⁺ cells to $92 \pm 5\%$ post-expansion (Fig. 3B). The T-cell activation marker CD28 was also up-regulated from $78 \pm 6\%$ to $93 \pm 7\%$ in CD4⁺ T cells (Fig. 3A) and from $45 \pm 8\%$ to $80 \pm 12\%$ in CD8⁺ T cells (Fig. 3B). We also evaluated naive and memory (CD45RA⁺/CD45RO⁺) subsets in expanded T cells. CD45RA⁺ naive T cells were remarkably decreased from $40 \pm 11\%$ in pre-expansion CD4⁺ cells to $6 \pm 4\%$ of post-expansion CD4⁺ T cells (Fig. 3A) and $69 \pm 5\%$ in pre-expansion CD8⁺ T cells to $15 \pm 6\%$ post-expansion (Fig. 3B). In contrast, CD45RO⁺ memory T cells were largely increased from $50 \pm 10\%$ to $93 \pm 3\%$ in CD4⁺ cells (Fig. 3A) and from $39 \pm 6\%$ to $91 \pm 4\%$ in CD8⁺ T cells (Fig. 3B). Thus, the major population of HER-2/*neu* – specific T cells post-expansion is predominantly composed of CD25⁺CD28⁺CD45RA⁻CD45RO⁺ T lymphocytes.

Ex vivo expansion does not increase T regulatory cells in tumor antigen – specific T-cell lines. To determine whether the IL-2 used in T-cell expansion induced the growth of T regulatory cells, we evaluated the expression of CD25 and FOXP3 on the CD4⁺ cells in pre-expanded PBMC, and the cells were stimulated with HER-2/*neu* peptides and cultured with IL-2 and IL-12 (day 12) in two experiments. The CD25⁺ cells (% CD4⁺) were greatly increased after the culture period

(range: before culture, 1.4-4.6%; after culture, 16.5-20.8%; $P = 0.022$). However, the FOXP3⁺ cells (% CD4⁺) did not increase (range: before culture, 0.18-1.42%; after culture, 0.24-1.85%; $P = 0.18$). We further evaluated the T regulatory cells in expanded T cells (day 24; Fig. 3C). The CD25⁺ cells (% CD4⁺) were greatly increased after the expansion period (pre-expansion, 7.1 ± 1.6 ; after expansion, 76 ± 12 ; $P = 0.004$). Again, the FOXP3⁺ cells (% CD4⁺) did not increase (pre-expansion, 1.5 ± 0.5 ; after expansion, 1.8 ± 2.5 ; $P = 0.79$). Representative density plots are shown in Fig. 3D.

Functional HER-2/neu-specific CD8⁺ T cells are also expanded after in vitro culture with T helper peptides. Our previous work showed that HER-2/neu-specific CD8⁺ T cells could be elicited using HER-2/neu T helper peptides, which contain encompassed HLA-A2-binding motifs (18). Phenotypic analysis (above) showed an expansion of CD8⁺ T cells; thus, we questioned whether those T cells could represent a population responding to HER-2/neu class I epitopes. Figure 4 shows both IFN- γ production and granzyme B secretion in response to stimulation with three HER-2/neu HLA-A2-restricted epitopes from HER-2/neu T helper peptide stimulated T-cell cultures derived from the three patients who were HLA-A2*. Although T-cell lines were generated using the T helper epitopes as a pool, individual responses to the class I peptides could be detected. HER-2/neu class I peptides elicited both IFN- γ and granzyme B secretion in post-expansion cultures derived from patient 0756 (Fig. 4A and D), patient 0601 (Fig. 4B and E), and patient 4723 (Fig. 4C and F). No significant responses were found in pre-expansion cells.

Antigen-specific enrichment is required for expanding tumor antigen-specific T cells from previously vaccinated patients. To examine whether antigen enrichment through *in vitro* stimulation was necessary for expanding antigen-specific T cells from vaccinated patients, we compared the cell growth, phenotype, and specificity between antigen-enriched cells and cells that were simply directly expanded with anti-CD3/28 beads. PBMC from five vaccinated patients were either stimulated with two or three HER-2/neu Th peptides or left unstimulated before nonspecific expansion. At the end of culture, the response to single HER-2 peptides was measured using IFN γ ELISPOT.

As expected, the total expansion of T cells without antigen enrichment (59.3 ± 26.4) was nearly thrice higher than that of antigen enriched cells (23.3 ± 10.3) due to greater numbers of cells at the start of expansion (Fig. 5A). The phenotype of expanded cells without antigen enrichment was similar to that of antigen-enriched cells (data not shown). The precursor frequencies of tumor-specific T cells in 10^6 cells from non-antigen-enriched cells (161 ± 128) were significantly lower than that from antigen-enriched cells ($1,552 \pm 432$, $n = 14$; $P = 0.003$), as shown in Fig. 5B.

The magnitude of immunity achieved after active immunization predicts the extent of ex vivo expansion. We have shown that HER-2/neu-specific T cells can be readily expanded from vaccine primed patients but not from unvaccinated patients. We next questioned whether the magnitude of immunity achieved after active immunization could predict the extent of *ex vivo* expansion. The mean of the highest post-vaccine response (stimulation index) against three peptides used to expand HER-2/neu-specific T-cell lines significantly correlated with the precursor frequencies of *ex vivo* expanded T cells ($R^2 = 0.7674$, $P = 0.0004$; Fig. 6). As expected, the patients

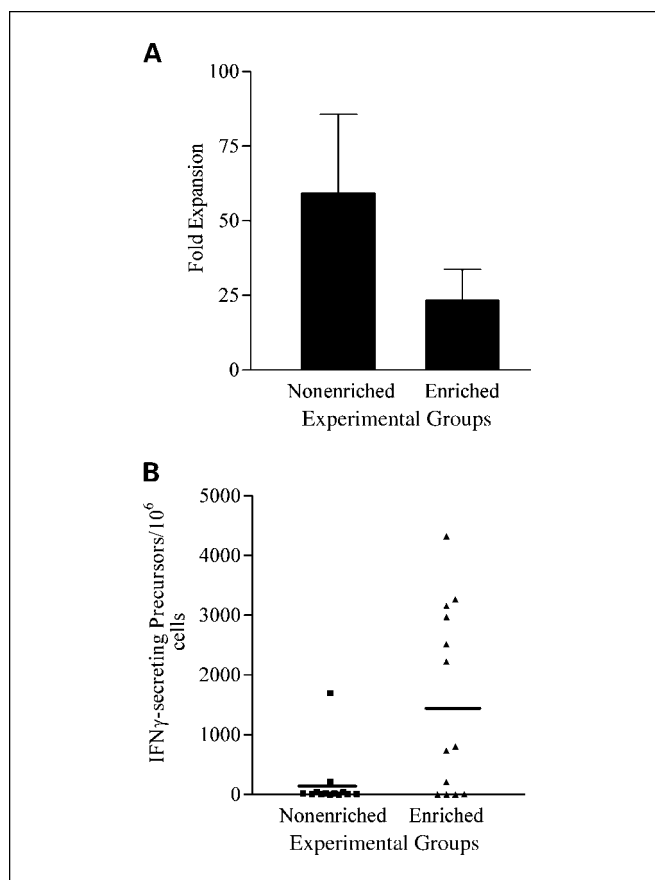


Fig. 5. Antigen enrichment is required for expanding tumor antigen-specific T cells from previously vaccinated patients. **A**, T-cell growth (fold expansion) stimulated with anti-CD3/28 beads without tumor peptide enrichment (nonenriched) and with tumor peptide enrichment (enriched). Columns, mean of five vaccinated patients; bars, SE. **B**, precursor frequencies of HER-2/neu-specific T cells in expanded cultures from tumor antigen-enriched (\blacktriangle) versus non-antigen-enriched cultures (\blacksquare). Points, individual HER-2/neu peptide-specific response. The solid lines indicate the mean precursor frequencies for the groups.

that generated the highest precursor frequencies in expanded T-cell products were the patients had highest post-vaccine response (Fig. 2B).

HER-2/neu peptide-specific T-cell lines, expanded ex vivo for therapeutic use, recognize HER-2/neu protein. Example data are shown from two of the lines specific for HER-2/neu peptides evaluated with recombinant extracellular domain protein (Fig. 7). The T-cell line derived from patient 8474 was expanded by stimulation with p42, p98, and p328. The response to the extracellular domain protein at 98 ± 5 IFN- γ secreting spots per 10^5 cells was significantly higher than the irrelevant antigen control, insulin-like growth factor binding protein peptide (14 ± 3 ; $P = 0.0001$). Another T-cell line derived from patient 0601 was expanded by stimulation with p42, p369, and p927. IFN- γ secretion specific for the extracellular domain protein (161 ± 25) was also significantly higher than that of the irrelevant antigen control, myoglobin (6 ± 1 ; $P = 0.002$).

Discussion

Adoptive T-cell therapy, or the infusion of large numbers of tumor antigen-specific T cells, for the treatment of established

tumors is a promising therapeutic strategy (29). Indeed, clinical responses can be induced by the infusion of antigen-specific T cells even in patients who have disease refractory to standard treatment. Recent results have shown that almost 50% of refractory advanced stage melanoma patients can achieve a clinical response with the infusion of polyclonal antigen-specific T-cell lines consisting of expanded tumor-infiltrating lymphocytes (9). Unfortunately, relatively, few studies have been published evaluating the clinical efficacy of adoptive T-cell therapy, largely due to the technical difficulties in obtaining the tumor antigen-specific T cells in numbers likely to impart a therapeutic effect. A few of the obstacles to the expansion of tumor antigen-specific T cells include the inability to generate T cells from the peripheral blood due to very low starting T-cell precursor frequencies, the need to derive the T cells from tumors where antigen-specific T cells are found in greater numbers but where immunosuppressive T regulatory cells also accumulate, and the concomitant expansion of T regulatory cells with IL-2 that may serve to inhibit the function of tumor antigen-specific T cells that have been expanded *ex vivo* (30, 31). In this study, we evaluated the feasibility of expanding tumor-specific T cells *ex vivo* from PBMC following *in vivo* priming with a HER-2/*neu* vaccine. Data presented here show that prior immunization greatly facilitates *ex vivo* T-cell expansion. Furthermore, continued short-term antigen-specific enrichment in culture, before nonspecific T-cell expansion, greatly increases the number of antigen-specific T cells for potential infusion. In addition, expansion of T regulatory cells was not evident using the peripheral blood as a source of antigen-specific T cells. Finally, the magnitude of immunity achieved during active immunization predicts the extent of *in vitro* expansion.

Methods developed to expand tumor-specific T cells from the peripheral blood have been problematic for several reasons. As stated, in most cancers, the endogenous tumor-specific T-cell precursor frequency is quite low (32). A minimal number of antigen-specific T cells in the initial culture requires extended culture periods with multiple *in vitro* stimulations. Such an approach has the potential to preferentially elicit effector memory T cells. Effector memory cells may persist *in vivo* only for a limited time (33). Furthermore, stimulation of low-

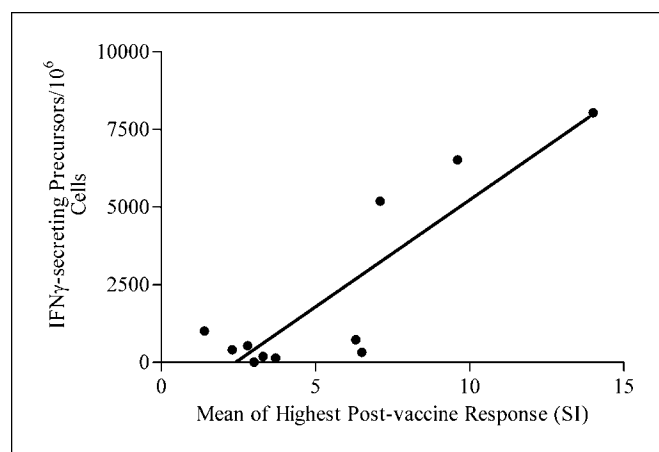


Fig. 6. The magnitude of immunity achieved after active immunization predicts the extent of *ex vivo* expansion. X-axis, post-vaccine responses [stimulation index (SI)]. Y-axis, precursor frequencies in response to peptides in expanded T-cell lines.

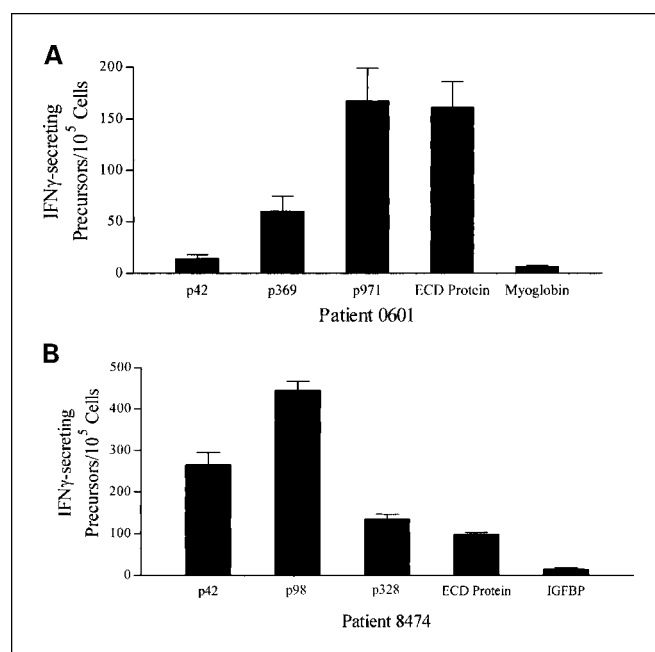


Fig. 7. HER-2/*neu* peptide-specific T-cell lines, expanded *ex vivo* for therapeutic use, recognize HER-2/*neu* protein. The expanded T-cell lines were derived from two vaccinated patients (patients 0601 and 8474). Columns, mean of spots per 10^5 cells from six replicates; bars, SE.

frequency T cells, specific for self-tumor antigens, requires the use of professional antigen-presenting cells: either dendritic cells or antigen-presenting cells engineered for efficient presentation (34). Greatly increasing T-cell precursors by active immunization allows rapid significant expansion in culture without the use of professional antigen-presenting cells. Finally, simply nonspecifically expanding T cells directly from the peripheral blood of cancer patients is not enough to generate large numbers of tumor antigen-specific T cells. Studies that have attempted nonspecific expansion without a specific enrichment step to augment the antigen-specific T-cell population have met with little clinical success (35).

Expanding T cells *in vitro* with IL-2 and IL-12 did not result in the elaboration of T regulatory cells. Recent studies have shown that T regulatory cells may preferentially accumulate at the site of the tumor rather than circulate in the peripheral blood (36–39). Indeed, studies in the *neu* transgenic mouse model of breast cancer show that the level of T regulatory cells in the peripheral blood or spleen is no different between tumor-bearing mice and mice without tumors. However, close to 20% of the CD4⁺ T cells in the tumors of *neu* transgenic mice express FOXP3, indicative of T regulatory cells (40). IL-2 is often used a growth factor for the propagation of tumor-specific T cells *ex vivo*. Recently, IL-2 has been shown to readily expand immunosuppressive T regulatory cells *in vivo* (41–43). Data presented here would suggest that despite the use of IL-2 to expand the cultures the expansion of endogenous T regulatory cells was negligible. Potentially, the use of peripheral blood as the source of T cells limits the starting number of T regulatory cells compared with tumor-infiltrating lymphocytes where T regulatory cells are more abundant. Furthermore, we and others have previously established that the addition of IL-12 to cell culture enhances both the function and number of

antigen-specific T cells (25, 44). Recently, it has been shown that IL-12 can induce CD4⁺CD25⁻ T-cell activation in the presence of T regulatory cells (45). Thus, expansion of tumor-specific T cells without concomitant expansion of T regulatory cells is possible.

The potency of the vaccine in inducing immunity *in vivo* is an important component of successful expansion. The greater the level of immunity induced by vaccination, the greater the number of antigen-specific T cells generated in culture. Vaccination, however, may achieve more than simply increasing the starting number of tumor-specific T cells. Active immunization against self-tumor antigens may also influence the character of the T cells available for expansion. Studies have shown that immunization may allow the outgrowth of high-affinity tumor-specific T cells that, in general, constitute only a small minority of the endogenous response (46).

Adoptive T-cell therapy can not be done without the *ex vivo* generation of large numbers of tumor-specific T cells (29). The difficulty in obtaining such cells has significantly limited

clinical application of this therapeutic strategy. Tumor-specific T cells can be easily expanded *ex vivo* following *in vivo* priming with a vaccine in patients with breast and ovarian cancer. This approach provides an effective method to generate a large number of polyclonal tumor antigen-specific cells for therapeutic use and will allow the generation of therapeutic T cells from the blood rather than tumor or lymph nodes. This is especially important for treating diseases where the ability to harvest metastatic disease may be difficult, such as in breast cancer. The use of cancer vaccines as the initial step to prime tumor antigen-specific T cells for clinical infusion represents a combination immunotherapeutic approach that may be more clinically effective in patients with advanced stage established cancers than the use of vaccination alone.

Acknowledgments

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