Published in final edited form as:

Cancer Immunol Immunother. 2014 February ; 63(2): 101–109. doi:10.1007/s00262-013-1489-4.

HER-2/neu vaccine-primed autologous T-cell infusions for the treatment of advanced stage HER-2/neu expressing cancers

Mary L. Disis^{1,4}, Yushe Dang¹, Andrew L. Coveler¹, Edmond Marzbani¹, Zhong C. Kou², Jennifer S. Childs¹, Patricia Fintak¹, Doreen M. Higgins¹, Jessica Reichow¹, James Waisman³, and Lupe G. Salazar¹

¹Tumor Vaccine Group, Center for Translational Medicine in Women's Health, University of Washington, Seattle, WA

²BioMed Immunotech, Inc., Tampa, FL

³City of Hope, Duarte, CA

Abstract

This Phase I study evaluated the feasibility of expanding HER-2/neu (HER2) vaccine-primed peripheral blood T-cells ex vivo and assessed the safety of T-cell infusions. Eight patients with HER2⁺ treatment refractory metastatic cancers were enrolled. T-cells could be expanded to predefined parameters in 7 patients (88%). Ninety-two percent of adverse events were grades 1 or 2. Three of 7 patients developed infusion related inflammatory reactions at their disease sites. HER2 specific T-cells significantly increased in vivo compared to pre-infusion levels ($p=0.010$) and persisted in 4/6 patients (66%) over 70 days after the first infusion. Partial clinical responses were observed in 43% of patients. Levels of T-regulatory cells in peripheral blood prior to infusion ($p<0.001$), the level of HER2 specific T-cells in vivo ($p=0.030$), and development of diverse clonal T-cell populations ($p<0.001$) were associated with response. The generation of HER2 vaccine primed autologous T-cells for therapeutic infusion is feasible and well tolerated. This approach provides a foundation for the application of T-cell therapy to additional solid tumor types.

Keywords

HER2; adoptive T-cell therapy; Phase I; clinical trial; vaccine

Introduction

The infusion of ex vivo expanded TIL has resulted in objective clinical responses in approximately 50% of patients with metastatic melanoma [1] which could be further increased by myeloablative preparative regimens [2]. The need for TIL as a T-cell source has limited the extension of this approach to cancers such as breast, lung, and colorectal cancer where metastatic lesions are visceral or bony and harvesting TIL would be difficult. Peripheral blood has not been commonly exploited as a source of T-cells for adoptive T-cell therapy as tumor specific T-cells are found in higher numbers in the cancer as TIL compared

⁴To whom correspondence should be addressed: Mary L. Disis, Tumor Vaccine Group, Center for Translational Medicine in Women's Health, 850 Republican Street, Box 358050, University of Washington, Seattle, WA 98195-8050. Phone (206) 616-1823, FAX: (206) 685-3128, ndisis@uw.edu.

Conflicts of Interest

All other authors do not have any conflicts to disclose.

to the peripheral blood and more readily expanded [3]. Moreover, the culture conditions needed to expand rare T-cells from the peripheral blood have not been well developed, especially for antigen specific CD4⁺ T-cells.

Studies by our group have shown that patients with metastatic breast cancer can be immunized against HER2 resulting in significant increases in HER2-specific peripheral blood T-cells as well as a broadening of the immune response to additional antigens expressed by the tumor, i.e. epitope spreading [4]. We developed T-cell culture methods that would enrich for and preserve the epitope spreading elicited with vaccination [5, 6]. Data presented here demonstrates the feasibility of expanding HER2 specific T-cells from the peripheral blood of previously vaccinated patients and evaluates the safety and immunogenicity of the approach.

Methods

Patient population

After informed consent, patients were enrolled into the IRB approved trial from 2003–2011 (NCT00228358). Major enrollment criteria were: (1) treatment refractory HER2⁺ cancer, (2) lesions (>1cm) which could be quantitatively assessed by imaging, (3) previously received HER2 vaccinations, and (4) not received standard therapies for at least 1 month prior to the first T-cell infusion.

Study design

The trial was a phase I, 2-arm study. For safety concerns, cells were administered as an inpatient dose escalation with limits of 1.0×10^9 (dose 1), 1.0×10^{10} (dose 2) and 1.0×10^{11} (dose 3) total cells given after administration of a single dose of cyclophosphamide (600mg/m²) (Patients A–D) or denileukin diftitox (ONTAK) (18 mg/kg) (Patients E–H) 24 hours prior to the first infusion. The ability to expand T-cells was defined as feasible if a minimum expansion (1-log less than the maximum dose) was achieved in the majority of expansions in 80% of subjects enrolled. A specific dose was defined as safe if 4 of 5 patients in a study arm experienced no dose-limiting toxicity (DLT). Toxicity was evaluated according to CTEP CTCAE v3.0 criteria.

Secondary objectives included the extent HER2 specific T-cell immunity could be boosted with infusion and persist and clinical response as measured by the comparison of pre- and post treatment imaging using RECIST criteria. Target lesions were defined as the largest measurable lesion prior to treatment, although measurements were made on all lesions during the course of the study. Immune parameters were assessed prior to infusion and at various time points after the active treatment period had ended. Concurrent use of bisphosphonates was allowed (Patients B,C,D,E,H).

Planned enrollment was 10 patients, 5 receiving conditioning with cyclophosphamide and 5 with ONTAK. After 3 patients were enrolled on the ONTAK arm of the study the manufacturer of the drug discontinued production and, due to the inability to complete the study as designed, the trial was closed with 8 patients enrolled.

Expansion of T-cells

PBMC were purified from leukaphereses using Ficoll-Hypaque density gradient centrifugation. After washing, PBMC were aliquoted and cryopreserved in plasmalyte-A (Baxter) with 10% DMSO (Sigma-Aldrich) and 10% human serum albumin (CSL Behring LLC) and stored in liquid nitrogen. At the time of clinical expansion, cells were cultured over a 24-day according to previously published methods [5, 6]. In brief, PBMC were

stimulated with a peptide pool of three HER2 MHC class II epitopes (Genemed Synthesis Inc) to which the patient had generated the greatest magnitude cellular immune response after the vaccination (10ug/ml each peptide) [7, 8]. IL-12 (10ng/ml) and IL-2 (10u/ml) were added to the cultures on days 4 and 8 and the cultures were subsequently expanded with CD3/CD28 beads on day 12 in IL-2 for 12 days. HER2 reactive T-cells increased approximately 10-fold during expansion [5]. Release criteria included: sterility, >70% CD3⁺ T-cells and >70% viable (Suppl. Table 1). Cell products were administered at 10± 3 day intervals.

ELISPOT

A 3-day IFN- γ ELISPOT assay was used to determine the frequencies of HER2 specific T-cells in the products using published methods [5]. Antigens evaluated included nine HER2 MHC class II epitopes (10ug/ml), HER2 intracellular domain (ICD); 1ug/ml (SignalChem) and extracellular domain (ECD); 1ug/ml (Sino Biological) proteins. These peptides and proteins were either the immunizing antigens in the patients' vaccine or antigens to which they had developed intramolecular epitope spreading [7]. Three HER2 specific MHC class I epitopes, p369–377, p689–p697, and p971–979, were used to interrogate T-cells from 4 HLA-A2⁺ patients. Data are presented as the mean IFN- γ spots/million T-cells calculated from six-well replicates subtracted from no-antigen wells (Corrected (c) IFN- γ spots/10⁶). The estimated total number of HER2 specific IFN- γ -secreting T-cells infused in each patient was calculated by: total number of cells infused $\times$ cIFN- γ HER2 spots per 10⁶/10⁶. A 10-day IFN- γ ELISPOT was used assess responses in PBMC before and after T-cell infusions using previously described methods [4]. Data is expressed as corrected spots/10⁶ cells (cIFN- γ HER2 spots/10⁶).

Flow cytometry

Antibodies used included CD8 FITC, CD4 PE-Cy7, CD3 PerCP/Cy5.5, CD279 (PD-1) APC (Biolegend). The levels of T-regulatory cells (Treg) in all specimens was assessed as we have published [5]. Data are expressed as the percentage of FOXP3⁺CD4⁺ T-cells of all CD4⁺ T-cells. The frequency of myeloid derived suppressive cells (MDSC) was examined in PBMC obtained before and after infusions using HLA DR-FITC, CD33 PerCP/Cy5.5, and CD11b PE antibodies (Biolegend). Data are expressed as the percentage of HLA DR^{low}, CD33⁺, CD11b⁺ MDSC in the total PBMC population. The stained cells were acquired with FACS Canto flow cytometer (BD Bioscience) and analyzed using FlowJo software.

T-cell receptor (TCR) repertoire diversity analysis

CD4⁺ and CD8⁺ T-cell subsets from cell products and PBMC obtained pre- and post-infusion, were separated using magnetic microbeads coated with an anti-CD4 or CD8 antibody (Invitrogen). The purity of each T-cell subpopulation was >95% as determined by flow cytometry. Total RNAs were isolated from the purified CD4⁺ and CD8⁺ T-cell subsets using a total RNA isolation kit (Qiagen). TCR V β repertoire analysis was performed using SuperTCRExpress human CDR3 diversity determination kit (BioMed Immunotech) [9].

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). The differences between two groups (pre-infusion vs. post-infusion; responding patients vs. non-responding patients) were analyzed using a two-tailed Student's t-test. Strengths of the relationship between the changes of tumor size after T-cell therapy and other factors were analyzed by Pearson's product moment correlation. A p<0.05 was considered statistically significant.

Results

Clinical expansion of HER2 vaccine-primed T-cells from the peripheral blood of advanced stage cancer patients is feasible

One HER2⁺ ovarian cancer patient and 7 HER2⁺ breast cancer patients were enrolled (Table 1). T-cells were expanded from mononuclear cells obtained by leukapheresis a median of 1.8 months prior to the first infusion. Four patients received all 3 planned T-cell infusions and 4 patients received only two infusions due to the low number of cells collected at the time of initial leukapheresis. T-cell products met or exceeded minimal requirements for cell expansion in 7 of 8 patients (88%). The mean ($\pm$ SE) fold increase in the growth of cells from 2 or 3 expansions for each subject, is shown in Figure 1A. For the group as a whole, the median fold increase in growth of cells from the start to the end of the culture was 8.3, range 0.2–22.1 (Fig. 1A, Suppl. Table 1). Cell products from 1 patient could not be adequately expanded ex vivo. She received the two low yield products that were generated and returned to her primary care physician with no further scheduled imaging or laboratory evaluations. The median total number of T-cells infused for successful expansions was $10,127 \times 10^6$, range $1,692$ – $47,207 \times 10^6$ with a median (range) of CD3⁺ 99% (74–100%), CD4⁺ 38% (13–46%) and CD8⁺ T-cells 58% (49–80%) (Suppl. Table 1).

We examined the frequency of several populations of IFN- γ producing HER2 specific T-cells within the infused product using material derived from the first or second expansion for the 7 remaining patients. The mean cumulative number of IFN- γ secreting T-cells per million cells for each category of antigen analyzed, for each subject, is shown in Figure 1B. For all products, the median (range) of HER2 epitope specific T-cells, for peptides that were used to stimulate the cultures, was 206×10^6 (undetectable–1,492) T-cells. The median (range) of HER2 protein specific T-cells, for both the HER2 ECD and ICD, was 211×10^6 (31 – $1,702 \times 10^6$) T-cells. The ability of the T-cells to respond to recombinant protein processed and presented by autologous APC is an in vitro indicator of potential in vivo activity. The median (range) of HER2 epitope specific T-cells, for peptides which represented epitope spreading from the original vaccination, was 366×10^6 (85 – $2,240 \times 10^6$) T-cells. Vaccination with HER2 Class II peptides, designed to stimulate CD4⁺ T-cell responses, has been shown to induce HER2 specific CD8⁺ T-cells [8]. Four of the patients were HLA-A2⁺ which allowed assessment of the expansion of class I HER2 specific T-cells. Mean responses are shown in Figure 1C. For HLA-A2⁺ patients the median (range) of HER2 class I epitope reactive T-cells was $1,527 \times 10^6$ (80 – $7,428 \times 10^6$). Figure 1D depicts the estimated total number of HER2 specific T-cells, of any measured specificity, that were infused during the two or three infusions. For all products, a median (range) of 36×10^6 (1 – 265×10^6) HER2 specific T-cells were infused.

The majority of adverse events associated with the infusion of HER2 vaccine primed autologous T-cells are Grades 1 and 2

One hundred and forty three adverse events (AE) were collected during the trial and none were classified as DLTs (Table 2, Suppl. Table 2). Of all AE, 71% were grade 1, 21% grade 2 with 8% grades 3 or 4. The most common and significant AE were those expected from treatment with either cyclophosphamide, ONTAK, or related to disease progression (Table 2, Suppl. Table 2). Significant pulmonary toxicity has been reported with the infusion of HER2 specific chimeric antigen receptor modified T-cells [10]. Such toxicity was not observed on this study, 6 pulmonary associated AEs were reported, all grade 1.

Three patients had evidence of potential infusion induced inflammation at the site of metastases. An ovarian cancer patient developed severe abdominal pain consistent with peritonitis two weeks after her third infusion. Imaging revealed increased ascites and

edematous mesentery. The CA-125 had risen from 61 to 101U/ml. Evaluation revealed no infectious etiology and she returned to her primary physician where, in the absence of additional therapy, her symptoms resolved and the CA-125 declined to 69U/ml over 1 month. The second patient, with extensive lymph node metastases, developed increased tenderness and swelling in a right supraclavicular lymph node beginning 7 days after her last infusion. Symptoms resolved without treatment over a period of 2 weeks. A final patient with disease progression after treatment, in a solitary liver metastasis, had evidence of a 30% increase in tumor size at imaging 21 days after her last infusion. The true extent of progression was obscured by a concomitant marked increase in peritumoral edema.

T-cell expansion, persistence and diversity in vivo after infusion of HER2 vaccine primed autologous T-cells

We examined the magnitude of the IFN- γ response to the HER2 antigens used to stimulate the T-cell products in the peripheral blood of patients after infusion. There was significant increase of these T-cells in vivo, compared to levels assessed prior to expansion, in the majority of the patients; mean $\pm$ SEM: post infusion, $622 \pm 99 \times 10^6$ vs. pre-expansion, $206 \pm 95 \times 10^6$ PBMC; $p=0.010$ (individual mean values Fig. 2A). The IFN- γ T-cell responses for all HER2 antigens in each individual patient at time points ranging from 10 to 280 days after the first T-cell infusion are shown in Supplemental Figure 1 (persistent lymphopenia precluded immune assessment at day 20 in all but two patients). T-cell responses to all HER2 antigens could be maintained at elevated levels compared to baseline, in the majority of patients (4/6, 66%), over 70–280 days after the first infusion; post infusion mean $\pm$ SE: $3072 \pm 882 \times 10^6$ vs. pre-infusion $1539 \pm 565 \times 10^6$ PBMC (Suppl. Fig. 1). In three patients, HER2 specific T-cells continued to augment in vivo greater than 100 days from the first infusion (Suppl. Fig. 1).

Mean levels of FOXP3⁺ CD4⁺ are shown for each patient (Fig. 2B). As a group, there was no significant difference between pre-infusion and post-infusion Treg levels (mean $\pm$ SEM: pre-infusion, $2.5 \pm 0.4\%$ vs. post-infusion $3.5 \pm 0.8\%$), $p=0.282$ (Suppl. Fig. 2A). In general, levels of Treg in the product were not increased by the culture method (mean $\pm$ SEM: 1.6 ± 0.3) as compared to pre-infusion levels, $p=0.115$. The number of Treg in the peripheral blood after infusions had been completed were not significantly different between cyclophosphamide and ONTAK pre-conditioned groups, $p=0.110$. There was no significant difference in the level of MDSC between pre- and post- infusion PBMC in the group, $p=0.260$ (Fig. 2C, example, Suppl. Fig. 2B).

Broadening of the TCR repertoire and the development of multiple clonal species over a short period of time has been associated with epitope spreading in exacerbations of autoimmune disease [11]. As expected, the majority of the repertoire from both CD4⁺ and CD8⁺, in pre-expansion PBMC demonstrated a polyclonal distribution (Suppl. Fig. 3). However, after infusion in some patients, multiple TCR clonal populations [12] developed in specific CD4⁺ and CD8⁺ T-cell populations (Table 3, Supplemental Fig. 3). The median (range) number of clonal TCR identified in pre-infusion peripheral blood was 4 (1–14) while post infusion the median number was 10 (3–17).

Immune parameters associated with clinical response

All patients had sites of soft tissue metastases. The most common sites of metastatic lesions were liver (75%), lymph node (50%), and bone (50%). Seventy five percent of patients had more than one site of metastasis (median 2, range 1–4). All patients had measurable disease documented by standard imaging techniques a median of 5 days prior to the first T-cell infusion. Imaging performed a median of 21 days after the last infusion was used to calculate response of target lesions. The overall response rate in the 7 evaluable patients was

43% (0 CR and 3 PR (8, 4 and 3 months duration)) with reductions in target lesions ranging from 57–77% (Fig. 3A). In all 3 responders all lesions responded, i.e. no mixed responses. In patient C, however, a new bone lesion developed after the first infusion. The remainder of the patients demonstrated progression in target lesions, ranging 25–50%.

We performed univariate analyses evaluating quantitative immune parameters between responding and non-responding patients and assessed significant associations (Suppl. Table 3). The % of Treg in the peripheral blood of the patients prior to T-cell infusion was associated with response, with higher levels of Treg found in those patients who progressed ($p<0.001$, $R^2=-0.989$) (Fig. 3B). Treg levels in the infused product or found in the peripheral blood after infusion gave no such association (Suppl. Table 3). Two immune parameters assessed, in vivo, after T-cell infusions had been completed were also correlated with response. The greater the number of T-cells specific for the epitopes used to stimulate the T-cell cultures in the peripheral blood, the more likely a clinical response was observed ($p=0.030$, $R^2=0.642$) (Fig. 3C). The development of new TCRV β species demonstrating clonality was also associated with those patients who demonstrated tumor regression ($p<0.001$, $R^2=0.967$) (Fig. 3D).

Discussion

The infusion of tumor-competent T-cells allows T-cells to be activated outside the immune suppressive environment of an existing cancer and has the potential of increasing the number of T-cells over what can be achieved with vaccination alone. Data presented here demonstrates expansion of HER2 vaccine-primed autologous T-cells is feasible and infusions are associated with minimal toxicity, that HER2 specific T-cells can be augmented in vivo by adoptive transfer and may persist at elevated levels for months in some patients. We additionally explored immune biomarkers that may be associated with clinical response.

Vaccine-priming prior to leukapheresis to obtain antigen specific peripheral blood T-cells for ex vivo expansion has been studied for the reconstitution of immunity to infectious agents [13, 14]. Investigators evaluated the ability to reconstitute protective immunity to *S. pneumoniae* by either collecting unprimed T-cells or by vaccinating patients to obtain peripheral blood T-cells for ex vivo expansion and infusion [13]. T-cell products derived from patients who had been vaccinated prior to transplant, when administered in the first few weeks post-transplant, resulted in the generation of high-levels of antigen specific CD4⁺ T-cells which persisted for months after treatment. Moreover, antigens associated with robust precursor frequencies, such as CMV, were also observed to expand in vivo, potentially due to a bystander effect of the proliferative environment induced by lymphopenia and the infusion of large numbers of highly activated T-cells. The translation of these findings to the adoptive transfer of tumor antigen specific T-cells derived from peripheral blood has not been as successful. In a study of metastatic melanoma, in patients who had been previously immunized with a gp100 vaccine, significant levels of gp100 specific T-cells could be generated by ex vivo culture and high levels of antigen specific CD8⁺ T-cells could be detected in the peripheral blood after transfer [15]. However, there were no demonstrable clinical responses. The investigators proposed several reasons for the lack of efficacy. Vaccine induced T-cells may be highly restricted in antigen specificity thus have only a minor impact on the tumor. Moreover, studies of both the product and peripheral blood demonstrated that Treg were elevated, as many as 25% of CD4⁺ T-cells expressed FOXP3 potentially limiting T-cell efficacy [16]. In contrast to melanoma, a highly immunogenic tumor, breast cancer induces low level cellular immunity [17]. Regulatory cells may be actively attempting to limit the function of the significant numbers of tumor specific T-cells already present in the peripheral blood and tumor of melanoma patients. Similar to the studies in infectious disease, we also observed the concurrent expansion of a

number of HER2 specific T-cells directed against epitopes not used to stimulate the cell cultures. Studies by our group have demonstrated that HER2 vaccination elicits both inter- and intramolecular epitope spreading. Patients develop robust immunity to additional proteins expressed by breast cancer such as p53 and topoisomerase II alpha [4]. We did not have sufficient material to evaluate levels of immunity to antigens other than HER2, but presumably elevated levels of T-cells specific to additional antigens could be present in the T-cell products.

We did not observe the pulmonary toxicity observed with the use of HER2 specific CAR [10]. After infusion of 10^{10} HER2-CAR, a patient developed respiratory distress with pulmonary infiltrate presumably due to the secretion of inflammatory cytokines by T-cells as well as cross-reactivity to lung epithelial cells expressing lower levels of HER2 by the high avidity CAR. Vaccine primed autologous T-cells are, most certainly, of a lower avidity than CAR in interaction with tumor cells or antigen presenting cells. However, the lower avidity of response, while limiting autoimmunity, may also enhance persistence and function of the T-cells in vivo. Recent studies have demonstrated that higher avidity T-cells are those most likely to be tolerized in the tumor environment and lose function [18, 19]. We did note inflammatory reactions associated with T-cell infusion in 3 patients. Investigators have reported 20% of patients developed inflammation at sites of disease after transfer of EBV specific T-cells [20]. The reactions we observed could indicate the potential for T-cell induced toxicity, but also underscore the potential of the infused T-cells to traffic to the site of tumor and function.

In studies of adoptive T-cell therapy, efficacy of treatment has been associated with the magnitude of response achieved and the persistence of the T-cells in vivo [21, 22]. We observed in vivo augmentation of the Th1 response. In the T-cell product, approximately 1:5,000 cells (median) were T-cells specific for the stimulating HER2 antigens which significantly increased in vivo to a median of 1:1,500 PBMC. In patients with persistent immunity, the median number increased further to 1:600 PBMC specific for stimulating HER2 antigen. The development of multiple clonal populations over a short period of time, and temporally related to T-cell infusions, suggests antigen recognition. Skewing of the T-cell repertoire has been observed in active autoimmune disease such as thyroiditis [11] and has been identified in patients with cervical cancer where experiments demonstrated that the clonal V β species elicited recognized and lysed autologous tumor [23]. We do not know if the clonal populations we observed are HER2 specific or represent immunity to different antigens, but evidence suggests recognition of multiple epitopes and antigens is associated with tumor regression after adoptive transfer TIL in patients with metastatic melanoma [24]. We explored associations of response with immune biomarkers collected during the study. The number of Treg in the peripheral blood prior to T-cell infusion was a predictor of response. The negative prognostic impact of pre-infusion peripheral blood Treg cells has been documented for TIL based therapy by other investigators [25]. The magnitude of antigen specific T-cells both in the product and in vivo and the diversity of the T-cell response were also associated with clinical outcome. These immunologic correlates provide a basis for exploration of biomarkers of response in future optimized protocols.

There is substantial evidence that Type I T-cells play an important role in immune surveillance and even patients with advanced stage disease can derive clinical benefit from a robust endogenous Type I T-cell response [26]. In only a minority of patients is Type I immunity elicited by exposure to the tumor. Adoptive T-cell therapy, using vaccine-primed peripheral blood T-cells, may provide a therapeutic approach that would allow the delivery of Type I antigen specific T-cells to advanced stage patients with a variety of solid tumor types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Mr. and Mrs. Gavin Herbert, a grant from the Arnold and Mabel Beckman Foundation, Gateway, and NIHUL1TR000423. Most importantly, we acknowledge the invaluable contribution of the patients who volunteered to participate in this study.

Mary L. Disis is an inventor on patents held by the University of Washington that pertain to data presented in this manuscript. Zhong C. Kou is the founder of Biomed Immunotech, Inc. which holds proprietary technology on TCR analysis.

References

1. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol*. 2005; 23(10):2346–2357. [PubMed: 15800326]
2. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol*. 2008; 26(32):5233–5239. [PubMed: 18809613]
3. Tjin EP, Konijnenberg D, Krebbers G, Mallo H, Drijfhout JW, Franken KL, van der Horst CM, Bos JD, Nieweg OE, Kroon BB, et al. T-cell immune function in tumor, skin, and peripheral blood of advanced stage melanoma patients: implications for immunotherapy. *Clin Cancer Res*. 2011; 17(17):5736–5747. [PubMed: 21750202]
4. Disis ML, Wallace DR, Gooley TA, Dang Y, Slota M, Lu H, Covelev AL, Childs JS, Higgins DM, Fintak PA, et al. Concurrent trastuzumab and HER2/neu-specific vaccination in patients with metastatic breast cancer. *J Clin Oncol*. 2009; 27(28):4685–4692. [PubMed: 19720923]
5. Dang Y, Knutson KL, Goodell V, dela Rosa C, Salazar LG, Higgins D, Childs J, Disis ML. Tumor antigen-specific T-cell expansion is greatly facilitated by in vivo priming. *Clin Cancer Res*. 2007; 13(6):1883–1891. [PubMed: 17363545]
6. Knutson KL, Disis ML. IL-12 enhances the generation of tumour antigen-specific Th1 CD4 T cells during ex vivo expansion. *Clin Exp Immunol*. 2004; 135(2):322–329. [PubMed: 14738463]
7. Disis ML, Gooley TA, Rinn K, Davis D, Piepkorn M, Cheever MA, Knutson KL, Schiffman K. Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. *J Clin Oncol*. 2002; 20(11):2624–2632. [PubMed: 12039923]
8. Knutson KL, Schiffman K, Disis ML. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. *J Clin Invest*. 2001; 107(4):477–484. [PubMed: 11181647]
9. Kou ZC, Pühr JS, Rojas M, McCormack WT, Goodenow MM, Sleasman JW. T-Cell receptor Vbeta repertoire CDR3 length diversity differs within CD45RA and CD45RO T-cell subsets in healthy and human immunodeficiency virus-infected children. *Clin Diagn Lab Immunol*. 2000; 7(6):953–959. [PubMed: 11063505]
10. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther*. 2010; 18(4):843–851. [PubMed: 20179677]
11. Okajima M, Wada T, Nishida M, Yokoyama T, Nakayama Y, Hashida Y, Shibata F, Tone Y, Ishizaki A, Shimizu M, et al. Analysis of T cell receptor Vbeta diversity in peripheral CD4 and CD8 T lymphocytes in patients with autoimmune thyroid diseases. *Clin Exp Immunol*. 2009; 155(2):166–172. [PubMed: 19040601]
12. Langerak AW, Groenen PJ, Bruggemann M, Beldjord K, Bellan C, Bonello L, Boone E, Carter GI, Catherwood M, Davi F, et al. EuroClonality/BIOMED-2 guidelines for interpretation and

- reporting of Ig/TCR clonality testing in suspected lymphoproliferations. *Leukemia*. 2012; 26(10): 2159–2171. [PubMed: 22918122]
13. Rapoport AP, Stadtmauer EA, Aqui N, Badros A, Cotte J, Chrisley L, Veloso E, Zheng Z, Westphal S, Mair R, et al. Restoration of immunity in lymphopenic individuals with cancer by vaccination and adoptive T-cell transfer. *Nat Med*. 2005; 11(11):1230–1237. [PubMed: 16227990]
 14. Stadtmauer EA, Vogl DT, Luning Prak E, Boyer J, Aqui NA, Rapoport AP, McDonald KR, Hou X, Murphy H, Bhagat R, et al. Transfer of influenza vaccine-primed costimulated autologous T cells after stem cell transplantation for multiple myeloma leads to reconstitution of influenza immunity: results of a randomized clinical trial. *Blood*. 2011; 117(1):63–71. [PubMed: 20864577]
 15. Powell DJ Jr, Dudley ME, Hogan KA, Wunderlich JR, Rosenberg SA. Adoptive transfer of vaccine-induced peripheral blood mononuclear cells to patients with metastatic melanoma following lymphodepletion. *J Immunol*. 2006; 177(9):6527–6539. [PubMed: 17056585]
 16. Cesana GC, DeRaffele G, Cohen S, Moroziewicz D, Mitcham J, Stoutenburg J, Cheung K, Hesdorffer C, Kim-Schulze S, Kaufman HL. Characterization of CD4+CD25+ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. *J Clin Oncol*. 2006; 24(7):1169–1177. [PubMed: 16505437]
 17. Disis ML, Knutson KL, Schiffman K, Rinn K, McNeel DG. Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. *Breast Cancer Res Treat*. 2000; 62(3):245–252. [PubMed: 11072789]
 18. Zhu Z, Singh V, Watkins SK, Bronte V, Shoen JL, Feigenbaum L, Hurwitz AA. High-avidity T cells are preferentially tolerized in the tumor microenvironment. *Cancer Res*. 2013; 73(2):595–604. [PubMed: 23204239]
 19. Brentville VA, Metheringham RL, Gunn B, Durrant LG. High avidity cytotoxic T lymphocytes can be selected into the memory pool but they are exquisitely sensitive to functional impairment. *PLoS One*. 2012; 7(7):e41112. [PubMed: 22829916]
 20. Comoli P, Pedrazzoli P, Maccario R, Basso S, Carminati O, Labirio M, Schiavo R, Secondino S, Frasson C, Perotti C, et al. Cell therapy of stage IV nasopharyngeal carcinoma with autologous Epstein-Barr virus-targeted cytotoxic T lymphocytes. *J Clin Oncol*. 2005; 23(35):8942–8949. [PubMed: 16204009]
 21. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C, et al. A human memory T cell subset with stem cell-like properties. *Nat Med*. 2011; 17(10):1290–1297. [PubMed: 21926977]
 22. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res*. 2011; 17(13):4550–4557. [PubMed: 21498393]
 23. Pilch H, Hohn H, Neukirch C, Freitag K, Knapstein PG, Tanner B, Maeurer MJ. Antigen-driven T-cell selection in patients with cervical cancer as evidenced by T-cell receptor analysis and recognition of autologous tumor. *Clin Diagn Lab Immunol*. 2002; 9(2):267–278. [PubMed: 11874862]
 24. Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, Robbins PF, Sette A, Appella E, Rosenberg SA. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol*. 1995; 154(8):3961–3968. [PubMed: 7706734]
 25. Yao X, Ahmadzadeh M, Lu YC, Liewehr DJ, Dudley ME, Liu F, Schrumph DS, Steinberg SM, Rosenberg SA, Robbins PF. Levels of peripheral CD4(+)FoxP3(+) regulatory T cells are negatively associated with clinical response to adoptive immunotherapy of human cancer. *Blood*. 2012; 119(24):5688–5696. [PubMed: 22555974]
 26. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012; 12(4):298–306. [PubMed: 22419253]

Figure 1. Clinical scale expansion of HER2 vaccine-primed T-cells from the peripheral blood of advanced stage cancer patients is feasible

(A) Fold T-cell expansion (y-axis) in individual patients (x-axis). Bars represent the mean ($\pm$ SEM) of 2 or 3 expansions. Dotted line indicates the mean. (B) Mean cumulative HER2 specific IFN- γ -secreting T-cells per million cells (y-axis) for individual patients (x-axis) responding to culture stimulating peptides (white), HER2 proteins (grey) and other HER2 peptides not used to expand the cultures, i.e. epitope spreading (black). (C) Mean cumulative HER2 specific IFN- γ -secreting T-cells per million cells (y-axis) responding to three HER2 class I epitopes (from 1st or 2nd cell product) for individual patients (x-axis). (D) The estimated total HER2 specific T-cells infused (y-axis) for an individual patient (x-axis). Dotted line indicates the mean.

Figure 2. HER2 specific immunity and immune regulation after infusion of HER2 vaccine primed autologous T cells

(A) Mean HER2 specific IFN- γ responses (y-axis), to culture stimulating peptides, in PBMC of patients (x-axis) before (white) and after (black) infusion. Post infusion samples were analyzed greater than 40 days after the first infusion with the exception of Patient E taken 10 days after infusion. (B) % Foxp3⁺CD4⁺ cells of all CD4⁺ cells (y-axis) pre-infusion (white) vs. post-infusion (black) and in T-cell products (grey) for all patients (x-axis). (C) % of HLADR^{low}CD11b⁺CD33⁺ cells (y-axis) in patient PBMC (x-axis) pre-infusion (white) and post-infusion (black).

Figure 3. Immunologic parameters associated with clinical response

(A) % of change of tumor size from baseline (y-axis) after T-cell infusion in individual patients (x-axis) ordered by magnitude of response. Percent of change of tumor size from baseline (y-axis) with (B) % FOXP3⁺CD4⁺ cells of all CD4⁺ cells pre-infusion (x-axis), (C) Mean cIFN- γ response to stimulating HER2 epitopes/10⁶ PBMC post infusion (x-axis); and (D) Number of TCRV β clonal populations generated after infusion (x-axis).

Table 1

Patient Demographics

Patient Characteristics (8)	Median Range	No. of Patients	%
Disease			
Breast		7	
Ovarian		1	
Age, years			
Median	52		
Range	35 – 71		
Time from metastatic diagnosis, months			
Median	98		
Range	74 – 216		
Time from last chemotherapy, months			
Median	1.5		
Range	1 – 12		
Time from last HER2 vaccine, months			
Median	39		
Range	14–120		
HER2 status			
IHC 3+		0	0
IHC 2+ and/or FISH+		3	38
IHC 1+		0	0

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; IHC, immunohistochemistry; FISH, fluorescent in-situ hybridization

Table 2

Adverse Events

Adverse Events (n=143)				
Event	All AEs		Possibly, Probably, or Definitely Related	
	No.	% of All AEs	No.	% of All Related AEs
Most Common				
Lymphopenia	10	7%	9	9%
Anemia	9	6%	7	7%
Leukopenia	8	6%	7	7%
Nausea	7	5%	5	5%
Fatigue	7	5%	5	5%
Headache	6	4%	4	4%
Hypoalbuminemia	6	4%	4	4%
Abdomen pain	5	3%	0	0%
Hypokalemia	4	3%	3	3%
Constipation	4	3%	2	2%
AE grading				
1	101	71%	70	71%
2	30	21%	19	19%
3	7	5%	5	5%
4	5	3%	4	4%
5	0	0%	0	0%

Abbreviation: AE, adverse event.

Table 3

Changes in Clonality/Diversity of TCR V β Repertoire Following T Cell Infusion. The TCR V β repertoire clonality detections within CD4 and CD8 T cell populations were performed using specific PCR amplifications in CDR3 region and Genescan assay on DNA analyzer.

Subject	Clonal TCRV β repertoire			
	CD4		CD8	
	Pre-infusion	Post-infusion	Pre-infusion	Post-infusion
A		V β s 1,7,9,11,12,14,15,16,20,21	V β 15	V β s 4,11,15
B	V β 2	V β s 1,2	V β 16	V β s 1,7,11,15,16,20,22,23
C	V β 16	V β s 1,8,11,16,23	V β s 1,5,7,15,16,24	V β s 1,4,5,9,12,13,14,15,16,18,20,24
D	V β s 8,13	V β s 8,13, 24	V β s 1,3,9,11,14,15,16,18,23	V β s 1,3,9,11, 12,14,15,16,24
E			V β s 1,3,7	V β s 1,7, 22
G	V β s 1, 6, 8,11,12,14,16,22,23	V β s 4,6,16,23	V β s 2,5,7,16,24	V β s 2,7,16
H	V β s 4,12,16	V β 2	V β 21	V β s 1,3

* Subtype of TCR V β s in **bold** indicate new clonal population generated after T cell infusion.