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ORIGINAL ARTICLE

Immunotherapy with IL-10- and IFN-γ-producing CD4 effector cells modulate "Natural" and "Inducible" CD4 TReg cell subpopulation levels: observations in four cases of patients with ovarian cancer

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Abstract Adoptive T cell therapy for cancer patients optimally requires participation of CD4 T cells. In this phase I/II study, we assessed the therapeutic effects of adoptively transferred IL-10- and IFN-γ-producing CD4 effector cells in patients with recurrent ovarian cancer. Using MUC1 peptide and IL-2 for ex vivo CD4 effector cell generation, we show that three monthly treatment cycles of autologous T cell restimulation and local intraperitoneal re-infusion-modulated T cell-mediated immune responses that were associated with enhanced patient survival. One patient remains disease-free, another patient experienced prolonged survival for nearly 16 months with recurrent disease, and two patients expired within 3–5 months following final infusion. Prolonged survivors showed elevated levels of systemic $CD3^+CD4^+CD25^+$ and $CD3^+CD4^+CD25^-$ T cells when compared to that of pre-treatment levels and similarly treated short-term survivors. Such cell populations among these patients contained variable levels of "Inducible" Tr1 ($CD4^+CD25^-FoxP3^-IL\text{-}10^+$) and "Natural" ($CD4^+CD25^+CD45RO^+FoxP3^+$) TReg cell numbers and ratios that were associated with prolonged and/or disease-free survival. Moreover, peptide-restimulated T cells from these patients showed an elevation in both IFN-γ production, memory cell phenotype, and select TNF family ligands associated with enhanced T cell survival and apoptosis-inducing activities. This suggests that intraperitoneally administered Th1-like cells, producing elevated levels of IL-10, may require and/or induce differential levels of distinct systemic TReg subpopulations that influence, in part, long-term tumor immunity and enhanced memory/effector CD4-mediated therapeutic potentials. Furthermore, treatment efficacy and enhanced memory cell phenotype did not appear to be dependent on TReg cell numbers but upon ratios of "Inducible" and "Natural" TReg subpopulations.

Keywords Tumor immunity · Regulatory T cells · Th1 effector cells · TNF superfamily · T effector/memory cells · Adoptive T cell immunotherapy · IL-10 · IFN-γ · Recurrent ovarian cancer

Abbreviations

Th1	$CD4^+$ T cells producing IFN-γ
TReg	Regulatory T cells
Foxp3	Forkhead box protein p3
TR1	$CD4^+$ $CD25^-FoxP3^-$ T cells producing IL-10
nTRegs	Ag-experienced natural TReg $CD4^+CD25^+CD45RO^+FoxP3^-$ cells

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Introduction

Ovarian cancer is the leading cause for mortality among gynecologic malignancies and represents the fourth leading overall cause for cancer-related death in women [1]. Standard treatment relies on surgical debulking and platinum-based chemotherapy. Although most patients initially respond to treatment, the majority of women with advanced disease become resistant to such treatments and experience terminal relapse [2]. There is currently no known treatment capable of achieving a cure for women with recurrent ovarian cancer in whom adjuvant therapy has been previously administered. Thus, the evaluation and subsequent characterization of new therapeutic strategies is needed [3].

CD4 T cells play a central role in virtually every aspect of immunity, including the antitumor response, and have been suggested to be a key missing component in current cancer immunotherapies [4]. Human Th1 effector cells are a subpopulation of CD4 T cells that are characterized by their capacity to secrete IFN-γ and IL-10 [5–9]. These cells can mediate and/or assist in tumor rejection by directly killing tumor cells via TNF family-related lytic pathways (i.e., TRAIL and FasL), up-regulation of HLA Class I and II expression on tumor cells, inducing angiogenesis inhibitors and promoting DC-dependent and/or -independent T cell activation or tolerance [3, 10–16]. Such antitumor responses have been associated, in part, with both Th1 cell-derived IFN-γ and/or IL-10-mediated mechanisms that contribute to tumor regression and immunity [5–7, 10, 11]. Moreover, a recent case study involving a single melanoma patient demonstrated that infusion of a Th1 cell clone, specific for the NY-ESO tumor-associated Ag, led to a durable remission [17], and thus provides an impetus to study the roles and immunomodulatory effects of such effector cells in patients with similarly aggressive diseases, such as recurrent ovarian cancer.

In addition to Th1 effector cells, CD4 T cell subpopulations have also been associated with regulatory T cells (TRegs) that control self-reactivity in autoimmune disease and immune response homeostasis at sites of inflammation and tumor growth [18–22]. TReg cells can be classified into two main subsets according to their origin and suppressive activity. Ag-experienced "Natural" CD4^{+} TReg effector cells (nTRegs), constitutively expressing FoxP3 and the activation markers CD25 and CD45RO (CD4^{+}CD25^{+}CD45RO^{+}FoxP3^{+}), originate in the thymus by high affinity interaction of the T cell receptor with Ag expressed on the thymic stroma [23, 24]. Such cells suppress the proliferation of effector T cells in a contact-dependent, cytokine-independent manner. In contrast, other types of TReg cells can be induced from naive CD4 cells in the periphery, such as IL-10-producing TR1 cells and TGF-β-producing Th3 cells [18, 25–27]. Such "induced" CD4^{+}CD25^{-} TReg subpopulations (iTReg) exert suppression mostly through soluble factors, and their suppressive function is not strictly associated with a high level of FoxP3 expression. In humans, both Natural and Inducible TReg cell subpopulations have been identified at increased frequencies in the peripheral blood and malignant effusions of patients with various types of cancers, including ovarian [24, 25, 28–31]. Although evidence that both "Inducible" and "Natural" TRegs may alter the clinical course of cancer progression has been described, their diverse roles, phenotypic heterogeneity, and effects on tumor immunity during adoptive immunotherapy with tumor-reactive effector cells in patients with epithelial cell-based tumors remain relatively undefined.

In our previous study, which marked one of a few clinical studies focused on the therapeutic effects of intraperitoneal T cell transfer, we investigated the generation, expansion, and therapeutic effects of autologous Th1-like effector cells as a potential immunotherapeutic strategy in patients with recurrent ovarian cancer [32]. We showed that three monthly treatment cycles of T cell restimulation and local–regional intraperitoneal re-infusion selectively modulated endogenous T cell-mediated immune responses in some patients that were associated with diminished or stabilized serum CA125 tumor marker levels. In the current study using the same cohort, we extend our observations in these patients to more definitively characterize IFN-γ and IL-10 production among transferred T cells and investigate the effects of such Th1-like effector cell transfer on both "Inducible" TR1 and "Natural" FoxP3^{+} TReg cell subpopulations at various stages of treatment. To our knowledge, this is the first report that investigates multiple cycles of adoptively transferred Th1 cell therapy and their relationship with different TReg cell subpopulations and memory T cell responses in patients with recurrent ovarian cancer. This study offers insight into Th1-mediated mechanisms that enhance Th1/memory T cell responses following T cell-based immunotherapy in cancer patients and further suggests a potential role for co-therapeutic approaches targeting modulation, and not depletion, of the TReg cellular network in such patients.

Materials and methods

Patients

In this Phase I/II study, seven patients with recurrent epithelial ovarian cancer confined to the peritoneal cavity were enrolled on protocol through the Harrington Cancer

Center (Amarillo, TX) after obtaining informed consent. All patients, ranging from 47 to 70 years of age, were previously treated by standard surgery and chemotherapy with cis- or carboplatin- and paclitaxel-containing regiments. Following standard treatments, CT scans and pathology of biopsied samples prior to immunotherapy confirmed recurrent disease. None of these patients had received other cancer therapies within 4–6 weeks of protocol entry. All patients received chemotherapy with either cisplatin or gemcitabine following completion of immunotherapy. Four patients completed the proposed three cycles of adoptive T cell immunotherapy, whereas three patients developed local occlusion or rupture of the intraperitoneal port that resulted in discontinued treatment. Characteristics and clinical course summaries for patients completing three cycles of adoptive T cell immunotherapy are described in Table 1. All studies were carried out with approval of the institutional review board of the Texas Tech University School of Medicine and under IND from the Food and Drug Administration (FDA).

MUC1 mucin peptide

The 20mer MUC1 peptide GSTAPPAHGVTSAPATAPAP was synthesized by American Peptide Inc. (Sunnyvale, CA). The orientation is a single repeat of the mucin 1 peptide and shown to be optimal for the stimulation of human mononuclear cells from patients with adenocarcinoma [33–35].

Generation of MUC1 peptide-stimulated effector T cells

Generation of MUC1 peptide-stimulated effector T cells has been previously described [32, 34]. Briefly, peripheral blood mononuclear cells (PBMC) from eligible ovarian cancer patients were obtained via leukaphereses. Cells were adjusted to 2×10^6 cells/ml in serum-free AIM-V (Registered TM) lymphocyte medium (Life Technologies GIBCO-BRL, Grand Island, NY) and maintained in a 37°C humidified 5% CO_2 atmosphere. Cells within culture bags were stimulated with MUC1 peptide (1 μg/ml) on days 0 and 7. Human IL-2 (Cetus, Nutley, NJ) was added twice per week at 100 IU/ml for cell expansion. Twice weekly, cells were counted, diluted to 2×10^6 cells per ml with more media to maintain lymphocyte proliferation. On day seven, 2 ml of supernatant were collected, centrifuged at $400 \times g$ for 10 min, and sent for sterility testing. After 8 days, MUC1-stimulated T cells were harvested from culture bags and prepared for patient treatment. Cells were washed twice in normal saline and resuspended in 5% albumin/normal saline solution. Cells and supernatants from cultures prior to (Day 0) or following (days 3 and 8) restimulation with peptide and IL-2 were cryopreserved for future functional and phenotypic analysis. Cryopreserved cell preparations showed a 93–97% viability range after thawing.

Adoptive T cell immunotherapy and treatment scheme

Adoptive immunotherapy with autologous MUC1 peptide-stimulated T cells was performed on patients with residual recurrent ovarian cancer following standard surgery and chemotherapeutic protocols. Eligible patients underwent leukaphereses for each treatment cycle. Collected PBMC were then expanded ex vivo with MUC1 peptide and IL-2. The effector T cells were administered regionally via an intraperitoneal port-a-catheter and repeated monthly for a total of three cycles of T cell transfer. The number of T cells ranged from 10^8 to 10^9 cells per infusion (i.e., $1–4 \times 10^8$ cells/m^2). Patients were evaluated by magnetic resonance imaging (MRI) or computed tomography (CT)

Table 1 Characteristics and clinical course summary of patients completing 3 cycles of therapy

Patient no.	Patient age (years)	Pre-study therapy	Pre-study histology	Pre-study tumor nodule number (size)[a]	Disease status	Survival (months)
OV1	70	Resection chemotherapy	Poorly differentiated/epithelial papillary serous adenocarcinoma	Miliary-type nodules in multiple areas of abdomen and ascites	Recurrent	3
OV2	61	Resection chemotherapy partial resection	Moderately differentiated/epithelial not otherwise specified adenocarcinoma	Right pelvis (1–2 cm) midline pelvic (3–4 cm)	Recurrent	>84[b]
OV3	61	Resection Chemotherapy resection	Poorly differentiated/epithelial papillary serous adenocarcinoma	2–3 Nodules upper abdomen (<1 cm)	Recurrent	5
OV7	59	Resection chemotherapy	Poorly differentiated/epithelial papillary serous adenocarcinoma	Multiple nodules in multiple areas of abdomen (5–6 cm range)	Recurrent	16

[a] Determined by CT scan just prior to immunotherapy. [b] *DFS* disease-free survival

before and after the completion of therapy. Disease responses were determined by the comparison of pre-treatment and post-treatment images. In addition, individual serum CA-125 levels were determined by ELISA at various time points over the next 200 days following treatment initiation and compared with pre-treatment CA125 levels.

Flow cytometric analysis

Single cell suspensions of peptide-stimulated PBMC were washed three times in a fluorescent antibody buffer (FAB) consisting of 1% human serum albumin and 0.02% sodium azide in 0.01 M phosphate-buffered saline, pH 7.2. Immune cell populations were phenotyped by their expression of surface markers using direct immunofluorescence staining techniques [32, 36]. Lymphocytes (10^6), pre-treated with polyclonal human IgG (Sigma Inc) to block FcR, were mixed with 100 µl of FAB containing 1 µg of various mAbs conjugated to either PE, FITC, PE-CY5, or APC. The mAbs used include anti-CD4 (eBioscience, San Diego, CA. Clone HIS51), anti-CD8 (eBioscience. Clone 53-2.1), anti-CD45RO or anti-CD45RA (Pharmingen), anti-CD3 (eBioscience), or anti-CD25 (eBioscience, San Diego, CA). Stained cell preparations were incubated for 20 min on ice then washed three times in FAB and analyzed by multiparameter flow cytometry using a Becton–Dickinson FACscalibur (San Jose, CA). One hundred thousand cells were analyzed per sample with dead cells excluded on the basis of forward light scatter. Surface marker analysis was performed using Cell Quest Software (Becton–Dickinson), and the percent positive and absolute cell numbers were determined. For intracellular staining, cells were labeled with antibodies to specified cell surface markers as described above. Following incubation, brefeldin A (10 µg/ml) was added to cultures to retain cytoplasmic markers. Subsequently, cells were fixed with 2% paraformaldehyde followed by intracellular staining in permeabilization buffer containing 0.5% saponin and 1% BSA in PBS, and either human anti-FoxP3, anti-IL-10, or anti-IFN-γ mAbs (BD Pharmingen). Cells were washed and resuspended in 1% BSA/PBS solution and analyzed by flow cytometry as described above.

Comparative analysis of human gene expression levels by RT–PCR

Human inflammatory or common cytokine mRNA expression levels were quantitated using Pathway Specific Array Systems purchased from SuperArray Bioscience Corp. (Frederick, MD). Total RNA from PBMCs obtained either prior to (Day 0) or following peptide stimulation for 3 or 8 days was extracted by tissue homogenation in TRIzol reagent (GIBCO). Experimental RNA samples were converted into first-strand cDNA templates using the RT First Strand Kit (Superarray Corp.). Templates were then mixed with instrument-specific RT qPCR Master Mixes and dispensed into wells containing pre-dispensed gene-specific primer sets. Relative gene expression levels and threshold cycle values (Ct) were determined with the Bio-Rad iCycler (BioRad Labs, Hercules, CA). Calculations were performed using the 2^-ΔCt method of analysis according to manufacturer's instructions. Data are expressed as either Average Raw Ct values (where Ct values of 35 or greater are equal to 0), Average ΔCt values (Average Ct (gene of interest)−Average Ct (housekeeping genes)), or as fold changes (test sample/control sample) in gene expression [32, 36].

Data presentation and statistical analysis

In select studies, the absolute cell numbers were calculated. Lymphocyte numbers were obtained from freshly isolated and cultured PBMCs by coulter counter. Depending on the cell subpopulation of interest and their level of sequential gating following flow cytometric analysis, absolute cell numbers were derived by multiplying the cell percentages at each layer of gating × the coulter counter-derived lymphocyte cell number per ml of PBL. For statistical analysis, the Student's *t* test was used for paired comparisons and provided by the PRISM Graph Pad statistical software package. ANOVA analysis was used for the comparison of three or more matched groups. Statistical significance was defined as a *P* value less than 0.05 for all analysis.

Results

Phenotypic characterization of adoptively transferred MUC1 peptide-stimulated effector T cells

Patients underwent leukaphereses at various time intervals prior to and following adoptive T cell transfer for collection of PBMCs. Cells from such patients were stimulated with MUC1 peptide and IL-2 for 8 days as described in "Materials and methods". Following restimulation, generated effector T cells were harvested, characterized, and evaluated for MUC1 Ag reactivity in vitro. Previously, we have shown that such freshly generated human effector cells were predominantly CD4 T cells, demonstrated MUC1 cytolytic potential and produced significantly greater amounts of supernatant-derived IFN-γ when compared to that of pre-stimulation levels. Moreover, there were no significant differences in either the CD4/CD8 expansion rates or functional potentials among corresponding group cultures and/or treatment cycles [32].

In the current study, we extended our observations to directly assess CD4 T cell activation and cytokine production at the single cell level within these cultures. Using multiparameter flow cytometry, freshly generated effector T cell populations were predominantly $CD3^{+}CD4^{+}$ (>87%), whereas $CD3^{+}CD8^{+}$ T cells were routinely lower (<10%). Moreover, such CD4 cells co-expressed up-regulated levels of CD25 and CD45RO (Fig. 1a, b). As shown in Fig. 1c, $CD4^{+}CD25^{+}$ $CD45RO^{+}$ donor effector cells, among patients undergoing 3 treatment cycles of PBMC restimulation and re-infusion, showed no significant ($P > 0.05$; ANOVA) differences in the frequencies of such cells at each treatment cycle among either individual patients or the four patients utilizing this 8-day restimulation strategy. Since human Th1 cells have been shown to produce both IFN-γ and IL-10 [5, 8, 9], intracellular cytokine staining showed that CD4 effector T cells expressed substantial levels of IFN-γ with lower levels of IL-10 (Fig. 1b). As shown in Fig. 1d, individual patients showed no significant ($P > 0.05$) differences in the mean frequency of $CD4^{+}CD25^{+}CD45RO^{+}$ cells producing IFN-γ for all three cycles with all patients producing similarly elevated levels ($P > 0.05$; ANOVA). In contrast, patients OV1 and OV3 showed substantial ($P < 0.05$) decreases in IL-10 production among corresponding cells when compared to that of patients OV2 and OV7 (Fig. 1e). Furthermore, the mean IL-10/IFN-γ cell frequency ratios among the former were significantly ($P < 0.05$) lower when compared to the latter (Fig. 1f). Collectively, this suggested that restimulation and expansion of systemic ovarian cancer patient effector T cells with MUC1 peptide and IL-2 can effectively generate functionally differentiated $CD3^{+}CD4^{+}CD45RO^{+}$ Th1 cells that not only produced IFN-γ, but also substantially different levels of IL-10 ex vivo.

Clinical evaluation and therapeutic efficacy among patients receiving three cycles of MUC1-stimulated CD4 effector T cell transfer

Patients underwent leukaphereses at various time intervals prior to and following adoptive T cell transfer for collection of PBMCs. Following restimulation and expansion with MUC1 peptide and IL-2, freshly generated autologous effector T cells were harvested and administered via an intraperitoneal port-a-catheter as described in Materials and Methods. Treatment was conducted at monthly intervals for up to three cycles of T cell transfer. Four patients completed the proposed three cycles of adoptive T cell immunotherapy, whereas three patients developed local occlusion or rupture of the intraperitoneal port that resulted in discontinued treatment (<3 treatment cycles) and death due to disease progression. Characteristics and clinical course summaries for patients completing three cycles of adoptive T cell immunotherapy are described in Table 1. All subjects had confirmed recurrent ovarian cancer following resection and chemotherapy consisting of cis- or carboplatin and paclitaxel-containing regiments. Initially, patient OV1 had a complete response to treatment; however, approximately 6 months later, the patient had disease recurrence. Subsequently, she received topotecan, oral etoposide, and intravenous liposomal doxorubicin with no response. Pre-study CA-125 levels for all patients varied from normal (<35 units/ml normal range) for patient OV2 to over a thousand units/ml (32). CT scans and pathology of biopsied samples prior to immunotherapy confirmed recurrent disease (Table 1). None of the patients received other cancer therapies within 4–6 weeks of protocol entry. There was no toxicity except for grade 1 abdominal pain in patient OV2 at the time of infusion. All patients received chemotherapy with either cisplatin or gemcitabine following completion of immunotherapy. Of the four patients completing 3 cycles of adoptive T cell transfer, one patient remains disease-free (OV2), another patient survived for nearly 16 months with recurrent disease and death (OV7). In contrast, short-term surviving patients treated with CD4 effector cells possessing lower IL-10/IFN-γ cell ratios (i.e., OV1 and OV3) showed progressive disease and expired within 3–5 months following T cell therapy.

Treatment with autologous MUC1-stimulated CD4 effector cell transfer differentially enhanced endogenous $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ T cell subpopulation levels among long-term surviving patients

Since co-expression of CD25 on $CD4^{+}$ T cells has been previously linked to specific cell subpopulations associated with either immune cell activation or immunoregulation [24], we next assessed and characterized such CD4 T cell populations in peripheral blood of ovarian cancer patients receiving T cell transfer. As shown in Fig. 2a, long-term surviving patients (OV2 and OV7) showed elevated levels of systemic $CD4^{+}CD25^{+}$ T cell numbers when compared to that of corresponding T cell populations in short-term survivors (OV1 and OV3). However, in long-term surviving patient OV2, such CD4 T cell populations were substantially elevated when compared to that of corresponding pre-treatment levels and that of patient OV7 at similar treatment times. In contrast, the latter showed elevated levels of $CD4^{+}CD25^{-}$ when compared to that of the former (Fig. 2b). Patients OV1 and OV3 showed no differences in either T cell subpopulation when compared to either respective pre-treatment levels or each other (Fig. 2a, b).

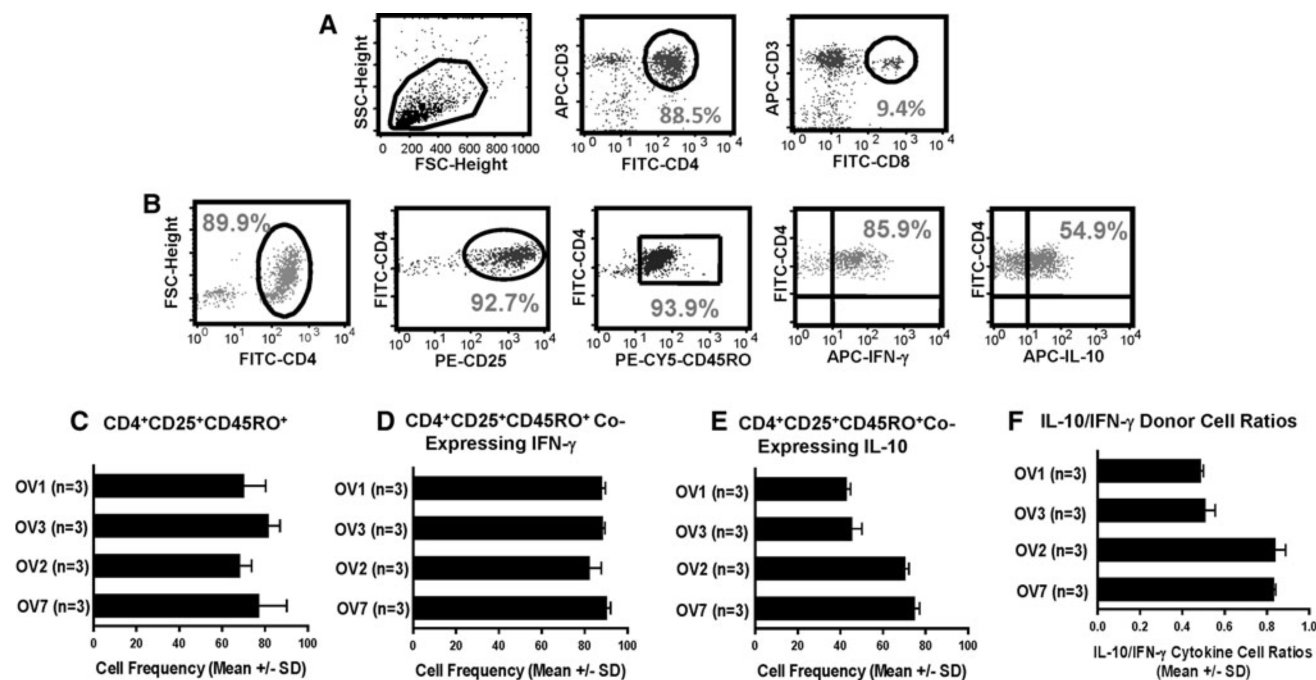

Fig. 1 Adoptively transferred MUC1 peptide-stimulated CD4 effector T cells produce IFN-γ and differential levels of IL-10. Human MUC1 peptide-stimulated effector T cells were generated as described in "Materials and methods". **a** Eight-day cultured cells were harvested and labeled with FITC-anti-CD4, anti-CD8, and APC-CD3 mAbs. Lymphocytes were distinguished by their forward light scatter/side scatter profiles and gates set on CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ cells and assessed by multiparameter flow cytometry. In **b**, cells were labeled with FITC-anti-CD4, PE-anti-CD25, PE-CY5-CD45RO, and either APC-anti-IFN-γ or IL-10 mAbs. Gates were set on CD4$^+$CD25$^+$CD45RO$^+$ cells, and T cells co-expressing either IFN-γ or IL-10 were assessed using multiparameter flow cytometry. Data shown are from a representative experiment showing the percentages of cells expressing specified cell markers and CD4$^+$CD25$^+$CD45RO$^+$ T cells co-expressing IFN-γ or IL-10. In **c**, mean donor cell frequencies of infused CD4$^+$CD25$^+$CD45RO$^+$ effector T cells for each patient undergoing 3 treatment cycles of PBMC restimulation and re-infusion. In **d** and **e**, mean donor cell frequencies of CD4$^+$CD25$^+$CD45RO$^+$ T cells co-expressing either IFN-γ or IL-10, respectively. Data are expressed as the mean cell frequency $\pm$ SD of 3 independent cell generation and infusion procedures for each patient. **e** Comparative analysis assessing IL-10/IFN-γ donor CD4 effector cell ratios. Data are expressed as the mean IL-10/IFN-γ donor cell ratios $\pm$ SD of 3 independent cell generation and infusion procedures for each patient

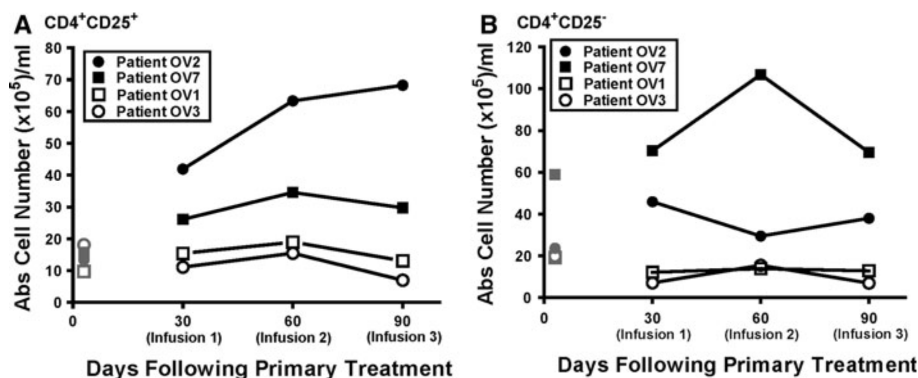

Fig. 2 Autologous MUC1-stimulated CD4 effector cell therapy enhances systemic CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cell levels among treated long-term surviving patients. PBMC from patients treated with 3 cycles of adoptive T cell transfer therapy were obtained prior to therapy and at monthly intervals following each treatment cycle. Cells were labeled with FITC-anti-CD4 and PE-anti-CD25 mAbs. Total lymphocyte populations were distinguished by their forward light scatter/side scatter profiles and gates set on CD4$^+$/CD25$^+$ or CD4$^+$/CD25$^-$ cells, and the percentages of these populations were assessed by multiparameter flow cytometry. Data shown are the absolute cell numbers of systemic CD4$^+$/CD25$^+$ (**a**) and CD4$^+$/CD25$^-$ (**b**) and were calculated as the percentages of positive staining cells $\times$ the total number of lymphocytes per ml of PBL for each patient. Individual points represent pre-treatment cell population levels for each patient tested

Autologous MUC1 peptide-stimulated CD4 effector cell transfer enhances systemic "memory/effector" $CD4^+CD25^+FoxP3^-CD45RO^+$ T cell levels among long-term surviving patients

Memory $CD4^+CD25^+$ T cell responses were assessed using flow cytometry and the following gating strategy (Fig. 3a). As shown in Fig. 3, systemic $CD4^+CD25^+FoxP3^-$ T cell population numbers co-expressing the memory marker CD45RO were markedly higher among patients OV2 and OV7 when compared to that of patients OV1 and OV3. Moreover, long-term surviving patient OV2 (disease-free) had greater cell numbers and frequencies of such cell populations when compared to that of long-term surviving patient OV7 manifesting disease (Fig. 3b, c). This suggested that treatment with autologous MUC1-stimulated Th1 cells differentially increased both the cell number and frequency of systemic $CD4^+CD25^+FoxP3^-$ T cell subpopulations co-expressing CD45RO (memory/Ag-experienced) among long-term surviving patients when compared to that of corresponding short-term survivors.

Multiple treatment cycles with autologous CD4 effector cell therapy "shift" endogenous "Inducible" TR1 and "Natural" $FoxP3^+$ TReg cell subpopulation levels and proportions among long-term surviving patients

Since it has been reported that Inducible Tr1 and Natural $FoxP3^+$ TReg subpopulations are functionally distinct T cell subpopulations that may have a significant impact on effector/memory T cell responses and disease progression/regression within cancer patients [18, 20, 24–28], we next assessed their systemic levels among patients receiving multiple cycles of Th1 cell transfer. Using multiparameter flow cytometry, we enumerated at the single cell level the presence of Tr1 ($CD4^+CD25^-FoxP3^-IL\text{-}10^+$) and Natural Effector ($CD4^+CD25^+CD45RO^+FoxP3^+$) TRegs at monthly intervals following each treatment cycle. As shown in Fig. 4a–d, treated long-term surviving patients (OV2 and OV7) showed elevated levels of both systemic Inducible TR1 and Natural $FoxP3^+$ TReg cell subpopulations when compared to that of corresponding short-term surviving patients (OV1 and OV3). Both patients OV7 and OV2 showed elevated levels of TR1 cells when compared to that of corresponding pre-treatment levels with the former having greater cell numbers and frequencies (Figs. 4c, 5). However, in contrast to patient OV7, patient OV2 showed a marked and progressive decrease in both the cell number and frequency of Natural TRegs co-expressing FoxP3 (Figs. 4a, 6). Consequently, this resulted in both progressively lower and higher levels of systemic Inducible TR1/Natural $FoxP3^+$ cell ratios with different rates of occurrence that correlated with prolonged and disease-free survival, respectively (Fig. 4e). Corresponding cell ratio levels for short-term survivors remained nearly uniform with negligible differences at each of the time points tested following treatment

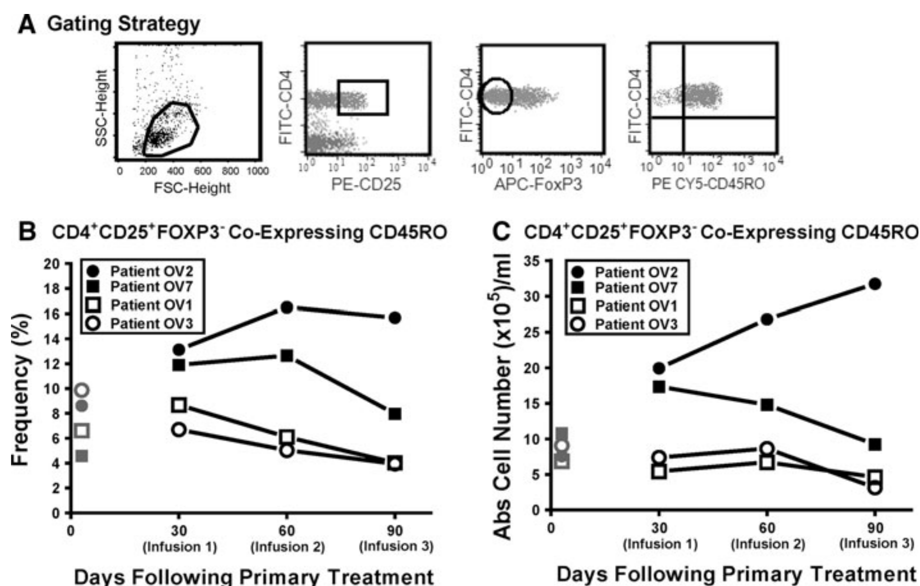

Fig. 3 Treatment with autologous MUC1-stimulated CD4 effector cell therapy enhances systemic memory/effector T cell subpopulation levels in long-term surviving patients. PBMCs from patients were obtained as described in Fig. 2. Cells were labeled with anti-CD4, anti-CD25, anti-FoxP3, and anti-CD45RO mAbs and assessed by multiparameter flow cytometry. The gating strategy for these experiments are shown in **a**. Gates were set on $CD4^+CD25^+FoxP3^-$ cells co-expressing CD45RO and the frequency of cells co-expressing CD45RO for each patient were determined (**b**). In **c**, absolute cell numbers were calculated as described in the "Materials and methods". Individual points represent pre-treatment cell population levels for each patient tested

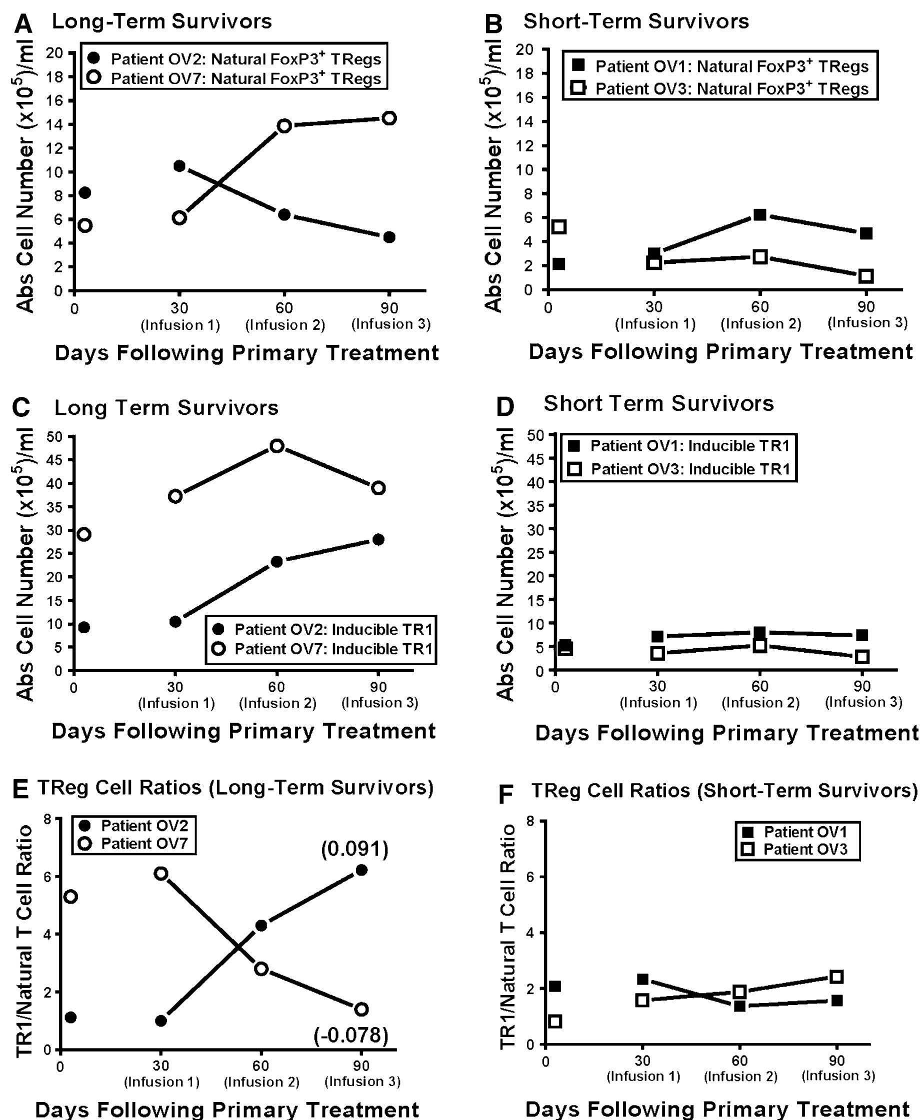

Fig. 4 Autologous MUC1-stimulated CD4 effector cell therapy modulates systemic "Inducible" TR1 and "Natural" $FoxP3^+$ TReg cell subpopulation levels and proportions in long-term surviving patients. PBMCs from patients treated with 3 cycles of T cell transfer were obtained prior to therapy and at monthly intervals following each treatment cycle. Cells were restimulated with MUC1 peptide and IL-2 for 72 h, harvested, and labeled with anti-CD4, anti-CD25, anti-FoxP3, and either anti-IL-10 or CD45RO mAbs as described in "Materials and methods". Gates were set on either "Natural Ag-experienced" TReg $CD4^+CD25^+CD45RO^+FoxP3^+$ T cell subpopulations (**a**, **b**) or "Inducible" TR1 $CD4^+CD25^-FoxP3^-IL\text{-}10^+$ cells (**c**, **d**) and assessed among either long-term or short-term surviving patients by multiparameter flow cytometry. Absolute cell numbers were calculated as described in the "Materials and methods". Individual points represent pre-treatment cell population levels for each patient tested. Systemic Inducible TR1/Natural Ag-experienced T cell ratios were assessed among long-term (**e**) and short-term (**f**) surviving patients following therapy. *Numbers in parenthesis* are the rates of cell subpopulation occurrence over the treatment period and were determined and quantified by the slope of the line for each patient and suggests changes in cell population ratios per unit time

(Fig. 4f). Collectively, such a "shift" and/or difference in the cell numbers, occurrence rates, and proportions of either Inducible TR1 or Natural TReg subpopulations may promote and influence, in part, more effective antitumor responses among patients following autologous T cell therapy.

Treatment with MUC1-stimulated CD4 effector cell transfer enhances systemic IFN-γ-secreting CD4 T cells among long-term surviving patients

$CD4^+CD25^+FoxP3^-$ T cells were next assessed for IFN-γ production in peripheral blood of patients receiving

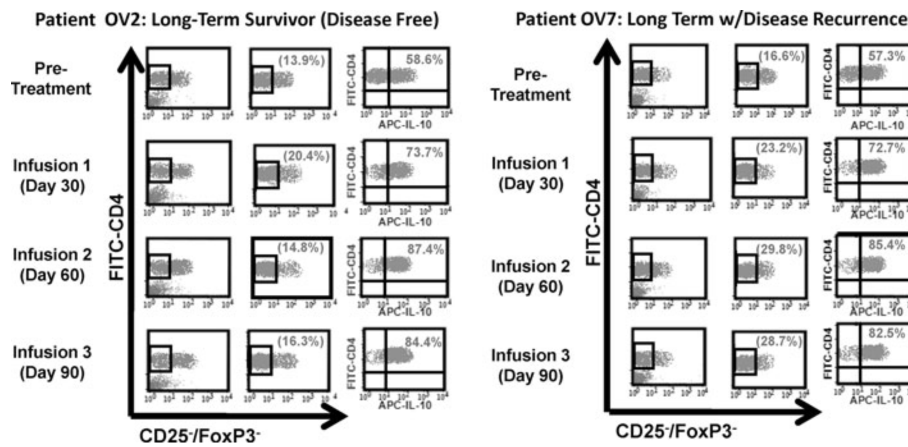

Fig. 5 MUC1 peptide-stimulated CD4 effector cell transfer influences the levels of systemic "Inducible" Regulatory T cell subpopulations co-expressing IL-10 in long-term surviving patients. PBMCs from long-term surviving patients were obtained and restimulated as described in Fig. 4. Gates were set on CD4$^+$CD25$^-$FoxP3$^-$ populations and cells co-expressing IL-10 were assessed by multiparameter flow cytometry for patients OV2 (*left panel*) and OV7 (*right panel*). *Numbers* represent the percentages of CD4$^+$CD25$^-$FoxP3$^-$ T cells co-expressing IL-10. *Numbers in parenthesis* represent the frequency of FoxP3$^-$ cells among CD4$^+$CD25$^-$populations for each patient at specified time points

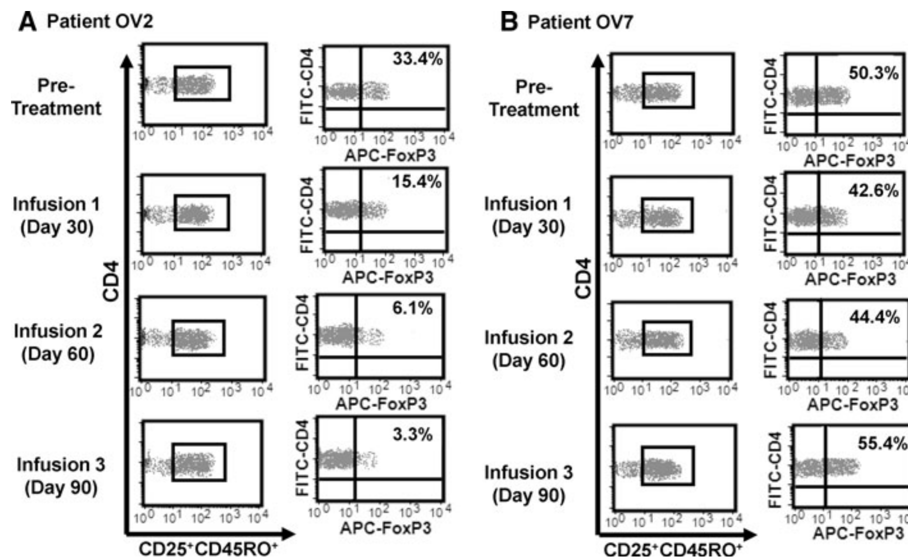

Fig. 6 Autologous MUC1 peptide-stimulated CD4 effector cell therapy modulates systemic levels of "Ag-experienced" Natural TReg cell subpopulations co-expressing FoxP3 in treated long-term surviving patients. PBMCs from long-term surviving patients were obtained and restimulated as described in Fig. 4. Cells from patients OV2 (**a**) and OV7 (**b**) were harvested and labeled with anti-CD4, anti-CD25, anti-CD45RO, and anti-FoxP3 mAbs. Gates were set on CD4$^+$CD25$^+$CD45RO$^+$, and cells co-expressing intracellular FoxP3 were assessed by multiparameter flow cytometry. *Numbers* represent the percentages of CD4$^+$CD25$^+$CD45RO$^+$ T cells co-expressing FoxP3

multiple cycles of CD4 effector cell transfer by intracellular cytokine staining and flow cytometry. As shown in Fig. 7, long-term surviving patients showed elevated cell numbers and frequencies of such cells when compared to that of short-term survivors. Moreover, disease-free patient OV2 had greater cell numbers and frequencies of such cells when compared to that of patient OV7 experiencing prolonged survival and recurrent disease (Fig. 7a, b).

Therapeutic efficiency of CD4 effector cell transfer correlates with the up-regulation of distinct TNF family gene expression levels among endogenous MUC1-stimulated CD4 T cells

Since effective CD4-mediated antitumor responses have been associated with cell expression of various TNF superfamily ligand/receptor complexes, we next assessed

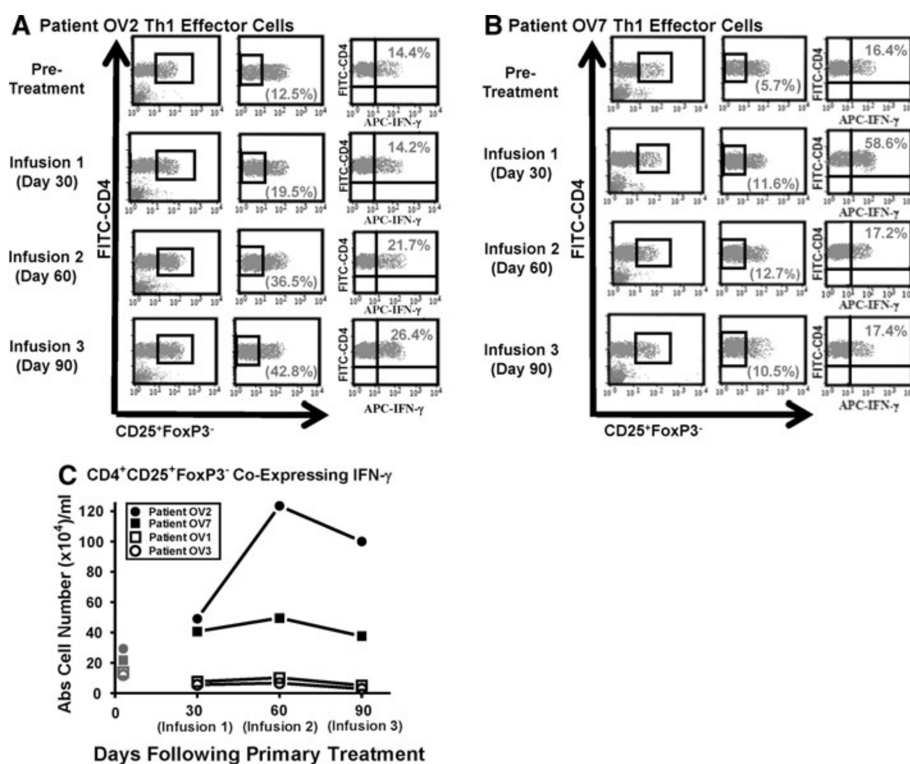

Fig. 7 Treatment with autologous MUC1-stimulated CD4 effector cell transfer enhances systemic IFN-γ-secreting CD4 T cells among long-term surviving patients. PBMCs from long-term surviving patients were obtained and restimulated as described in Fig. 4. Cells were labeled with anti-CD4, anti-CD25, anti-FoxP3, and anti-IFN-γ mAbs. Gates were set on $CD4^{+}CD25^{+}FoxP3^{-}$ populations, and cells co-expressing IFN-γ were assessed by multiparameter flow cytometry among long-term surviving patients OV2 (**a**) and OV7 (**b**). *Numbers* represent the percentages of $CD4^{+}CD25^{+}FoxP3^{-}$ T cells co-expressing IFN-γ. *Numbers in parenthesis* represent the frequency of $FoxP3^{-}$ cells among $CD4^{+}CD25^{+}$ populations for each patient at specified time points. In **c**, absolute cell numbers were calculated as described in the "Materials and methods". Individual points represent pre-treatment cell population levels

gene expression levels of select TNF-related genes associated with either CD4-mediated tumor cell killing, persistence/survival, and co-stimulation [37]. Peripheral blood mononuclear cells were obtained from patients prior to (Day 0) and 1 month following final treatment (Day 120 post-treatment) and restimulated with MUC 1 peptide and IL-2 for 72 h. Using SuperArray RT-PCR (Frederick, MD), we show that all treated patients had elevated gene expression levels of the TNF-related gene associated with direct tumor cell killing, LT-α (Table 2). However, only patient OV2 (disease-free survival) had comparatively elevated levels of FasL, TRAIL, and TNF when compared to that of other patients. In contrast, corresponding long-term surviving patient OV7 (with recurrent disease) had elevated survival/co-stimulatory-related gene expression levels of OX-40L and CD27-L, whereas patient OV2 selectively showed elevated levels of LIGHT. Short-term survivors (patients OV 1 and OV3) showed negligible levels of corresponding genes associated with prolonged survival and/or co-stimulation (Table 2). This suggested that peptide-restimulated $CD4^{+}$ T cells from long-term survivors are equipped for enhanced survival/persistence and can potentially facilitate direct CD4-mediated killing of tumor cells in vivo following treatment completion.

Discussion

Adoptive T cell therapy is a viable treatment for cancer patients and optimally requires participation of CD4 T cells. In this study, which marks one of a few clinical studies investigating treatment by local intraperitoneal T cell transfer, we assessed the therapeutic effects of adoptively transferred IFN-γ- and IL-10-producing CD4 effector cells in patients with recurrent ovarian cancer. We show that three monthly treatment cycles of autologous T cell restimulation and local–regional intraperitoneal re-infusion modulated both systemic memory and IFN-γ-producing CD4 effector T cell subpopulation levels that were associated with enhanced patient survival. Moreover, these patients also contained differentially elevated levels of Inducible Tr1 and Natural TReg cell subset numbers with progressively decreased and increased Inducible/Natural TReg cell ratios that were further associated with

Table 2 TNF super family gene expression levels associated with enhanced T cell survival and killing potentials among ovarian cancer patients following treatment with three cycles of adoptive T cell transfer

Function	Gene	Treatment time	Patient OV2	Patient OV7	Patient OV1	Patient OV3
Anti-apoptosis (survival)	OX-40-L	Pre-treatment	1.48	1.42	1.46	1.70
		Post-treatment	0.41	10.70	1.62	1.31
	CD30-L	Pre-treatment	0.14	0.20	0.12	0.15
		Post-treatment	0.63	0.37	0.10	0.17
	CD27-L	Pre-treatment	2.84	0.88	0.93	0.40
		Post-treatment	1.25	13.74	1.28	1.21
	LIGHT	Pre-treatment	0.27	0.62	0.32	0.95
		Post-treatment	20.88	0.75	0.23	0.14
	CD40-L	Pre-treatment	0.01	1.30	1.06	0.70
		Post-treatment	2.01	1.11	1.33	1.01
Pro-apoptosis (killing)	Fas-L	Pre-Treatment	0.98	1.08	0.39	0.73
		Post-treatment	3.77	1.16	1.46	2.08
	TRAIL	Pre-treatment	0.26	0.63	1.39	0.54
		Post-treatment	3.28	0.38	0.30	0.54
	TNF	Pre-treatment	1.96	0.86	0.74	0.80
		Post-treatment	3.49	1.53	1.04	1.67
	LT-a	Pre-treatment	6.81	5.39	6.66	5.73
		Post-treatment	3.24	10.56	7.07	5.93

PBMC were obtained from patients prior to (Day 0) and 1 month following final treatment (Day 120 post-treatment) and stimulated with MUC1 peptide and IL-2 for 72 h. Cells were harvested, and total RNA was isolated and first-strand cDNA were prepared as described in "Materials and methods". Template cDNA was characterized in triplicate using the human Common Cytokine or Inflammatory Cytokine PCR Arrays with the RT SYBR Green/fluorescein PCR master mix on the Bio-Rad iCycler. The raw Ct (cycle threshold) values were calculated by the instrument and converted into the Avg ACt and/or Relative Gene Expression Level (ΔCt = Ct (gene of interest)—Avg Ct (house keeping genes) by the AACt method. Comparison of fold changes in gene expression between either peptide-stimulated pre (Day 0)- and post (Day 120)-treated patient PBMC to that of pre-treated non-stimulated patient PBMC (Day 0) were calculated using the Ct method in the PCR Array Data Analysis template. Shaded regions indicate comparative differences in gene fold-expression levels (i.e., greater than 3-fold up-regulation) among corresponding genes in both long- and short-term surviving patients

prolonged and disease-free survival, respectively. Lastly, we observed that adoptively transferred Th1-like cells derived from long-term survivors consistently produced significantly elevated levels of IL-10 when compared to that of corresponding cell preparations from short-term survivors. Such disproportionate production levels in antigen-experienced CD4 T cell-derived IL-10 and IFN-γ were associated with enhanced patient antitumor immune responses and clinical responses and may suggest a potentially important determinant in the balance between effective and ineffective immunotherapeutics.

The immunoenhancing and therapeutic roles of IFN-γ in facilitating and enhancing the immune response in cancer patients has been well documented. Generally, while playing a dominant role as a feedback regulator to inhibit active immune responses and establish and/or maintain immune response homeostasis [5–11, 38–41], differentially localized levels of IL-10 may also enhance select immune functions [6, 38, 39, 41]. Several clinical studies on patients with various autoimmune disorders have shown that administration of IL-10 appeared to be immunosuppressive, whereas elevated doses induced an increase in patient IFN-γ production and a marked elevation in their immune stimulatory capacities [38, 42–47]. Similarly, in tumor therapy studies, it has been shown that IL-10 has stimulatory effects on both the innate and adaptive immune responses that also include the cytotoxic and proliferative capacity of Ag-specific CD8 and NK effector T cells [38, 46–51]. This suggested that the clinical experience with human IL-10 can be either anti-inflammatory or immunostimulatory and may further appear to be dependent on cytokine dose. In certain viral infections, where T cells undergo oligoclonal expansion of effector T cells that may result in excessive and/or functionally reduced differences in effective memory T cell generation and stability, IL-10 has been shown to be a potent inhibitor of such "memory T cell inflation" [52]. This limit on memory T cell accumulation at various stages of the immune response may be actually efficacious to clinical outcomes among cancer patients by enhancing the quality and quantity of memory/effector T cell populations at sites of tumor progression or dormancy [53, 54]. Conceivably, IL-10 derived from elevated levels of Th1 cells following T cell transfer may have immunoenhancing and/or immunostimulatory effects that augment

antitumor immunity and promote patient survival. Furthermore, we speculate that lower levels of T cell-derived IL-10, as seen in short-term surviving patients, may drive immunosuppressive functions while potentially higher and sustained levels as detected in long-term survivors may facilitate and/or generate IFN-γ-induced immune activation events that elicit clinically relevant results.

It has become clear that TR1 cells are generated in an Ag-specific fashion and can therefore be defined as "inducible" TRegs, whereas FoxP3^{+} TRegs are primarily "natural" occurring cells that are selected in the thymus, are specific for self Ags, and probably represent a separate T cell lineage [18, 20, 23–25, 27, 28]. Moreover, the time at which these two distinct TReg subsets play a role in the modulation of the antitumor response may also be different. Natural FoxP3^{+} TRegs are recruited and activated early during an immune response to control its magnitude, whereas adaptive TR1 cells, which are induced upon repeated Ag stimulation, may act later to dampen the immune response and to restore and maintain tolerance or homeostasis. Consequently, the relative levels and ratios of TR1 verses FoxP3^{+} Natural TReg cell subpopulations may be an important determinant in the balance between effective and ineffective antitumor responses. In our study, we observed that TR1/Natural TReg cell ratios over the three treatment periods were inversed among the prolonged survivor (patient OV7) and disease-free (patient OV2) patient. In the former, such cell ratios declined with treatment time whereas in the latter, such corresponding ratios progressively increased. This further correlated with our observations that patient OV2 (disease-free) had comparatively greater levels of both endogenous IFN-γ-producing CD4 effector and CD4 memory T cell populations when compared to patient OV7 (prolonged survival with recurrent disease). Collectively, this may suggest that treatment-induced modulation and/or elevation of select TReg cell subset proportions, with their distinct functional kinetics and phenotype, can influence endogenous Th1 and/or memory cell generation and maturation that may further contribute to tumor-free patient responses and enhanced cancer patient survival.

Aside from their diverse biological properties and mechanisms of actions, it has also been shown that TReg cell subsets also respond differently to various cytokines [18, 20, 23–25, 27, 28]. Recent studies have shown that IFN-γ can further modulate and facilitate Natural TReg cell subpopulation function and phenotype by induction, conversion, and maintenance of FoxP3 expression in such cells [55–58]. Moreover, it has been suggested that there is an inherent plasticity in the functional repertoire of T cells where under select conditions, memory T cell populations can convert into Natural FoxP3 TRegs via IFN-γ-dependent mechanisms, which can further modulate Ag-specific immune responses and the clinical course of disease progression [59, 60]. Alternatively, others have shown that IL-10 can amplify one regulatory T cell subpopulation while actively suppressing another [25, 39–42, 47]. For example, it has been shown that TR1 cells are induced under conditions of Ag stimulation via an IL-10-dependent process in vivo [7, 25]. Aside from its potential effects on select TReg cell subpopulations, it has been suggested that IL-10 possesses an immunosuppressive role against Th1-mediated immune responses that may aid in avoiding uncontrolled chronic inflammation and potentially deleterious immunopathology at sites of tumor growth and progression [5–9, 27, 41, 47]. It is thus conceivable that heightened or variable levels of either IL-10 or IFN-γ derived from adoptively transferred Th1 cells and/or ongoing T cell-induced antitumor responses may have opposing regulatory effects on different effector/memory or TReg cell subpopulations that subsequently affect the balance between effective and ineffective antitumor responses in either long-term verses short-term patient survival.

Effective patient responses to CD4-mediated therapies may be dependent, in part, on their capacity to directly mediate tumor cell killing via TNF superfamily-mediated mechanisms [3, 4, 10, 11, 37]. Our study showed that all treated patients had elevated gene expression levels of the TNF-related gene associated with direct tumor cell killing, LT-α. However, only patient OV2 (disease-free survival) had comparatively elevated levels of FasL, TRAIL, and TNF when compared to that of other patients. This suggested that following multiple treatment cycles, the disease-free surviving patient had an enhanced systemic capacity to mediate direct tumor cell lysis in vivo. Another observation with therapeutic relevance was the presence of select co-stimulatory TNF superfamily ligand/receptor genes associated with T cell co-stimulation, expansion, and persistence [37]. Again, differences in such gene expression levels were noted among MUC1 peptide-restimulated T cells from patients experiencing prolonged survival. Patient OV7, with recurrent disease, had elevated gene expression levels of OX-40L and CD27-L (CD70) ligands. Interestingly, both human CD4 and CD8 T cells can express both of these TNF ligands and their receptors, which suggests that T cell–T cell interactions in this patient may exist and potentially influence the development of effector T cell populations [18, 37, 61–63]. Alternatively, it has been shown that both OX-40 and CD27 receptors are also expressed on both mouse and human natural and induced TReg cells [37]. Thus, although such TNF family member receptors have been linked to prolonged TCR-induced T cell survival and persistence among conventional effector T cells, it has been suggested that OX-40L

interaction with OX-40-expressing TReg cell subsets can selectively destabilize and/or attenuate TReg-mediated suppression and subsequent enhancement of effector T cell function and their immune responses [18, 64, 65]. In contrast, disease-free patient OV2 showed elevated levels of LIGHT following MUC1 peptide T cell restimulation. LIGHT expression has been shown to be essential for memory T helper cell-mediated activation of DCs and further suggested to be involved in the maintenance or reactivation of secondary Th1 responses [37, 66, 67]. We speculate that the presence of such TNF superfamily ligand/receptor signaling among various T cell subsets could influence the development of memory/effector and TReg cell subpopulations within the cellular repertoires from responding verses non-responding cancer patients receiving T cell immunotherapy [68].

The role of regulatory T cells in cancer is disputed [69, 70]. In both ovarian and breast cancer patients, either systemic or local FoxP3 expression is associated with a poor prognosis and overall survival [30, 31, 71–73]. Curiel and colleagues reported that the presence of high numbers of $CD4^{+}FoxP3^{+}$ T cells in malignant ascites of ovarian carcinoma correlated with tumor staging and reduced survival [29]. In colorectal cancer, several investigators did not find any differences between patients with high or low TReg cell infiltration [74], whereas others have found an improved survival associated with a high density of local and systemic $FoxP3^{+}$ cells suggesting no major immunosuppressive role of such cells in colorectal cancer [75]. Moreover, it has been suggested that the presence and levels of various TReg cell subsets in cancer patients may be beneficial to survival [69, 76, 77]. In either instance, it has been suggested that induction of lymphopenia before adoptive T cell transfer improves homeostatic proliferation and persistence of transferred cells by eliminating, in part, $CD4^{+}CD25^{+}$ TRegs [4, 78, 79]. Our studies among ovarian cancer patients treated with multiple cycles of adoptively transferred autologous MUC1-specific Th1 cells, enhanced patient antitumor responses and survival that appeared to correlate with differential levels of distinct $CD4^{+}$ TReg subpopulations. We suggest that treatment efficacy and heightened memory/Th1 effector cell phenotype did not appear to be dependent, in part, on particular TReg cell numbers but upon ratios of Inducible and Natural TReg subpopulations. Moreover, our studies using intraperitoneal local–regional treatment offer insight into Th1-mediated mechanisms that enhance memory T cell responses in ovarian cancer patients and further suggest that TReg modulation and not depletion may heighten antitumor responses following CD4 T cell-based immunotherapy.

Lastly, we have shown that adoptive transfer with CD4 effector T cells, producing elevated levels of both IFN-γ and IL-10, modulated select T cell-mediated immune responses that were associated with enhanced patient survival. However, it is worth noting that all patients in this study received standard cancer treatments that included surgery and chemotherapy both prior to and following adoptive T cell transfer. The combination of immunotherapy and conventional cancer therapies has been shown to increase the therapeutic index within patients by reducing the tumor load and enhancing the possibility for developing effective adoptively transferred and/or endogenously generated effector cell responses to residual disease. The disease-free survivor, demonstrating long-term remission (patient OV2), showed relatively normal levels of serum CA125 at the initiation of immunotherapy that suggested a lower tumor burden when compared to that of other patients. This corroborates the findings by others that adoptive T cell immunotherapy works best when tumor burden is minimal and can be further enhanced, in part, by concurrently treating patients with conventional modalities. Thus, such a combinatorial approach may have contributed, in part, to a better T effector-to-tumor cell ratio under minimal disease burden and/or reduced tumor-associated immune suppression in this patient. Alternatively, in the subject with prolonged survival and recurrent disease (patient OV7), it may be speculated that immunotherapy initially controlled her microscopic and/or residual disease; however, ineffective responses to standard chemotherapy with time may have unleashed tumor growth that subsequently reduced her immune-mediated therapeutic potentials that resulted in progressive disease and death. In either instance, this suggests that the impact of standard treatment modalities should not be disregarded and experimental CD4-mediated immune therapies should probably be administered in conjunction with, rather than, in place of such conventional treatments.

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Conflict of interest None of the authors have any potential financial conflict of interest related to this manuscript.

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