Breast Cancer Res Treat (2018) 168:57–67
https://doi.org/10.1007/s10549-017-4570-4

CLINICAL TRIAL

Serial immunological parameters in a phase II trial of exemestane and low-dose oral cyclophosphamide in advanced hormone receptor-positive breast cancer

Maryann Kwa[1] · Xiaochun Li[1] · Yelena Novik[1] · Ruth Oratz[1] · Komal Jhaveri[2] · Jennifer Wu[1] · Ping Gu[1] · Marleen Meyers[1] · Franco Muggia[1] · James Speyer[1] · Alyssa Iwano[1] · Maryam Bonakdar[1] · Lina Kozhaya[3] · Ece Tavukcuoglu[1] · Bahar Budan[1] · Roy Raad[1] · Judith D. Goldberg[1] · Derya Unutmaz[1,3] · Sylvia Adams[1]

Received: 18 October 2017 / Accepted: 4 November 2017 / Published online: 9 November 2017

Abstract

Background and purpose Resistance to endocrine therapies in hormone receptor (HR)-positive breast cancer is a significant challenge. Prior studies have shown that low-dose oral cyclophosphamide can transiently deplete regulatory T cells (Tregs) and improve anti-tumor immunity. We investigated the combination of exemestane with cyclophosphamide in patients with advanced HR-positive breast cancer and assessed changes in circulating immune cell subsets.

Methods This was a single-arm phase II trial of exemestane with cyclophosphamide in patients with metastatic HR-positive/HER2-negative breast cancer who had progressed on prior endocrine therapy (ClinicalTrials.gov: NCT01963481). Primary endpoint was progression-free survival (PFS) at 3 months (RECIST 1.1). Secondary objectives included median PFS, objective response rate, duration of response, and safety. Circulating Tregs ($FOXP3^{+}Helios^{+}$) and other immune cell subsets were monitored during treatment and compared with healthy controls.

Results Twenty-three patients were enrolled. Treatment was well tolerated, without grade 4/5 toxicities. Objective responses were seen in 6/23 patients (26.1%; 95% CI 10.2–48.4%) and were durable (median 11.6 months). Three-month PFS rate was 50.1% (95% CI 33.0–76.0%); median PFS was 4.23 months (95% CI 2.8–11.7). No treatment-related decrease in Tregs was observed. However, elevated baseline levels of Naïve Tregs [greater than 2.5 (the median of the naïve Tregs)] were associated with relative risk of disease progression or death [hazard ratio 11.46 (95% CI 2.32–56.5)]. In addition, the baseline levels of Naïve Tregs (adj-$p = 0.04$), Memory Tregs (adj-$p = 0.003$), CD4 + Central Memory T cells (adj-$p = 0.0004$), PD-1 + CD4 + Central Memory T cells (adj-$p = 0.008$), and PD-1 + CD4 + Effector Memory T cells (adj-$p = 0.009$) were significantly greater in the patients than in the healthy controls; the baseline levels of %CD4 + Naïve T cells (adj-$p = 0.0004$) were significantly lower in patients compared with healthy controls ($n = 40$).

Conclusion Treg depletion was not observed with low-dose cyclophosphamide when assessed by the specific marker FOXP3 + Helios +; however, baseline naïve Tregs were associated with 3-month PFS. Exemestane/cyclophosphamide combination had favorable safety profile with evidence of clinical activity in heavily pretreated patients.

Keywords Exemestane · Cyclophosphamide · Immune phenotyping · Regulatory T cells (Tregs) · Immunotherapy · Breast cancer

Introduction

The majority of breast cancers are hormone receptor (HR) positive, expressing the estrogen receptor (ER), progesterone receptor (PR), or both, and are human epidermal growth factor receptor 2 (HER2) negative. Endocrine therapy is

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10549-017-4570-4) contains supplementary material, which is available to authorized users.

✉ Sylvia Adams
sylvia.adams@nyumc.org

[1] Laura and Isaac Perlmutter Cancer Center, NYU Langone Medical Center, 160 East 34th Street, New York, NY 10016, USA

[2] Memorial Sloan Kettering Cancer Center, New York, NY, USA

[3] Jackson Laboratory for Genomic Medicine, Farmington, CT, USA

the mainstay of treatment for advanced HR-positive/HER2-negative breast cancer. Sensitivity to therapy is usually suggested by a high expression level of ER and/or PR in tumors, a long disease-free or progression-free interval, and metastatic disease confined to nonvisceral sites. Despite advances in endocrine therapy, the majority of patients with metastatic HR-positive breast cancer will experience disease progression due to tumor resistance to endocrine agents, making the care of this population a clinical challenge. Treatment in the second-line setting with single-agent nonsteroidal (anastrozole or letrozole) or steroidal (exemestane) aromatase inhibitors, fulvestrant, or tamoxifen has demonstrated only modest activity [1–5]. Combination therapies to circumvent endocrine resistance include agents targeting aberrations in the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway. One such agent is everolimus, which, in combination with exemestane (BOLERO-2 trial), demonstrated an improvement in progression-free survival (PFS) in patients with HR-positive/HER2-negative advanced breast cancer who were resistant to endocrine therapy. Everolimus was approved by the Food and Drug Administration (FDA) at the time of the conception of the current trial [6, 7]. The combination of exemestane and everolimus had an improved PFS of 7.8 months compared with 3.2 months for exemestane alone (HR 0.45; 95% CI 0.38–0.54; $p < 0.0001$), and response rates by central assessment were 12.6 and 2.1%, respectively. However, there was no difference in overall survival (OS), and toxicity was significantly higher in those receiving the combination. Approximately two-thirds of the patients treated with everolimus (66.8%) discontinued the study drug because of toxicity.

Evading anti-tumor immunity is a hallmark for the development and progression of cancer. Malignancies may use various mechanisms to suppress anti-tumor immunity, thereby avoiding effective tumor destruction by the host immune system. One of these mechanisms may be the expression of programmed cell death protein 1 (PD-1) and an abundance of regulatory T cells (Tregs) within tumor beds [8–11]. Immunotherapy, especially the checkpoint inhibitors with anti-PD-1/PD-L1 antibodies, has shown preliminary efficacy and durable responses in metastatic breast cancer. Triple-negative breast cancer (TNBC) and HR-positive/HER2-negative subtypes, in particular, appear to respond to this therapeutic approach [12–15].

In malignancy, Tregs can inhibit the anti-tumor immune response by suppressing the generation and function of adaptive T cell responses, as well as by blunting the innate arm of immunity [10, 16]. Patients with cancer have been shown to have an increased number of circulating and tumor-infiltrating Tregs, which is associated with a worse prognosis [17–19]. In preclinical and clinical studies of cancer, the frequent administration ("metronomic" scheduling) of low-dose cyclophosphamide, a nitrogen mustard alkylating agent, has been shown to selectively deplete Tregs as well as to attenuate their function leading to loss of suppression [20, 21]. Low-dose cyclophosphamide can also enhance effector T cell activity and unmask preexisting endogenous tumor antigen-specific T cells, leading to an improvement in anti-tumor immune responses [22–24]. Several small studies have demonstrated the efficacy of low-dose cyclophosphamide as a single agent in chemotherapy-refractory solid tumors, including breast cancer, and suggested transient modulation of circulating Tregs and restoration of anti-tumor immunity [22, 23]. It has also been shown in preclinical and clinical studies that estrogen can cause the expansion of Tregs, leading to immune tolerance [25, 26], therefore making HR-positive breast cancer an ideal disease type in which to study Treg-modulating therapies.

We conducted an open-label phase II trial to evaluate the clinical and immunomodulatory activity of the combination of low-dose oral cyclophosphamide and exemestane in patients with HR-positive/HER2-negative advanced breast cancer who had progressed on prior endocrine therapy. We hypothesized that cyclophosphamide would induce the inhibition of circulating Tregs and, due to improved anti-tumor immunity, result in durable responses in this patient population.

Materials and methods

Patients

Postmenopausal women or men with histologically confirmed breast cancer that was ER positive and/or PR positive, and HER2 negative with disease that is metastatic ($T_{any}N_{any}M_1$) were eligible. Women were defined as postmenopausal if they were at least 60 years of age, had undergone bilateral oophorectomy, or were younger than 60 years of age and had cessation of regular menses for at least 12 consecutive months with no alternative pathologic or physiological cause and had serum levels of follicle-stimulating hormone and estradiol in the postmenopausal range. All patients must have had either disease progression during at least one line of endocrine therapy for metastatic disease or recurrent disease while or within 12 months of receiving adjuvant endocrine therapy. Prior treatments allowed included nonsteroidal aromatase inhibitor (anastrozole or letrozole), tamoxifen, fulvestrant, or combinations. Prior chemotherapy in the adjuvant and/or metastatic setting was also allowed. Prior treatment with exemestane was not allowed, except in patients who started the combination of exemestane plus everolimus but discontinued everolimus due to toxicity within 4 weeks of initiating treatment.

All patients had to have measurable disease as per response evaluation criteria in solid tumors (RECIST), version 1.1, or bone-only lytic or mixed lytic and blastic lesions that could be accurately assessed by computed tomography (CT) for progression. Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2. They had adequate bone marrow reserve (absolute neutrophil count $\geq$ 1200/mcL; platelets $\geq 100 \times 10^9$/L; hemoglobin $\geq$ 9 g/dL), hepatic function (AST/ALT, bilirubin, and alkaline phosphatase levels $\leq$ 2.5 the upper limit of normal value), and renal function (serum creatinine $\leq$ 1.5 the upper limit of normal value). Patients with uncontrolled brain metastases or symptomatic visceral spread who were at risk for life-threatening complications were excluded.

Study design

In this single-arm, single-institution, open-label, phase II study, all patients received exemestane (25 mg oral daily) and cyclophosphamide (50 mg oral daily) (ClinicalTrials.gov identifier: NCT01963481). Each treatment cycle was 4 weeks (28 days). Treatment continued until objective demonstration of disease progression, unacceptable toxic effects, or withdrawal of consent. Reduction in the daily dose of exemestane was not allowed. Cyclophosphamide was held for grade 3 or higher cystitis, neutropenia, anemia, and/or thrombocytopenia and could be resumed when there was a reduction in the severity of adverse events to grade 2 or lower. Patients were allowed to continue exemestane alone if cyclophosphamide was discontinued due to toxicity. Compliance with both oral medications was monitored by medication diary entry by patients and reviewed by study personnel at all study visits. The study was approved by the New York University Institutional Review Board (IRB). Written informed consent was obtained from all subjects prior to enrollment in the study. Blood samples from healthy controls were obtained from the New York Blood Center. All healthy controls used in the study were anonymous.

Procedures

Imaging with computed tomography (CT) of the chest and abdomen/pelvis or 18F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) of the skull base to the mid thighs was performed at screening within 6 weeks before the start of study treatment and then repeated every 12 weeks while on therapy. Adverse events were recorded and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 4.0. History, physical exam, and blood drawn for hematologic, biochemical, and immunological analyses were performed at the beginning of each treatment cycle.

Immune analysis of peripheral blood was performed at baseline and at 1 and 3 months. At each time point, a sample of 40 ml of peripheral blood was drawn from the patients for immune studies. Buffy coats from 40 healthy individuals were obtained anonymously from the New York Blood Center for comparison. Peripheral Blood Mononuclear Cells (PBMCs) from patients and healthy donors' peripheral blood were isolated using Ficoll-Hypaque (GE Healthcare) density gradient centrifugation and then cryopreserved.

Flow cytometric studies

Cryopreserved PBMCs were thawed and then evaluated with multi-color flow cytometry. Cells were first stained with fixable viability dye (eBioscience) in PBS for 30 min, washed, and then stained with fluorescent-conjugated antibodies at 4 °C for 30 min in PBS buffer containing 2% FCS and 0.1% sodium azide. For intracellular staining, the cells were fixed and permeabilized using FOXP3 intracellular staining kit (eBioscience) and stained with antibodies for transcription factors forkhead box P3 (FOXP3) and Helios (Biolegend) as described. The following antibodies were also used for human T cell surface molecules: CD3, CD4, CD8, CD27, CD45RO, and PD-1 (all from Biolegend). The stained cells were analyzed using an LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star). Singlet lymphocytes were gated based on forward and side scatter properties. Dead cells were excluded based on viability dye staining. All T cell subsets were analyzed as the percentage of parent populations indicated in the results or figure legends.

The following 14 immune parameters were evaluated in PBMC: %Naïve Tregs (CD4 + CD45RO-FOXP3 + Helios +); %Memory Tregs (CD4 + CD4 5RO + FOXP3 + Helios +); %CD4 + Central Memory T Cells (CD45RO + CD27 +); %CD4 + Effector Memory T cells (CD45RO + CD27-); %CD4 + Naive T Cells (CD45RO-CD27 +); %CD8 + Central Memory T Cells (CD45RO + CD27 +); %CD8 + Effector Memory T cells (CD45RO + CD27-); %CD8 + Naive T cells (CD45RO- CD27 +); % CD4 + PD-1 + Central Memory T cells (CD4 + CD45RO + CD27 + PD-1 +); % CD4 + PD-1 + Effector Memory T cells (CD4 + CD45RO + CD27-PD-1 +); %CD4 + PD-1 + Naïve T cells (CD4 + CD45RO-CD27 + PD-1 +); % CD8 + PD-1 + Central Memory T cells (CD8 + CD45RO + CD27 + PD-1 +); %CD8 + PD-1 + Effector Memory T cells (CD8 + CD45RO + CD27-PD-1 +); and %CD8 + PD-1 + Naïve T cells (CD8 + CD45RO-CD27 + PD-1 +)

Study objectives

The primary objective was to estimate PFS at 3 months according to RECIST version 1.1. The secondary objectives were to estimate the median PFS, objective response rate, clinical benefit rate, and duration of response, to evaluate the safety of the regimen, as well as to summarize the distributions of the immune subsets at baseline and changes during treatment.

Sample size and statistical considerations

PFS was defined as the time from date of first study treatment until objective disease progression or death from any cause. Based on the data from BOLERO-2 [6, 7], we estimated PFS at 3 months with the combination regimen (exemestane plus everolimus) to be 75%, assuming that the distribution of PFS was exponential [calculations from PASS, NCSS, 2008, J. Hintze, Kaysville, Ut.]. With 23 patients, the null hypothesis would be rejected if the PFS rate at 3 months is 50% or less versus the alternative that the PFS rate at 3 months is 75% or greater with an alpha of 0.05 and a power of 80%.

The distributions of baseline characteristics and immune parameters at baseline were summarized with frequency distributions (frequency count and the percentage of patients in each category) for qualitative variables and with summary statistics for quantitative variables (mean, standard deviation, median, range, etc.). Secondly, the number of patients and their maximum grade adverse effects at least possibly related to treatment (drug safety) were summarized. Kaplan–Meier curves were used to estimate PFS at 3 months and median PFS along with 95% confidence intervals. Patients who dropped out of the study prior to disease progression or death (due to toxicity, noncompliance, or were lost to follow-up) were censored at the time of last study visit. Objective response rate (ORR: CR + PR) and clinical benefit rate [CBR: complete response (CR) + partial response (PR) + stable disease (SD)] were estimated with 95% confidence intervals. Finally, the distributions of 14 immune parameters were compared between breast cancer patients and healthy controls at baseline using two-sample *t* tests. Changes in each of the 14 immune parameters over time were investigated using mixed effects regression models, which considered both within-subject correlations and between-subject variation. The associations of the hazard of disease progression or death over the study observation period and these immune parameters were investigated using Cox proportional hazard models. In order to control for the False Discovery Rate (FDR), each of the evaluations of the immune parameters was adjusted for 14 independent analyses with adjusted (adj) *p* values based on the Benjamini and Hochberg (BH) method with a significance level of 0.05 [27]. Because of the differences observed in the age distributions of the breast cancer patients and healthy controls and the known variations in naïve T cells that are dependent on age [28], the immune comparisons were also conducted between patients and healthy controls with ages greater than 40.

Results

Patients

Twenty-three patients (22 postmenopausal females and 1 male) were enrolled between November 2013 and April 2015. Baseline patient characteristics are summarized in Table 1, Supplementary Table 2 and Supplementary Fig. 1. The median age was 54 years; 39% were Caucasian, 26% Asian, 22% African American, and 13% Hispanic. All patients had ER-positive and HER2-negative breast cancer, and 69.6% (16/23 patients) had visceral organ involvement. Eight patients had de novo metastatic breast cancer, and 15 patients had received prior adjuvant therapy. The median number of prior endocrine therapies was 2 (range 1–3), and the median number of prior lines of chemotherapy in the metastatic setting was 1 (range 0–5). All patients were menopausal, including four women rendered postmenopausal surgically. All patients were compliant with the oral study treatment. The median follow-up was 3 months (range 0.9–20.3 months).

Safety

Supplementary Table 1 provides a summary of the numbers of patients with maximum grade adverse events (at least possibly related to the study treatment). Overall, the regimen was very well tolerated, with only four grade 3 adverse events observed in 3 patients: fatigue ($n = 1$), myalgia and arthralgia ($n = 1$), and urinary tract infection ($n = 1$). There were no grade 4–5 adverse events. Grade 1 thrombocytopenia was noted in two patients who had prolonged therapy with cyclophosphamide (beyond 1 year). In these two cases, cyclophosphamide was stopped and exemestane was continued because both patients had evidence of disease stability for greater than 1 year (1 SD and 1 CR).

Efficacy

Twenty-two of the 23 patients were eligible for response assessment (one patient withdrew consent during the first week of treatment). At 3 months, 11 of the 22 patients had either a response or stable disease, and 11 patients experienced disease progression. No patients died during the study period.

Table 1 Patient demographic and tumor characteristics at baseline and treatment history ($n = 23$)

	Number (%)
Gender	
Female	22 (96%)
Male	1 (4%)
Age (years)	
Median	54
Range	31–77 (100%)
Mean	56.3
SD	12.4
Ethnicity	
Caucasian	9 (39%)
Asian	6 (26%)
African American	5 (22%)
Hispanic	3 (13%)
Pathology	
Invasive ductal	21 (91%)
Invasive lobular	2 (9%)
Estrogen receptor (ER) status	
Positive	23 (100%)
Negative	0
Progesterone receptor (PR) status	
Positive	18 (78%)
Negative	5 (22%)
Disease presentation	
Recurrent	15 (65%)
De novo metastatic	8 (35%)
Sites of metastases	
Bone only	6 (26%)
Bone, liver	2 (9%)
Bone, lymph nodes	1 (4%)
Bone, liver, lymph nodes	4 (18%)
Bone, lungs, lymph nodes	3 (13%)
Bone, liver, lungs, lymph nodes	2 (9%)
Lungs, lymph nodes	2 (9%)
Chest wall, lungs, lymph nodes	1 (4%)
Chest wall, lungs	1 (4%)
Orbit, colon	1 (4%)
Prior endocrine therapies/targeted therapies	
Median	2
Range	1–3 (100%)
Therapies	
Anastrozole	2
Letrozole	2
Tamoxifen	5
Fulvestrant	4
Everolimus	2
Anastrozole and fulvestrant	8
Letrozole and fulvestrant	5
Everolimus and exemestane	1
Fresolimumab (investigational)	1
Number of prior lines of chemotherapy in metastatic setting	
Median	1
Range	0–5 (100%)
Therapies	
Carboplatin	1
Capecitabine	7
Eribulin	3
Gemcitabine	3
Nab-paclitaxel	2
Paclitaxel	3
Paclitaxel and Bevacizumab	1
Pegylated liposomal doxorubicin	1
Vinorelbine	1

The 3-month PFS rate estimated from a Kaplan–Meier curve was 50.1% (95% CI 33.0–76.0%) and the median PFS was 4.23 months (95% CI 2.8–11.7 months) (Fig. 1).

The objective response rate (ORR) was 6/23 (26.1%; 95% CI 10.2–48.4%) in the intent-to-treat (ITT) population and 6/22 (27.3%; 95% CI 10.7–50.3%) in patients with assessable response status. There were 6 patients with objective responses (two complete responses (CR) and four partial responses (PR); Table 2). Importantly, for patients with an objective response, the median duration of response was 11.6 months (range 8.3–22.1). Responders ranged in age from 35 to 77 years, and their breast tumors were highly ER positive (> 95% by immunohistochemistry). All six patients had osseous metastases, and three

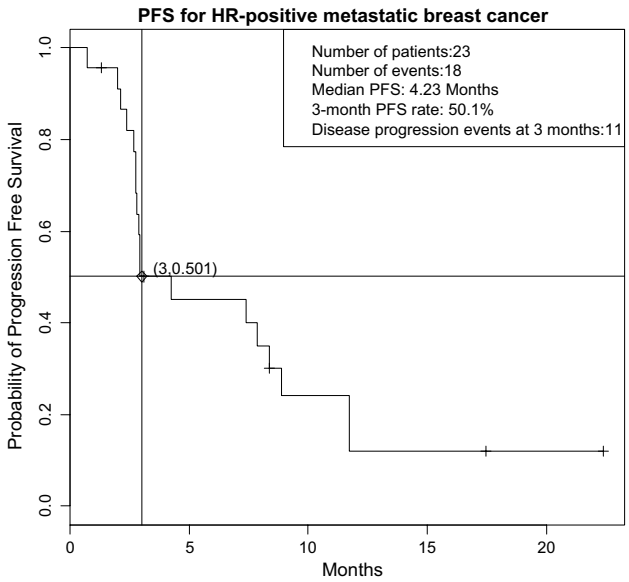

Fig. 1 Progression-free survival (PFS) (Kaplan–Meier curve)

Table 2 Efficacy results: all treated patients

Best overall response	Number of patients (%), $n = 23$
Complete response (CR)	2 (8.7%)
Partial response (PR)	4 (17.4%)
Stable disease (SD)	5 (21.7%)
Progression of disease (PD)	11 (47.8%)
Objective response	6 (26.1%, 95% CI 10.2–48.4)
Duration of response (months)	Median: 11.6
	Range: 8.3–22.1

of these patients also had liver metastases. Five of these six patients recurred after adjuvant endocrine therapy. Four had not received prior chemotherapy, one had one line of prior chemo-treatment, while the other patient had received three prior chemotherapy regimens for metastatic disease. The prior number of endocrine therapies in this group was 1 ($n = 2$), 2 ($n = 3$), and 3 ($n = 1$).

Five additional patients had stable disease with the median duration of stability of 7.3 months; the clinical benefit rate (CBR) was therefore 11/23 (47.8%; 95% CI 26.8–69.4%).

At time of data analysis, 21 patients are off the study due to either disease progression ($n = 18$), other reasons (1 withdrawal of consent, 1 geographic relocation, and 1 physician discretion), and two patients continued to receive treatment on study for over 2 years.

Immune profiling of T cell subsets

We first determined the proportion of thymus-derived naïve and memory Tregs by FOXP3 and Helios co-expression after gating on CD4 + naïve and memory T cells, respectively, using flow cytometry (Fig. 2). Figure 3 shows the distributions of the baseline levels of these immune parameters in patients and the corresponding healthy controls. We found that the baseline levels of Naïve Tregs (adj-$p = 0.04$), Memory Tregs (adj-$p = 0.003$), CD4 + Central Memory T Cells (adj-$p = 0.0004$), CD4 + CD45RO + CD27 + PD-1 + T cells (adj-$p = 0.008$), and CD4 + CD45RO + CD27- PD-1 + T cells (adj-$p = 0.009$) were significantly greater in patients than in the healthy controls. The baseline levels of %CD4 + Naïve T cells (adj-$p = 0.0004$) were significantly lower in patients than in the healthy controls. Because the healthy controls (age mean: 44.0; SD: 12.3) were younger than the breast cancer patients (age mean: 56.3; SD: 12.4) (Supplementary Table 2), we also compared the baseline immune parameters in patients and controls with ages greater than 40 (patients: $n = 21$, age mean 58.5; SD: 10.5; healthy controls: $n = 23$, age mean: 53.7, SD: 6.7) to reduce the confounding effects of age. In this subgroup of cases and controls of comparable ages, the observed results are similar to those for the entire group: the baseline levels of Naïve Tregs (adj-$p = 0.05$), Memory Tregs (adj-$p = 0.005$), CD4 + Central Memory T cells (adj-$p = 0.005$), and CD4 + CD45RO + CD27 + PD-1 + T cells (adj-$p = 0.05$) in cancer patients were significantly greater than those in the healthy controls, and the baseline levels of %CD4 + Naïve

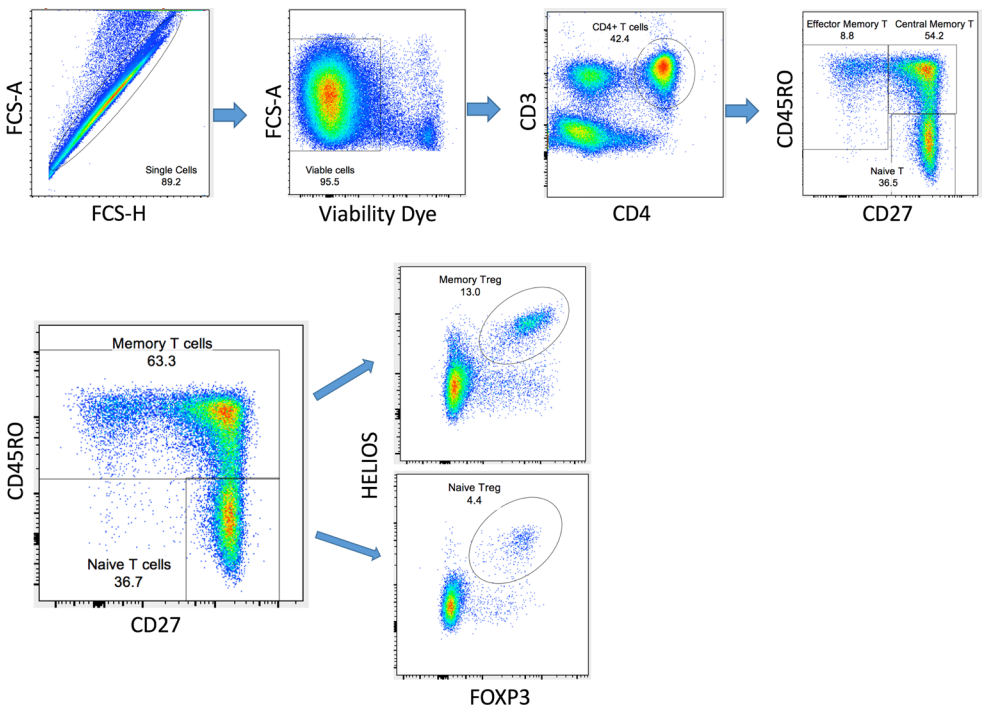

Fig. 2 Gating schema for FOXP3 + Helios + Naïve/Memory Tregs and CD4 + Central/Effector Memory T cells

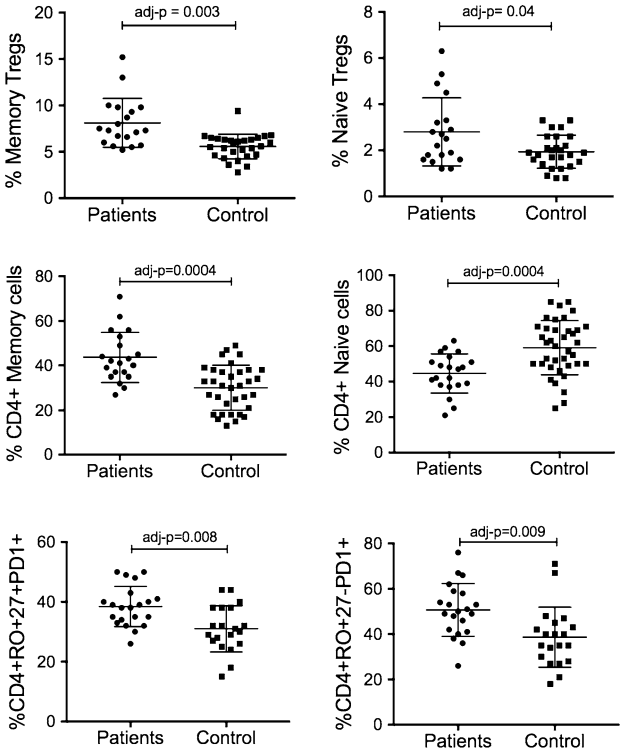

Fig. 3 Distributions of main T cell subsets that differed in baseline levels between patients and healthy controls (based on BH FDR method to adjust for multiple comparisons [27], adjusted *p* values shown)

T cells (adj-$p = 0.005$) in cancer patients were significantly less than those in the healthy controls.

Among the breast cancer patients, the following immune parameters changed significantly over the treatment course (Fig. 4): Naïve Tregs (adj-$p = 0.0014$), Memory Tregs (adj-$p = 0.013$), CD4 + Central Memory T Cells (adj-$p = 0.0056$), %CD4 + Naïve T Cells (adj-$p = 0.030$), CD4 + CD45RO + CD27 + PD-1 + T cells (adj-$p = 0.030$), and CD4 + CD45RO-CD27 + PD-1 + T cells (adj-$p = 0.0056$). Over 1 month, the changes in these immune parameters were as follows: Memory Tregs increased 0.34 (95% CI 0.19–0.48), CD4 + Central Memory T Cells increased 0.51 (95% CI 0.18–0.83), CD4 + Central Memory T Cells increased 1.73 (95% CI 0.74–2.72), %CD4 + Naïve T Cells decreased 1.73 (95% CI 0.39–3.07), CD4 + CD45RO + CD27 + PD-1 + T cells increased 0.68 (95% CI 0.16–1.20), and CD4 + CD45RO-CD27 + PD-1 + T cells increased 0.29 (95% CI 0.13–0.45).

Increased baseline levels of Naïve Tregs were significantly related to the hazard of disease progression during the study with adj-$p = 0.014$ (Cox proportional hazards regression models and the BH FDR method to adjust for multiple testing). The risk of disease progression or death was 1.90 (95% CI 1.30–2.79) times greater for a one percentage increase of naïve Tregs. The risk of disease progression or death in patients with naïve Tregs greater than 2.5 (the median of the naïve Tregs) was significantly higher than that in patients with naïve Tregs less than or equal to 2.5 with a hazard ratio of 11.46 (95% CI 2.32–56.5). Supplementary Table 3 summarizes the immune parameters at baseline and at 1 and 3 months for patients and healthy controls.

Discussion

In this phase II trial, we studied the clinical and immunological activity of the combination of exemestane with low-dose oral cyclophosphamide in patients with advanced HR-positive breast cancer who had progressed on prior endocrine therapy. Overall, in this pretreated population, the combination was very well tolerated with only few grade 3 adverse events (all expected), but no grade 4 or 5 adverse events. Objective responses were seen in 6/23 patients (26.1%) including patients with liver metastases, and all responses were long-lasting (8–22 months). The clinical benefit rate was 46. In comparison, the ORR were 12.6, 10.4, and 12% and the median PFS were 7.8, 9.5 months, and not reported in the exemestane and everolimus (BOLERO-2), palbociclib and fulvestrant (PALOMA-3), and single agent pembrolizumab administration in PD-L1-positive breast cancer (KEYNOTE-028) trials, respectively [6, 7, 13, 29].

Correlative analyses in our trial focused on comprehensive peripheral blood immunophenotyping with particular emphasis on Tregs and several important observations were made. Patients had significantly elevated baseline Naïve and Memory Treg levels as well as PD-1^+ $CD4^+$ Central Memory and PD-1^+ $CD4^+$ Effector Memory T cells when compared to healthy donors. In contrast to prior observations, however, serial assessments during treatment with continuous low-dose cyclophosphamide administered with exemestane did not show a decrease but significant increases in circulating Naïve and Memory Tregs. Baseline Naïve Tregs were identified as a predictive biomarker for clinical benefit.

In healthy donors, Tregs comprise approximately 2–4% of circulating $CD4^+$ T cells [30]. Tregs have physiologic immunosuppressive functions but also play a fundamental role in tumor immune evasion [31]. In cancer patients, Treg numbers have been shown to be increased in the peripheral blood, are prevalent in the tumor itself as well as in tumor draining lymph nodes, and are associated with worse prognosis [32, 33]. Strategies to deplete Tregs (at least transiently) in patients have been intensely studied, mainly in the setting of cancer vaccines in order to enhance T cell priming and function but also as single modality in advanced malignancies. In these studies, selective depletion of circulating Tregs, albeit transient, was seen with low-dose cyclophosphamide which may augment effector

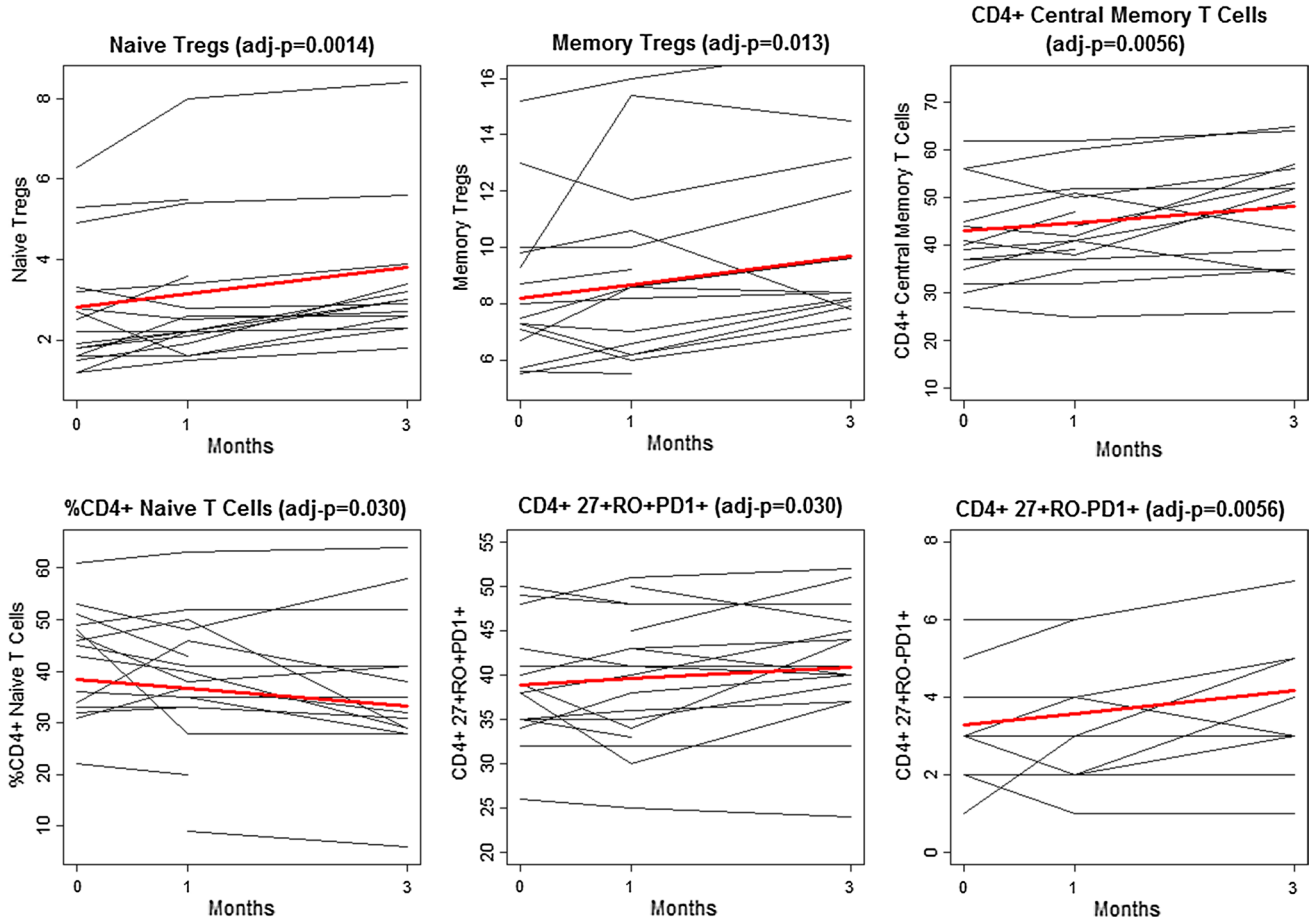

Fig. 4 Immunologic parameters that changed significantly over time (BH FDR method to adjust for multiple comparisons [27]; $n = 23$). *Note* For each of the above plots, the red line is the mean change over time obtained from a mixed effects model

T cell and innate anti-tumor immunity [22, 23, 34–37]. Ge et al. demonstrated in a small study of 12 advanced breast cancer patients that treatment with low-dose cyclophosphamide led to a significant reduction in circulating $CD25^+$ $FOXP3^+$ Tregs. After 14 days on treatment, Tregs were reduced by more than 40% but gradually recovered to pretreatment levels by day 84 [23]. Meanwhile, there was a significant increase in breast tumor-reactive effector IFN-γ + T cells that correlated with improved clinical outcome. In a randomized vaccine trial in renal cell cancer, treatment with low-dose cyclophosphamide was associated with reduced Tregs and vaccine-induced T cell responses, as well as increased overall survival in vaccinated patients [34]. Likewise, in another trial of cyclophosphamide administered the day before a breast cancer vaccine along with weekly trastuzumab, a decrease in peripheral Tregs was observed [35]. However, a study in advanced cancer patients, including breast cancer, did not demonstrate a decrease in Tregs when cyclophosphamide was given in combination with intratumoral Bacilli Calmette-Guerin (BCG) [38].

In our study, we confirmed prior observations that patients had significantly elevated baseline Treg levels when compared to healthy donors. However, serial assessments during treatment with continuous low-dose cyclophosphamide administered with exemestane did not show a decrease in circulating Treg percentages over time. We did, however, observe an increase in the proportion of naïve and memory Tregs over time with the combination. This increase could be associated with disease progression or represent a rebound, reactionary increase in an attempt to control immune activation, alternatively by stimulation caused by initial depletion of Tregs by treatment.

In cancer studies published to date, Tregs have been identified by co-expression of CD25 and FOXP3. FOXP3 is expressed on all Tregs and is a transcription factor that is important for the development and function of Tregs [39]. However, it is now well established that transforming growth factor-β (TGFβ) signaling can transiently upregulate FOXP3 activation of nonsuppressive T cells [40–42]. Thus, FOXP3 expression alone does not always distinguish a regulatory phenotype in human T cells. Similarly, CD25 may also be

transiently upregulated in recently activated conventional CD4 T cells, confounding Treg identification especially during chronic activation conditions. Recently, Helios, an Ikaros family zinc finger transcription factor, has been shown to represent natural Tregs that originate in the thymus [41, 43, 44]. Together with FOXP3, Helios can differentiate Tregs into two distinct populations, both with suppressive functions: Helios-positive(+) Tregs, which do not produce inflammatory cytokines such as IL-17, and Helios-negative(-) Tregs that do secrete IL-17 and other cytokines [45]. More importantly, Helios expression remains stable during T cell activation [43, 45]. Previously, we have shown that in comparison to CD25 and FOXP3 staining, co-expression of Helios with FOXP3 identified more Tregs within memory T cells, and that this changed the analysis of Tregs in HIV patients [46]. Therefore, we exclusively used these markers to define Tregs, which could be a significant difference compared to previous studies. It is important to note that another key difference in our study was that we separated Tregs into naïve and memory subsets and analyzed their frequencies within each compartment.

We observed that elevated levels of baseline thymus-derived Treg subsets that express FOXP3 and Helios transcription factors were associated with disease progression at 3 months, suggestive of a novel biomarker for prediction of disease progression after treatment. This finding is mechanistically interesting, because naïve Tregs represent the precursor for memory Tregs and likely replenish these by expansion. It is conceivable that higher naïve Treg frequency can more rapidly maintain or sustain Treg numbers within tumor tissue. It is also possible that higher immune activation results in much higher turnover of T cells and Tregs. Indeed, similar to naïve Treg frequency, central memory CD4 + T cells were also significantly higher in breast cancer patients compared to healthy controls, which could again reflect higher T cell turnover in these subjects. While our sample size is too small to be conclusive, these findings warrant larger prospective studies to determine the use of this immune profiling analysis as a potential predictive biomarker.

Another important finding from our immune parameter analysis is the significantly increased population of PD-1-expressing CD4 central memory T cells in patients. While the focus in anti-tumor immunity has been on effector or highly differentiated T cells that express immune checkpoint inhibitory receptors such as PD-1, there is emerging evidence that T cells of central memory phenotype play an important role in anti-tumor responses [47, 48] and were observed in some tumor-infiltrating lymphocytes (TILs) [49, 50].

In conclusion, in the 22 patients eligible for response assessment, the response rate was 27.3% with a lower confidence bound of 10.7% and a median PFS of 4.23 months suggesting modest clinical activity in a pretreated patient population. The toxicity profile of the combination was expectedly low, and we observed durable treatment responses in several patients. These findings suggest that this regimen could be considered in future clinical investigation in breast cancer, perhaps in combination with immunotherapeutic approaches. Agents such as anti-PD-1/PD-L1 antibodies, which have demonstrated anti-tumor efficacy in metastatic breast cancer [12–14], could be considered as combination partners, especially as patients demonstrated significantly higher percentages of PD-1 + central memory T cells compared with controls.

Acknowledgements We thank all the patients who participated in this study and their families, the NYU Clinical Trials Office, and grant support through the Perlmutter Cancer Center (NYUCI Core Grant P30CA016087) (SA, XL, JDG). We also thank Juan Laifalle, PhD, for providing critical reading and guidance for the manuscript.

Funding support Grant support was provided through the Perlmutter Cancer Center (NYUCI Core Grant P30CA016087) (for SA, XL, JDG).

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

References

1. Rose C, Vtoraya O, Pluzanska A et al (2003) An open randomised trial of second-line endocrine therapy in advanced breast cancer. Comparison of the aromatase inhibitors letrozole and anastrozole. Eur J Cancer 39:2318–2327
2. Chia S, Gradishar W, Mauriac L et al (2008) Double-blind, randomized placebo controlled trial of fulvestrant compared with exemestane after prior nonsteroidal aromatase inhibitor therapy in postmenopausal women with hormone receptor-positive, advanced breast cancer: results from EFECT. J Clin Oncol 26:1664–1670
3. Di Leo A, Jerusalem G, Petruzelka L et al (2010) Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. J Clin Oncol 28:4594–4600
4. Di Leo A, Jerusalem G, Petruzelka L et al (2014) Final overall survival: fulvestrant 500 mg versus 250 mg in the randomized CONFIRM trial. J Natl Cancer Inst 106:337
5. Thurlimann B, Robertson JF, Nabholtz JM, Buzdar A, Bonneterre J, Arimidex Study G (2003) Efficacy of tamoxifen following anastrozole ('Arimidex') compared with anastrozole following tamoxifen as first-line treatment for advanced breast cancer in postmenopausal women. Eur J Cancer 39:2310–2317
6. Baselga J, Campone M, Piccart M et al (2012) Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med 366:520–529
7. Yardley DA, Noguchi S, Pritchard KI et al (2013) Everolimus plus exemestane in postmenopausal patients with HR(+) breast cancer: BOLERO-2 final progression-free survival analysis. Adv Ther 30:870–884
8. Topalian SL, Weiner GJ, Pardoll DM (2011) Cancer immunotherapy comes of age. J Clin Oncol 29:4828–4836

9. Drake CG, Jaffee E, Pardoll DM (2006) Mechanisms of immune evasion by tumors. Adv Immunol 90:51–81
10. Ghiringhelli F, Menard C, Terme M et al (2005) CD4 + CD25 + regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med 202:1075–1085
11. Liu Z, Kim JH, Falo LD Jr, You Z (2009) Tumor regulatory T cells potently abrogate antitumor immunity. J Immunol 182:6160–6167
12. Nanda R, Chow LQ, Dees EC et al (2016) Pembrolizumab in patients with advanced triple-negative breast cancer: phase Ib KEYNOTE-012 Study. J Clin Oncol 34:2460–2467
13. Rugo H, Delord, J-P, Im S-A, et al. (2015) Preliminary efficacy and safety of pembrolizumab (MK-3475) in patients with PD-L1-positive, estrogen receptor-positive (ER+)/HER2-negative advanced breast cancer enrolled in KEYNOTE-028. San Antonio Breast Cancer Symposium (SABCS), Abstract S5-07
14. Adams S, Diamond, JR, Hamilton, EP, et al. (2016) Phase Ib trial of atezolizumab in combination with nab-paclitaxel in patients with metastatic triple-negative breast cancer (mTNBC). American Society of Clinical Oncology (ASCO) Annual Meeting, Abstract 1009
15. Adams S LS, Toppmeyer D, et al. (2017) Phase 2 study of pembrolizumab as first-line therapy for PD-L1-positive metastatic triple-negative breast cancer (mTNBC): preliminary data from KEYNOTE-086 cohort. J Clin Oncol 35 (Abstract 1088)
16. Ghiringhelli F, Larmonier N, Schmitt E et al (2004) CD4 + CD25 + regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 34:336–344
17. Woo EY, Chu CS, Goletz TJ et al (2001) Regulatory CD4(+) CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 61:4766–4772
18. Liyanage UK, Moore TT, Joo HG et al (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 169:2756–2761
19. Curiel TJ, Coukos G, Zou L et al (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10:942–949
20. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H (2005) Inhibition of CD4(+)25 + T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood 105:2862–2868
21. Berd D, Mastrangelo MJ (1987) Effect of low dose cyclophosphamide on the immune system of cancer patients: reduction of T-suppressor function without depletion of the CD8+ subset. Cancer Res 47:3317–3321
22. Ghiringhelli F, Menard C, Puig PE et al (2007) Metronomic cyclophosphamide regimen selectively depletes CD4 + CD25 + regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 56:641–648
23. Ge Y, Domschke C, Stoiber N et al (2012) Metronomic cyclophosphamide treatment in metastasized breast cancer patients: immunological effects and clinical outcome. Cancer Immunol Immunother 61:353–362
24. Dewan MZ, Vanpouille-Box C, Kawashima N et al (2012) Synergy of topical toll-like receptor 7 agonist with radiation and low-dose cyclophosphamide in a mouse model of cutaneous breast cancer. Clin Cancer Res 18:6668–6678
25. Prieto GA, Rosenstein Y (2006) Oestradiol potentiates the suppressive function of human CD4 CD25 regulatory T cells by promoting their proliferation. Immunology 118:58–65
26. Polanczyk MJ, Hopke C, Vandenbark AA, Offner H (2006) Estrogen-mediated immunomodulation involves reduced activation of effector T cells, potentiation of Treg cells, and enhanced expression of the PD-1 costimulatory pathway. J Neurosci Res 84:370–378
27. Benjamini YHY (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc 57:289–300
28. Thome JJ, Grinshpun B, Kumar BV et al (2016) Longterm maintenance of human naive T cells through in situ homeostasis in lymphoid tissue sites. Sci Immunol. https://doi.org/10.1126/sciimmunol.aah6506
29. Cristofanilli M, Turner NC, Bondarenko I et al (2016) Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. Lancet Oncol 17:425–439
30. Baecher-Allan C, Wolf E, Hafler DA (2005) Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4 + CD25 + T cells. Clin Immunol 115:10–18
31. Vignali DA, Collison LW, Workman CJ (2008) How regulatory T cells work. Nat Rev Immunol 8:523–532
32. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B (2003) Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res 9:606–612
33. Bates GJ, Fox SB, Han C et al (2006) Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 24:5373–5380
34. Walter S, Weinschenk T, Stenzl A et al (2012) Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med 18:1254–1261
35. Chen G, Gupta R, Petrik S et al (2014) A feasibility study of cyclophosphamide, trastuzumab, and an allogeneic GM-CSF-secreting breast tumor vaccine for HER2+ metastatic breast cancer. Cancer Immunol Res 2:949–961
36. Greten TF, Ormandy LA, Fikuart A et al (2010) Low-dose cyclophosphamide treatment impairs regulatory T cells and unmasks AFP-specific CD4 + T-cell responses in patients with advanced HCC. J Immunother 33:211–218
37. Lutz ER, Wu AA, Bigelow E et al (2014) Immunotherapy converts nonimmunogenic pancreatic tumors into immunogenic foci of immune regulation. Cancer Immunol Res 2:616–631
38. Audia S, Nicolas A, Cathelin D et al (2007) Increase of CD4 + CD25 + regulatory T cells in the peripheral blood of patients with metastatic carcinoma: a phase I clinical trial using cyclophosphamide and immunotherapy to eliminate CD4 + CD25 + T lymphocytes. Clin Exp Immunol 150:523–530
39. Miyara M, Yoshioka Y, Kitoh A et al (2009) Functional delineation and differentiation dynamics of human CD4 + T cells expressing the FoxP3 transcription factor. Immunity 30:899–911
40. Tran DQ, Ramsey H, Shevach EM (2007) Induction of FOXP3 expression in naive human CD4 + FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 110:2983–2990
41. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D (2009) Expression of GARP selectively identifies activated human FOXP3 + regulatory T cells. Proc Natl Acad Sci USA 106:13439–13444
42. Zhou AX, Kozhaya L, Fujii H, Unutmaz D (2013) GARP-TGF-beta complexes negatively regulate regulatory T cell development and maintenance of peripheral CD4 + T cells in vivo. J Immunol 190:5057–5064
43. Thornton AM, Korty PE, Tran DQ et al (2010) Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3 + T regulatory cells. J Immunol 184:3433–3441

44. Himmel ME, MacDonald KG, Garcia RV, Steiner TS, Levings MK (2013) Helios + and Helios-cells coexist within the natural FOXP3 + T regulatory cell subset in humans. J Immunol 190:2001–2008
45. Mercer F, Khaitan A, Kozhaya L, Aberg JA, Unutmaz D (2014) Differentiation of IL-17-producing effector and regulatory human T cells from lineage-committed naive precursors. J Immunol 193:1047–1054
46. Vetizou M, Pitt JM, Daillere R et al (2015) Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science 350:1079
47. Beckhove P, Feuerer M, Dolenc M et al (2004) Specifically activated memory T cell subsets from cancer patients recognize and reject xenotransplanted autologous tumors. J Clin Investig 114:67–76
48. Klebanoff CA, Gattinoni L, Torabi-Parizi P et al (2005) Central memory self/tumor-reactive CD8 + T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci USA 102:9571–9576
49. Meng Q, Liu Z, Rangelova E et al (2016) Expansion of tumor-reactive T cells from patients with pancreatic cancer. J Immunother 39:81–89
50. Liu Z, Meng Q, Bartek J Jr et al (2017) Tumor-infiltrating lymphocytes (TILs) from patients with glioma. Oncoimmunology 6:e1252894