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Autologous MUC1-Specific Th1 Effector Cell Immunotherapy Induce Differential Levels of Systemic TReg Cell Subpopulations That Result in Increased Ovarian Cancer Patient Survival

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Abstract

Adoptive T cell immunotherapy using autologous lymphocytes is a viable treatment for patients with cancer and requires participation of Ag-specific CD4 and CD8 T cells. Here, we assessed the immunotherapeutic effects of autologous MUC1 peptide-stimulated CD4⁺ effector cells following adoptive transfer in patients with ovarian cancer. Using MUC1 peptide and IL-2 for ex vivo CD4⁺/Th1 effector cell generation, we show that three monthly treatment cycles of peripheral blood T cell restimulation and intraperitoneal re-infusion selectively modulated endogenous T cell-mediated immune responses that correlated with diminished serum CA125 tumor marker levels and enhanced patient survival. One patient remains disease free, another patient survived long-term for nearly 16 months with recurrent disease and two patients expired within 3-5 months following final infusion. Although PBL from all patients showed elevated MUC1 cytolytic activity following therapy, such responses did not correlate with therapeutic efficacy. Long-term survivors showed elevated levels of systemic memory (CD45RO) and naïve (CD45RA) CD3/CD4/CD25⁺ T cells when compared to that of pre-treatment levels and similarly-treated short-term survivors. Such cells co-expressed different levels of Foxp3 and CTLA-4 that resulted in progressively lower systemic Foxp3/CTLA-4 memory T cell ratios that further correlated with disease-free survival. Lastly, these patients showed elevated levels of MUC1-specific T cells expressing the CCR5 and CCR1 chemokine receptors and the chemokine CCL4 associated with Th1 cell differentiation/memory. We suggest that effective

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immunotherapy with autologous MUC1-stimulated CD4⁺ effector cells induce differential levels of systemic “Ag-experienced” and “Ag-inexperienced” CD4/CD25⁺ TReg cell subpopulations that influence long-term tumor immunity in ovarian cancer patients.

Keywords

Tumor Immunity; Regulatory T Cells; Th1 Effector Cells; Chemokines; T Effector/Memory Cells; Adoptive T Cell Immunotherapy

Introduction

Adoptive T cell immunotherapy, which involves the ex vivo expansion and activation of select tumor Ag-specific T cells and their subsequent re-administration into cancer patients, has been shown to be effective for the treatment of patients with certain established malignancies [1-7]. Recent clinical studies have shown that selection, expansion and infusion of high-avidity tumor-reactive CD8⁺ T cell populations, derived from either peripheral blood or tumors of patients with late-stage disease, can induce either partial or complete tumor regression. These studies have shown that infused tumor peptide-specific CD8⁺ T cells can persist in cancer patients but may be ineffective and/or unresponsive to specific tumor antigen in vivo [8,9]. This may be due in part to alterations in T cell signal transduction [10], the presence of immunosuppressive cytokines and/or regulatory CD4⁺ T cells [8,9,11-12]. Alternatively, we and others have shown that endogenous CD4⁺ helper Th1 cells and/or their cytokines, such as IL-2 and IFN- γ , can augment tumor eradication by enhancing cell survival, persistence and therapeutic function of adoptively transferred tumor antigen-specific CD8 T cells [13-23]. In either instance, these observations provide a further impetus to characterize the role and effects of endogenous CD4⁺ effector T cell subpopulations and their antitumor responses within these patients that arise as a result of adoptive T cell immunotherapy.

Although the role of regulatory T cells (TRegs) in controlling self-reactivity in autoimmune disease has been described [24], there is evidence that TRegs have a significant impact on a patient's immune response to tumor progression and/or regression [25-27]. TReg cells can be classified into two main subsets according to their origin and suppressive activity [28-30]. “Natural” CD4⁺ TReg cells (nTRegs), co-expressing the activation marker CD25, originate in the thymus by high affinity interaction of the T cell receptor with Ag expressed on the thymic stroma. Such cells suppress the proliferation of effector T cells in a contact-dependent, cytokine-independent manner and constitutively express Foxp3 and/or CTLA-4 (CD152) surface Ag. In contrast, “induced” CD4/CD25⁺ TReg subpopulations (iTReg) exert suppression mostly through soluble factors (IL-10 and TGF- β) and their suppressive function is not strictly associated with high level Foxp3 or CTLA-4 expression. In humans, CD4/CD25⁺ TReg cells have been identified at increased frequencies in the peripheral blood and malignant effusions of patients with various types of cancers [26,27]. Although evidence that such TRegs may alter the clinical course of cancer progression has been described in tumors and malignant ascites of patients with ovarian cancer [31,32], their role, cellular interactions and effects on tumor immunity during adoptive immunotherapy in patients with epithelial cell-based tumors remains relatively undefined.

The epithelial mucin MUC1 is a large transmembrane glycoprotein that is expressed on the apical surfaces of healthy ductal epithelia. Upon malignant transformation, such cells produce hypo-glycosylated MUC1 that can aid in prognostic and potential therapeutic benefits in patients with various types of adenocarcinoma [33-35]. Moreover, this aberrant hypo-glycosylation could result in the exposure of various immunodominant T cell epitopes that consequently make this molecule an attractive immunotherapeutic target for the treatment of

many epithelial-based cancers including ovarian [36-41]. Utilizing a previously described MUC1 20mer peptide and IL-2 for ex vivo CD4/Th1-like effector cell generation and expansion [35,42,43], we investigated the therapeutic effects of adoptively transferred autologous MUC1 peptide-stimulated CD4⁺ effector T cells in patients with advanced stage ovarian cancer. We show that three monthly treatment cycles of T cell restimulation and intraperitoneal re-infusion selectively modulated endogenous T cell-mediated immune responses that correlated with diminished serum CA125 tumor marker levels and enhanced patient survival times. Of the four patients completing all cycles of therapy, one patient remains disease free (OV2), another patient survived long-term for nearly 16 months with recurrent disease and death (OV7) and two patients (OV1 and OV3) expired within 3-5 months following final infusion. Although PBL from all patients receiving T cell therapy showed elevated MUC1 cytolytic activity ex vivo, such clinical responses suggested that induced and heightened T cell-mediated cytolytic activity to MUC1 did not correlate with therapeutic efficacy. However, long term survivors showed elevated levels of systemic memory (CD45RO) and naïve (CD45RA) CD3/CD4/CD25⁺ T cells when compared to that of pre-treatment levels and similarly-treated short-term survivors. Moreover, both endogenous memory and naïve CD4⁺ effector T cell subpopulations co-expressed markedly different cell number and frequency levels of Foxp3 and CTLA-4 (CD152) that resulted in progressively lower systemic Foxp3/CTLA-4 T cell ratios that correlated with disease-free survival. Aside from differences in systemic “natural” TReg cell subpopulations, long-term surviving patients showed different expression levels of the effector T cell-derived chemokine ligand/receptor complexes CCL4/CCR5 or CCR1 and CCL1/CCR8 associated with Type 1- and Type 2-like T cell migration, differentiation and memory, respectively. Although TReg cells have been studied extensively in mouse cancer models, the role of regulatory T cells in human tumor immunity is less well studied. We discuss the potential regulatory effects of various endogenous CD4⁺ T cell subpopulations following adoptive Th1 cell therapy and their role in the generation of effective anti-tumor responses, disease recurrence and progression in patients with advanced stage ovarian cancer.

Materials and Methods

Patients

Seven patients, with residual-recurrent epithelial ovarian cancer, were enrolled through the Harrington Cancer Center (Amarillo, TX) and had given written informed consent releasing the use of peripheral blood for research purposes. All patients, ranging from 47-70 years of age, were previously treated by standard surgery and chemotherapy with cis- or carboplatin and paclitaxel (Taxol) containing regimens. Following standard treatments, pathology confirmed recurrent ovarian cancer. None of these patients had received other cancer therapies within 4-6 weeks of protocol entry. Four patients completed the proposed three cycles of adoptive T cell immunotherapy whereas, three patients developed local inflammation/obstruction at the intraperitoneal port that resulted in discontinued treatment (Table 1). All studies were done with approval of the institutional review board of the Texas Tech University School of Medicine.

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 stimulation of human mononuclear cells from patients with adenocarcinoma [42-44].

Generation of MUC1 peptide-stimulated effector T cells

Generation of MUC1-stimulated effector T cell cultures has been previously described [42-44]. 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% CO₂ 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 mls of supernatant were collected, centrifuged at $400 \times g$ for 10 minutes, 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.

Adoptive T cell immunotherapy and treatment scheme

Adoptive immunotherapy with autologous MUC1 peptide-stimulated T cells was performed on eligible patients with residual-recurrent ovarian cancer following standard surgery and chemotherapeutic protocols. Eligible patients underwent leukaphereses for collection of PBMC, which were then expanded ex vivo with tumor-associated MUC1 peptide and IL-2. The effector T cells were administered systemically 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 - 10^9 cells per infusion. Patients were evaluated by magnetic resonance imaging (MRI) or computed tomography before and after completion of therapy. Disease responses were determined by 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.

Assay for Cytolytic Activity

Cytolytic T cell activity was assessed by the standard XTT colorimetric cytotoxicity assay (Roche Diagnostics Corp., Indianapolis, IN) and performed according to manufacturer's instructions. Briefly, human HLA-A2⁺ MCF-7 breast cancer cell lines expressing hypo-glycosylated surface mucin were obtained from American Type Tissue Culture Inc. (Rockville, MD). Effector T cells were combined with tumor target cells (5×10^3 cells/well) at a 10:1 effector-to-target cell ratio in 96-well flat-bottom plates (Costar Corp.) and incubated for 4 hr at 37°C with 5% CO₂. Cultures were performed in triplicate and OD readings were assessed in a DYNATECH MR 5000 spectrophotometer (Dynex Technologies Inc., Chantilly, VA). Maximum release values were determined by incubation of targets in the absence of effectors, while wells for minimum release values contained no cells. Results are expressed as the percent lysis and calculated as follows: % lysis = $100 - ((\text{OD of CTL \& Target} - \text{OD of CTL Alone}) / (\text{OD of Target Alone} - \text{OD of Media Alone}) \times 100)$.

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 [45]. Lymphocytes (10^6), pretreated with polyclonal human IgG (Sigma Inc) to block FcR, were mixed with 100 µl of FAB containing 1 µg of either/or FITC-conjugated human anti-CD4 (eBioscience, San Diego, CA. Clone HIS51), FITC-conjugated human anti-CD8 (eBioscience. Clone 53-2.1), PE-CY5-conjugated human anti-CD45RO or anti-CD45RA (Pharmingen), APC-conjugated human anti-CD3 (eBioscience), or PE-conjugated human anti-FasL (CD178) mAbs and incubated for 20 min on ice. Stained cell preparations were 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 ug/ml) was added to cultures to retain cytoplasmic cytokines. 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 APC-conjugated human anti-Foxp3 or human anti-CTLA-4 (CD152) mAbs (BD Pharmingen). Cells were washed and resuspended in 1% BSA/PBS solution and analyzed by flow cytometry as described above.

Assay for cytokine-releasing activity

Detection for secreted cytokines from supernatants of human PBMC cultures following restimulation has been described previously [42-44]. Briefly, supernatants from cell cultures following restimulation with peptide and IL-2 were harvested after 8 days and assessed for human IFN- γ content by cytokine-specific ELISA kits (BD Pharmingen Inc., San Diego, CA). Values were obtained and compared to standard curves constructed with purified IFN- γ as per manufacturer's instructions.

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 were 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 $2^{-\Delta Ct}$ 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 (house keeping genes)) or as fold-changes (test sample/control sample) in gene expression [46,47].

Statistical Analysis

For statistical analysis the two-tailed Student's t-test, Linear Regression Analysis and nonparametric Mann-Whitney Rank Sum test were used and provided by the PRISM Graph Pad statistical software package. Statistical significance was defined as a *P value* less than 0.05 for all analysis.

Results

Phenotypic Characterization of Autologous MUC1-Peptide Stimulated Effector T Cells

Patients underwent leukaphereses at various time intervals prior to and following adoptive T cell transfer for collection of peripheral blood mononuclear cells. Cells from such patients were stimulated with MUC1 peptide and IL-2 for eight days as described in *Materials and Methods*. Following restimulation, generated effector T cells were harvested, characterized and evaluated for MUC1 Ag reactivity in vitro. Using multiparameter flow cytometry, freshly generated effector T cell populations were predominantly CD3/CD4⁺ (>87%) and co-expressed up-regulated levels of CD45RO and CD25 (Fig. 1A). In contrast, CD3/CD8⁺ T cells

were routinely lower (<10%) with substantially diminished levels of CD45RO and CD25. Both CD4 and CD8 effector T cells expressed down-regulated levels of CD178 (FasL). Moreover, CD4/CD8 T cell expansion ratios were routinely 6-8 times greater following restimulation (Day 8) when compared to that of pre-stimulation (Day 0) levels (Fig 1B). Differences in expansion rates among corresponding treatment cycles were not significant ($P > 0.05$).

To ascertain whether such human effector T cells were functional and demonstrated MUC1 cytolytic potential, freshly generated effector T cell populations ($n=4$ per time point) were washed and evaluated in a standard 4 hour XTT colorimetric tumor cytotoxicity assay. As shown in Figure 1C, effector T cells demonstrated (20-30% tumor cell killing at a 10:1 effector to target cell ratio) cytolytic activity to MUC 1-expressing human MCF-7 tumor cell targets in vitro following restimulation. Although not significant for corresponding treatment cycle groups following restimulation ($P > 0.05$), peptide-stimulated effector T cells from all patients showed statistically significant increased killing activity at all time points tested when compared to that of corresponding pre-stimulation levels. Moreover, such CD4 effector T cells produced statistically significantly greater amounts of IFN- γ protein following restimulation when compared to that of pre-stimulation levels (Fig 1D). In contrast, there were no significant differences in IFN- γ production among corresponding restimulated treatment cycles. 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⁺ T cells that produce substantial levels of the Type 1 cytokine IFN- γ .

Adoptively transferred autologous MUC1-stimulated CD4 effector T cells enhance therapeutic efficacy among ovarian cancer patients with late stage disease

Patients with residual-recurrent ovarian cancer underwent leukaphereses for collection of PBMC (Table 1). 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*. This was repeated for each patient at monthly intervals for up to three cycles of T cell transfer. As shown in Figure 2, of the four patients completing 3 cycles of adoptive T cell transfer, one patient remains disease free (OV2), another patient survived long-term for nearly 16 months with recurrent disease and death (OV7) and two patients (OV1 and OV3) expired within 3-5 months following final infusion. Since serum levels of CA125 Ag have been previously shown to be a reliable serum marker for assessment of ovarian tumor regression and/or progression [48,49], we assessed the serum levels of the four patients at various time intervals during treatment and up to 200 days following the first infusion. As shown in Figure 2A, Patient OV2 had low serum CA125 values, with a decline at 180 day and 200 days following treatment. Whereas patient OV7 showed an initial and maintained reduction in serum CA125 values (18,300 to 500 units per ml) during treatment. However, after 2 months following treatment with autologous MUC 1 peptide-stimulated CD4 effector T cells, serum CA125 levels progressively increased (Fig 2B). Subsequently, within 16 months, pathology and CT scans of patient OV7 confirmed recurrent ovarian cancer. In contrast, short-term surviving patients, OV1 and OV3 showed a progressive increase in serum CA125 levels that resulted in tumor progression and patient death within 3-5 months following T cell transfer (Fig. 2C and D).

Treatment with autologous MUC1-stimulated CD4 effector T cells enhance MUC1 CTL activity among ovarian cancer patients receiving multiple cycles of adoptive T cell transfer

Systemic cytolytic activity of T cells from peripheral blood of patients completing three cycles of adoptive immunotherapy with autologous MUC1-stimulated CD4 effector T cells were assessed 30 days following the last infusion (Day 120). Freshly isolated PBMC from treated patients were obtained and restimulated with MUC1 peptide and IL-2 for 72 hours as described

in *Materials and Methods*. Cytolytic activity to MUC1-expressing MCF-7 tumor cell targets were assessed in a 4 hour XTT tumor cytolytic assay. As shown in Figure 3, all patients showed significantly enhanced CTL activity to human MUC1-expressing MCF-7 tumor cell targets when compared to that of corresponding pre-treatment levels. Although peripheral blood T cells from all patients, treated with autologous MUC1-stimulated T cell transfer, showed elevated MUC1-lytic activity ex vivo, only two of four patients survived long term suggesting that induced functional T cell-mediated cytolytic activity to MUC1 bearing tumor cells did not correlate with therapeutic efficacy.

Treatment with autologous MUC1-stimulated CD4 effector T cells differentially enhanced endogenous CD4/CD25⁺ T cell subpopulations levels co-expressing CD45RO (memory) and CD45RA (naïve) among long-term surviving patients following T cell transfer

Since co-expression of CD25 on T cells has been previously linked with cell activation or immunoregulation that may promote and/or facilitate tumor regression or progression [26, 27], we next assessed and characterized such CD4 and CD8 T cell populations in peripheral blood of patients receiving T cell transfer. As shown in Figure 4A-D, all patients showed elevated levels of systemic CD3/CD4/CD25⁺ T cell numbers and frequencies when compared to that of corresponding CD8 T cell populations. However, in long-term surviving patients (OV2 and OV7), such CD4 T cell populations were substantially elevated when compared to both corresponding pre-treatment levels and that of short-term survivors (OV1 and OV3) following similar treatments. Patients OV1 and OV3 showed no differences in such T cell subpopulations when compared to respective pre-treatment levels (Fig 4C and D). Systemic CD3/CD4/CD25⁺ 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 (Figs 4E). Moreover, long-term surviving patient OV2 (without recurrent disease) had greater cell numbers and frequencies of such cell populations when compared to that of long-term surviving patient OV7 with recurrent disease (Figs.4E and 5). Similar results were shown in CD3/CD4/CD25⁺ T cell subpopulations co-expressing CD45RA (Figs 4F and 5). This suggested that treatment with autologous MUC1-stimulated CD4 effector T cells differentially increased both the cell number and frequency of systemic CD3/CD4/CD25⁺ T cell subpopulations co-expressing either CD45RO (memory/Ag-experienced) or CD45RA (naïve/Ag-inexperienced) among long-term surviving patients when compared to that of corresponding short-term survivors.

Treatment with autologous MUC1 peptide-stimulated CD4 effector T cells differentially modulate endogenous “Ag-experienced” CD4/CD25/CD45RO⁺ and “Ag-inexperienced” CD4/CD25/CD45RA regulatory T cell subpopulations co-expressing Foxp3 or CTLA-4

Since “natural” TReg cell (nTReg) function has been associated with and dependent on constitutive expression of either Foxp3 and/or CTLA-4 (CD152), we next extended our observations to assess their expression among the elevated levels of memory CD4/CD25/CD45RO⁺ and naïve CD4/CD25/CD45RA⁺ T cell populations in cancer patients receiving 3 cycles of MUC1 peptide-stimulated T cell therapy. Using multiparameter flow cytometer, we enumerated the presence of such endogenous TRegs at monthly intervals following each treatment. As shown in Fig. 6A-B and 7, long-term-surviving patients showed elevated levels of systemic CD4/CD25/CD45RO⁺ cells co-expressing CTLA-4 when compared to that of pre-treatment levels and corresponding short-term surviving patients (OV1 and OV3). However, patient OV2 showed a marked and progressive decrease in both the cell number and frequency of corresponding cells co-expressing Foxp3 when compared to that of patient OV7 (Figs 6D and 7). This resulted in a progressively lower rate of systemic Foxp3/CTLA-4 memory T cell ratios that correlated with long-term disease-free survival (Fig 6E and F). Similar results were observed for corresponding CD4/CD25/CD45RA⁺ T cell subpopulations (Fig 8A-D). Interestingly, long-term survivors consistently showed lower levels of systemic memory/naïve

CD4/CD25⁺ T cell ratios co-expressing either Foxp3 or CTLA-4 when compared to that of similarly-treated short-term surviving patients (Figs 8E and F). Collectively, such differences in the cell number, rate and frequency of either CD45RA “Ag inexperienced” and CD45RO “Ag-experienced” CD4/CD25⁺ T cells subpopulations co-expressing either CTLA-4 or Foxp3 may represent functionally-distinct nTReg cell subpopulations at different stages of differentiation and maturation that may, in part, promote and influence more effective antitumor responses among patients following autologous T cell therapy.

Adoptive T cell transfer with autologous MUC1-stimulated CD4 T cells enhance select chemokine receptor expression among endogenous MUC1-specific T cells from long-term surviving patients

Expression of the C-C chemokine receptor/ligand subfamily has been shown to mediate immune responses through the induction and participation of functionally-distinct immune T cell populations at sites of inflammation [50-52]. CCR5 is a C-C chemokine receptor that is expressed on T cells with memory/effector phenotype and has been associated with Th1-type responses in humans [50,52-54]. Using multiparameter flow cytometric analysis, we next assessed the levels of CCR5 receptor expression among systemic MUC1-specific CD3/CD4⁺ T cells from ovarian cancer patients following 3 cycles of adoptive T cell therapy. Peripheral blood mononuclear cells were obtained from patients prior to (Day 0) and one month following final treatment (Day 120 post treatment) and restimulated with peptide and IL-2 for 72 hours. As shown in Figure 9, all patients showed elevated levels of systemic CD3/CD4⁺ T cells co-expressing the surface chemokine receptor CCR5 when compared to that of corresponding pre-treatment levels. However, the frequency was comparatively 2-3 fold higher in patient OV2 (long-term survivor without recurrent disease) when compared to that of patients OV7 (long-term survivor with recurrent disease) and short-term survivors. Similarly, when using SuperArray RT-PCR (Frederick, MD), corresponding CCR5 gene expression levels were also profoundly elevated in patient OV2 following treatment with a nearly 250-fold increase in post-treatment versus pre-treatment levels (Table 2). Differences among OV7 and short-term surviving patients were negligible. When we extended our observations to other C-C chemokine receptors, all patients had similar expression levels of CCR2, CCR3, CCR4, CCR6, CCR7, CCR9, CXCR1, IL8RA and XCR1 following treatment (Table 2). However, in patient OV2, we show that CCR1 chemokine receptor expression among MUC1 peptide-restimulated CD4 T cells were nearly 5-fold greater when compared to that of similarly restimulated cells in patient OV7. In contrast, the latter showed a nearly 25- and 3-fold elevation in CCR8 and CCR2 expression, respectively, when compared to that of former (Table 2). Moreover, short-term survivors showed an elevation (>11-fold difference) in CCR8 when compared to that of patient OV2. This suggested that multiple cycles of adoptive T cell therapy with autologous MUC1 peptide-stimulated CD4 effector T cells induce differentially expressed chemokine receptors among endogenous T cells that are associated with memory/effector phenotype and Th1 (CCR5 and CCR1)/Th2 (CCR8) immune responses [50-52,54-56]. Such cells in the periphery can potentially regulate Ag-specific effector T cell activation/differentiation and movement in vivo that facilitate effective antitumor immune responses and enhance therapeutic efficacy.

Treatment with autologous MUC1-stimulated CD4 T effector cells modulate select endogenous MUC1-specific T cell-derived chemokines associated with therapeutic efficacy and long-term survival

Since endogenous MUC1-restimulated CD4 T cells among treated patients selectively exhibited elevated levels of CCR5, CCR1 and CCR8 following treatment, we next assessed the chemokine ligands associated with such receptors [50-52]. The chemokine receptors CCR5 (CCL3, CCL4, CCL5, CCL11, and CCL16 ligands), CCR1 (CCL3, CCL5, and CCL7 ligands) and CCR8 (CCL1 ligand) have been shown in some cases to share the same and/or multiple

chemokine ligands [50,51]. Thus in parallel studies, we investigated gene expression levels of the pro-inflammatory CCL chemokine ligand family following Ag-specific CD4 restimulation with peptide and IL-2. Cell cultures were harvested and RT-PCR performed as described above. Aside from all patients showing elevated levels of CCL2, CCL3 and CCL7, only long-term surviving patients OV2 and OV7 had elevated levels of CCL4 when compared to that of short-term survivors following treatment (Table 3). Moreover, patient OV7 (long-term survivor with disease recurrence) had a nearly 20-fold increase in CCL1 (ligand to CCR8) when compared to that of patient OV2 (long-term disease-free survivor). Pre- and post-treatment differences in these chemokines among short-term survivors were negligible (< 2.0 fold-increase from pre-treatment levels). Collectively, this suggested that aside from differences in TReg cell subpopulation numbers and ratios, long-term surviving patients receiving autologous MUC1-specific T cell transfer also showed different expression levels of select chemokine ligands (i.e. CCL1 and CCL4) and receptors (i.e. CCR5, CCR1 and CCR8) that may promote, in part, distinct cellular recruitment and effector cell functions at sites of tumor growth in vivo. Such cell recruitment and their kinetics in the periphery may determine long-term therapeutic responses that promote or facilitate enhanced patient survival.

Discussion

The adoptive cell transfer of ex vivo activated autologous tumor-reactive T cells is currently one of the most promising approaches for the treatment of patients with advanced cancers. Utilizing a previously described MUC1 20mer peptide and IL-2 for ex vivo CD4 effector cell generation and expansion, we investigated the therapeutic effects of adoptively transferred autologous MUC1 peptide-stimulated CD4⁺ effector T cells in patients with advanced stage ovarian cancer. Such restimulation procedures, utilizing ovarian cancer patient PBMC, resulted in the generation of functional CD3/CD4/CD25/CD45RO⁺ effector/memory T cells that produced substantial levels of the type 1 cytokine IFN- γ . We show that three monthly treatment cycles of T cell restimulation and intraperitoneal re-infusion selectively modulated endogenous T cell-mediated immune responses that correlated with diminished serum CA125 tumor marker levels and enhanced patient survival times. Although PBL from all patients receiving T cell therapy showed elevated tumor cytolytic activity ex vivo, only two of four patients survived long-term suggesting that induced T cell-mediated cytolytic activity to MUC1 did not appear to correlate with therapeutic efficacy. This is in agreement with others that have shown that adoptively transferred non-cytolytic and/or cytolytic CD4⁺ T cells can effectively eradicate tumors through multiple mechanisms independent of CTL lytic activity [57]. Moreover, we showed that long-term surviving ovarian cancer patients had elevated levels of both systemic “Ag-experienced” CD45RO (memory) or “Ag-inexperienced” CD45RA (naïve) CD3/CD4/CD25⁺ T cells following adoptive transfer of MUC 1 peptide-stimulated CD4/Th1 effector cells. Interestingly, such endogenous T cells contained various regulatory and effector cell subpopulations that correlated with enhanced therapeutic efficacy following T cell transfer. Such cellular dynamics were further associated with differences in the type of immune responses induced by multiple cycles of autologous T cell therapy. Thus, treatment with such IFN- γ -producing CD4 T cells may not only provide help for establishing and maintaining immune memory and/or effective tumor eradication through cytokine secretion but also modulate functionally and phenotypically-distinct cell subpopulations among various endogenous CD4⁺ T cells following treatment.

Recent animal studies have demonstrated that regulatory T cell-mediated immunosuppression is a critical tumor-immune evasion mechanisms and a main obstacle for successful tumor immunotherapy [26,27,30,58]. In humans, CD4/CD25 T cells have been identified at increased frequencies in the peripheral blood and malignant effusions of patients with ovarian cancer, where increased densities of such cells were predictive of poor survival [31,32]. In contrast, our studies showed that following autologous MUC1-reactive Th1 cell transfer, long-term

surviving ovarian cancer patients with late stage disease, exhibited increased levels of systemic Ag-experienced CD3/CD4/CD25⁺ T cells that correlated with increased patient survival. Moreover, such endogenous memory CD4/CD25⁺ effector T cell subpopulations co-expressed markedly different frequency levels of Foxp3 and CTLA-4 (CD152) that resulted in progressively lower systemic Foxp3/CTLA-4 T cell ratios and lower systemic CA125 tumor Ag levels among long-term surviving patients. Such differences may represent phenotypically distinct types of regulatory T cell subpopulations that may, in part, promote and influence more effective antitumor responses among patients following autologous Th1 effector cell therapy. Expansion of endogenous memory CD4/CD25⁺ T cells co-expressing CTLA-4 may concomitantly down regulate intrinsic Foxp3 expressing regulatory T cells responsible for effector T cell anergy and/or immune down regulation that result in a selective regulatory T subpopulation outgrowth associated with tumor regression or recurrence. For example, T cell-derived cell surface CTLA-4 may ligate CD80 and, to a lesser extent, CD86 expressed by other effector/regulatory T cells and directly initiate negative signals that results in suppression that further aids in maintaining homeostasis and/or effective antitumor immune responses [58, 59]. Another possible mechanism of CTLA-4 may involve the induction of the enzyme indolamine 2, 3-dioxygenase by interacting with CD80 and/or CD86 on dendritic cells [58, 60]. This enzyme catalyzes the conversion of typtophan to kynurenine and other metabolites, which can indirectly suppress the T cell persistence and function [58,61]. However, these potential CTLA-4 TReg-mediated mechanisms and their potential interaction with other T cells need to be further substantiated. Although we are not in a position to definitively address this in the current study, we propose that this shift in regulatory cell subpopulation dynamics may represent the accumulation of two independent and/or synergistic regulatory cell populations with distinct alternative pathways/mechanisms for effector/memory T cell regulation in vivo [26,62]. Such subpopulations may act to counter-balance each other and promote effective antitumor responses among long-term surviving ovarian cancer patients. Alternatively, CD25 co-expression among T cells has not only been associated with immunosuppression but also T cell activation [12,26,28]. Interestingly, the disease-free survivor (patient OV2) showed progressively greater proportions of memory CD4/CD45RO/CD25⁺ T cells that were either Foxp3⁻ or CTLA-4⁻ when compared to that of the long-term surviving patient with recurrent disease (patient OV7). Moreover, in human lymphocytes, both CTLA-4 and Foxp3 have been shown to be transiently upregulated following activation and their expression may not always correlate with suppressive function [63,64]. Thus, suggesting that both Tregs and activated effectors may both express these same markers. This may reflect enhanced levels of acutely activated endogenous effector/memory CD4 helper T cells that promote and maintain effective antitumor responses within these patients that result in disease-free long-term survival. Similarly, such “non-Treg” cells may also have a significant role in modulating TReg cell function and persistence. Although we have not definitively assessed the functional attributes of such cells in these patients, FoxP3 transcription factor and CTLA-4 remain the best characterized “natural” Treg specific molecules that control a number of quintessential characteristics of Tregs, including the transcriptional repression and/or upregulation of various key nuclear factors and effector molecules [64,65]. In either instance, we suggest that multiple cycles of autologous MUC1 peptide-stimulated T cell transfer, facilitate and/or promote changes in systemic immunoregulatory/effector T cell subpopulation dynamics that affect long-term disease-free survival in ovarian cancer patients. Investigations to further elucidate and characterize the phenotype, function and contributions of such memory cells in tumor progression and/or regression are in progress.

Another observation with therapeutic relevance was the presence and elevation of both memory and naïve CD4/CD25⁺ T cell subpopulations co-expressing either Foxp3 or CTLA-4 in patients following Th1 cell therapy. Our studies showed that long-term surviving patients consistently had lower levels of systemic memory/naïve CD4/CD25⁺ T cell ratios co-expressing either Foxp3 or CTLA-4 when compared to that of similarly treated short-term ovarian cancer patient

survivors. This may suggest that such treatment with autologous MUC1 Ag-specific Th1 effector cells induce and/or modulate proportions of systemic differentiated (Ag-experienced) and non-differentiated (Ag-inexperienced) TReg subpopulations in cancer patients that can influence and/or contribute to effective antitumor responses and long-term patient survival. As shown in patients with short-term survival, elevated proportions of systemic CD45RO memory TReg cells may contain an “Ag-primed” activation state that may require a low activation threshold that results in an increase in “active” suppression of ensuing antitumor responses [58]. Whereas, long-term surviving patients, containing greater proportions of systemic CD45RA naïve TReg cells, may require more TCR engagement and co-stimulation to accommodate higher activation thresholds and thus attenuate cell-mediated suppression by such populations. Furthermore, as mentioned above, the expression of activation markers among Ag-primed T cells, such as CD80 and CD86, may differentially activate or deactivate TReg cells via CTLA-4-mediated mechanisms [59,66-68]. Thus, Th1 effector cell therapy may affect systemic TReg effector cell levels of differentiation and maturation. Such quantitative and qualitative differences among memory and naïve TReg cell subpopulations may exist and affect both treatment efficacy and disease progression following T cell transfer [69-71].

Differences in systemic T cell dynamics among treated patients may further induce changes in the balance of inflammatory mediators, such as chemokines and their cellular receptors. In particular, the C-C chemokine receptor/ligand subfamily has been shown to mediate immune responses through the induction and participation of functionally-distinct immune T cell populations at sites of tumor growth and inflammation [50-52]. Aside from differences in the levels and differentiation states of systemic TReg cell subpopulations, all patients showed elevated levels of the chemokines CCL2, CCL3 and CCL7 following T cell restimulation. However, only long-term surviving ovarian cancer patients selectively up-regulated the chemokine CCL4 associated with type 1-like antitumor immune responses [50,52,72]. Moreover, the disease-free long-term surviving patient (patient OV2) showed markedly enhanced levels of the CCR5 and CCR1 chemokine receptors that are expressed on T cells with memory Th1 cell phenotype [50,53,54]. Whereas, corresponding T cells from the long-term survivor with recurrent disease (patient OV7) showed an elevation in the type 2-associated chemokine CCL1 and its corresponding Th2-related chemokine receptor CCR8 [50,52]. This appears to correlate with the findings from others that elevated CTLA-4 expression among CD4/CD25⁺ T cells, as seen in patient OV7, may contribute to the emergence of a “less effective” type 2-like T cell-mediated immune response that may further counter “more effective” type 1 antitumor responses [73-77]. Collectively, multiple cycles of adoptive T cell therapy with autologous MUC1 peptide-stimulated CD4/Th1 effector T cells induced differentially expressed chemokine ligand/receptor complexes that can potentially enhance Ag-specific T cell activation/differentiation and movement in vivo that facilitate effective antitumor immune responses and enhance therapeutic efficacy.

Lastly, it is worth noting that the disease-free survivor (patient OV2), demonstrating long term remission, showed the lowest levels of serum CA125 at the initiation of immunotherapy among all patients. This may indicate that adoptive T cell immunotherapy works best when tumor burden is minimal. Subsequently, this may be attributed, in part, to a better T effector:tumor cell ratio under minimal disease burden and/or due to less suppressive/tolerogenic tumor-mediated mechanisms to T effector cells in vivo.

In summary, as an important member of the Th1 cytokine family, IFN- γ has been shown to play an important role in T cell activation, migration and tumor eradication [78]. Although we do not directly address the role of IFN- γ in this study, we suggest that IFN- γ derived from adoptively transferred MUC1-specific Th1 cells may in part modulate select TReg cell subpopulations, such as Foxp3 and CTLA-4 [79,80]. Such modulation may further up-regulate antitumor responses in tumor-bearing patients by promoting memory cells that amplify type

1-like inflammatory responses through their ability to mediate a variety of chemokines and their receptors that facilitate select T cell subpopulation migration and immune surveillance in ovarian cancer patients. These results have significant clinical relevance for the understanding of the mechanisms by which such cellular immunotherapeutic strategies regulate immune responses and provide insight into the interplay of these cells in establishing long-term tumor immunity and effective tumor regression. In addition, others have demonstrated that depletion of CD4/CD25⁺ cells can lead to enhanced generation of T cells recognizing tumor-associated Ags that subsequently enhance antitumor responses in humans [81-85]. In contrast, we further hypothesize that “broad-spectrum” depletion of CD4/CD25 TReg cell populations may not always augment T cell-mediated antitumor effects by eliminating “inhibitory factors” but that these populations may actually enhance T cell responses and promote therapeutic efficiency among cancer patients. Notably, the quantitative and qualitative “balance” and not ablation among different TReg cell subpopulations may determine successful therapy in cancer patients following CD4⁺ T cell transfer.

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Abbreviations used in this paper

Th1	CD4 ⁺ T cells producing IFN- γ
Treg	Regulatory T cells
Foxp3	Forkhead box protein p3
CTLA-4	Cytotoxic T Lymphocyte—associated Antigen 4

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Figure 1. Phenotypic Characterization of MUC1-Peptide Stimulated Human Effector T Cells

Human MUC1 peptide-stimulated effector T cells were generated as described in *Materials and Methods*. (A) Cells were harvested and labeled with FITC-anti-CD4 or anti-CD8, APC-CD3, PE-CY5-CD45RO and PE-anti-CD25 or CD178 mAbs. Lymphocytes were distinguished by their forward light scatter/side scatter profiles and gates set on CD3/CD4⁺ or CD3/CD8⁺ and CD45RO and co-expression of either CD25 or CD178 within these subpopulations were assessed by multiparameter flow cytometry. Data shown are from a representative experiment showing the percentages of either CD3/CD4/CD45RO⁺ or CD3/CD8/CD45RO⁺ T cells co-expressing CD25 or CD178 surface antigens. In B, expansion rates among CD3/CD4 and CD3/CD8 T cells at either pre- or post-restimulation (day 8) show a marked elevation in CD3/CD4 T cells at all infusion time points following restimulation. Data are expressed as the mean CD4/CD8 ratio $\pm$ SEM of 4 independent experiments per infusion time point. In C, cytolytic activity was assessed in a standard XTT tumor cell cytotoxicity assay against MUC1-expressing MCF-7 at an E/T ratio of 10:1. Spontaneous release in all assays were $<15\%$. Data are expressed as the mean $\pm$ SEM of 4 independent experiments per infusion time point. D, human IFN- γ production among cultures prior to and following each restimulation with MUC1 peptide and IL-2 were determined by ELISA. Data are expressed as the mean $\pm$ the SEM of four patients per group. *, $p < 0.05$ for re-stimulation values vs pre-stimulation values; **, $P > 0.05$ for corresponding treatment cycle groups following restimulation.

Figure 2. Adoptively transferred MUC1 peptide-stimulated CD4 effector T cells modulate serum CA125 levels and enhance therapeutic efficacy among treated late-stage ovarian cancer patients Patients (n=4) underwent leukaphereses for collection of peripheral blood mononuclear cells. Following restimulation and expansion with MUC1 peptide and IL-2 as described in *Materials and Methods*, freshly generated autologous effector T cells were harvested and administered via an intraperitoneal port-a-catheter at monthly intervals and serum CA125 levels among each patient determined. Serum CA125 values were obtained by ELISA. Arrows indicate infusion times of T cell therapy. Individual points represent pre-treatment levels for each patient tested.

Figure 3. Treatment with autologous MUC1 peptide-stimulated CD4 effector T cells enhance systemic T cell-mediated MUC1 cytolytic activity among ovarian cancer patients receiving multiple cycles of adoptive T cell transfer

Systemic cytolytic activity of T cells from peripheral blood of patients completing three cycles of adoptive immunotherapy with autologous MUC1-stimulated CD4 effector T cells were assessed 30 days following the last infusion. Freshly isolated PBMC from pre- and post-treated (day 120) patients were obtained and re-stimulated with MUC1 peptide and IL-2 for 72 hours as described in *Materials and Methods*. Cells were harvested and cytolytic activity to MUC1-expressing MCF-7 tumor targets were assessed by a 4 hour XTT tumor cytolytic assay. Data are expressed as the mean +/- SEM of triplicate wells. * P value < 0.05.

Figure 4. Treatment with autologous MUC1 peptide-stimulated CD4 effector T cells differentially enhance systemic CD4/CD25⁺ T cell subpopulations co-expressing CD45RO (memory) and CD45RA (naïve) among long-term surviving patients following T cell transfer

Patients (n=4) underwent leukaphereses for collection of peripheral blood mononuclear cells. 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 at monthly intervals as described in Figure 2. At monthly intervals following each treatment, systemic PBMC were obtained and labeled with anti-CD3, anti-CD4 or CD8, and anti-CD25 mAbs. Gates were set on CD3/CD4⁺ or CD3/CD8⁺ and populations of cells co-expressing CD25 were assessed among long-term surviving (A and B) or short-term surviving (C and D) patients by multiparameter flow cytometry. Absolute cell numbers were calculated as the percentages of positive staining cells × the total number of mononuclear cells per ml of PBL for each patient. In E and F, gates were set on CD3/CD4/CD25⁺ co-expressing CD45RO (E) or CD45RA (F) and T cell subpopulations assessed by multicolor flow cytometry. Individual points represent pre-treatment cell population levels for each patient tested.

Figure 5. Long-term surviving patients show elevated levels of systemic CD3/CD4/CD25⁺ T cell subpopulations co-expressing CD45RO (memory) and CD45RA (naïve) following treatment with MUC1-peptide stimulated CD4 effector T cells

Freshly isolated PBMC from long-term surviving patients treated with 3 cycles of T cell transfer therapy were obtained as described in *Materials and Methods*. At monthly intervals following each treatment, systemic PBMC from patients OV2 (A) and OV7 (B) were obtained and labeled with anti-CD3, anti-CD4, anti-CD25 and either anti-CD45RO or anti-CD45RA mAbs. Gates were set on CD3/CD4/CD25⁺ T cells and CD45RO or CD45RA surface marker expression levels within these cell populations were assessed via multiparameter flow cytometry. Numbers represent the percentages of specified positive staining cells. In C, memory and naïve CD4/CD25⁺ T cell frequencies among long-term survivors were calculated by multiplying the percentages of gated CD3/CD4⁺ cells × the percentages of CD25⁺ cells × the percentages of cells staining for either CD45RO or CD45RA surface markers.

Figure 6. Treatment with autologous MUC1 peptide-stimulated CD4 effector T cells differentially modulate systemic memory CD4/CD25/CD45RO⁺ regulatory T cell subpopulations co-expressing Foxp3 or CTLA-4

Freshly isolated PBMC from ovarian cancer patients receiving 3 cycles of T cell transfer were obtained at monthly intervals following each treatment. Cells were obtained and labeled with anti-CD4, anti-CD25 and anti-CD45RO mAbs. Gates were set on memory CD4/CD25/CD45RO⁺ T cell subpopulations co-expressing either intracellular CTLA-4 (CD152) (A and B) or Foxp3 (C and D) and assessed among either long-term or short-term surviving patients by multiparameter flow cytometry. Absolute cell numbers were calculated as the percentages of positive staining cells $\times$ the total number of mononuclear cells per ml of peripheral blood for each patient. Individual points represent pre-treatment cell population levels for each patient tested. Systemic Foxp3/CTLA-4 memory CD4/CD25/CD45RO⁺ T cell ratios were assessed among short-term (E) and long-term (F) patient 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 suggest changes in cell population ratios per unit time.

Figure 7. Treated long-term surviving patients differentially modulate systemic memory “Ag-experienced” CD4/CD25/CD45RO⁺ regulatory T cell subpopulations co-expressing either Foxp3 or CTLA-4

Freshly isolated PBMC from long-term surviving patients treated with 3 cycles of T cell transfer therapy were obtained as described in *Materials and Methods*. At monthly intervals following each treatment, systemic PBMC from patients OV2 (A) and OV7 (B) were obtained and labeled with anti-CD4, anti-CD25 and anti-CD45RO mAbs. Gates were set on CD4/CD25/CD45RO⁺ and cells co-expressing either intracellular CTLA-4 (CD152) or Foxp3 were assessed by multiparameter flow cytometry. Numbers represent the percentages of CD4/CD25/CD45RO⁺ T cells co-expressing CTLA-4 or Foxp3.

Figure 8. Treatment with autologous MUC1 peptide-stimulated CD4 effector T cells differentially modulate systemic naïve “Ag-inexperienced” CD4/CD25/CD45RA⁺ regulatory T cell subpopulations co-expressing Foxp3 or CTLA-4

Freshly isolated PBMC from ovarian cancer patients receiving 3 cycles of T cell transfer were obtained at monthly intervals following each treatment. Cells were obtained and labeled with anti-CD4, anti-CD25 and anti-CD45RA mAbs. Gates were set on naïve CD4/CD25/CD45RA⁺ T cell subpopulations co-expressing either intracellular CTLA-4 (CD152) (A and B) or Foxp3 (C and D) and assessed among either long-term or short-term surviving patients by multiparameter flow cytometry. Absolute cell numbers were calculated as the percentages of positive staining cells $\times$ the total number of mononuclear cells per ml of peripheral blood for each patient. Individual points represent pre-treatment cell population levels for each patient tested. Systemic memory/naïve CD4/CD25⁺ T cell ratios co-expressing either CTLA-4 (E) or Foxp3 (F) were assessed at different time intervals for each patient following therapy.

Figure 9. Adoptive T cell transfer with autologous MUC1-stimulated CD4 T cells enhance CCR5 chemokine receptor expression on systemic MUC1-specific CD4⁺ T cells in patients treated with 3 cycles of therapy

Peripheral blood mononuclear cells were obtained from patients prior to (Day 0) and one month following final treatment (Day 120 post treatment) and restimulated with peptide and IL-2 for 72 hours. Cells were harvested and labeled with FITC-anti-CD4, APC-anti-CD3 and PE-anti-CCR5 mAbs. Lymphocytes were distinguished by their forward light scatter/side scatter profiles and gates set on CD3/CD4⁺ T cells and co-expression of CCR5 within these populations were assessed by multicolor flow cytometry. Data are shown as the percentages of CD3/CD4⁺ T cells co-expressing CCR5 surface antigen among long-term (A and B) and a representative short-term surviving patients (C).

Table 1

Patient characteristics and clinical course summary

Patient No.	Pre-study Therapy	Disease Status	Pre-study serum CA-125 (units/ml)	Complications with intraperitoneal port	NCI Common Toxicity Criteria	Discontinued Treatment (< 3 Treatment Cycles)
OV1	Resection Chemotherapy	Recurrent	1455	None	None	
OV2	Resection Chemotherapy Partial Resection	Recurrent	22	None	Grade 1 abdominal pain	
OV3	Resection Chemotherapy Resection	Recurrent	70	None	None	
OV4	Resection Chemotherapy	Recurrent	122	Occluded	None	X
OV5	Resection Chemotherapy	Recurrent	56	Occluded	None	X
OV6	Resection Chemotherapy	Recurrent	983	Ruptured	None	X
OV7	Resection Chemotherapy	Recurrent	18,300	None	None	

Table 2

CC Chemokine Receptor Gene Expression Among Ovarian Cancer Patients Following Treatment With Three Cycles of Adoptive T Cell Transfer*

Patient	Receptor Gene	AVG ΔC_t (Ct(GOI) - Ave Ct)		$2^{-\Delta\Delta C_t}$		Fold** Difference
		Post-Treatment (Day 120)	Pre-Treatment (Day 0)	Post-Treatment (Day 120)	Pre-Treatment (Day 0)	Post/Pre-Treatment
Patient OV2 (Disease-Free Long Term Survivor)	CCR1	1.66	4.21	0.3169	0.0540	5.86
	CCR2	4.36	4.40	0.0488	0.0474	1.03
	CCR3	9.58	10.01	0.0013	0.0010	1.35
	CCR4	4.87	5.24	0.0342	0.0265	1.29
	CCR5	3.69	11.60	0.0776	0.0003	240.85
	CCR6	6.97	7.25	0.0080	0.0066	1.22
	CCR7	2.33	2.51	0.1992	0.1756	1.13
	CCR8	11.50	9.23	0.0003	0.0017	0.21
Patient OV7 (Long-Term Survivor) with recurrence	CCR9	8.15	6.61	0.0035	0.0102	0.34
	CX3CR1	7.60	4.17	0.0052	0.0556	0.09
	IL8RA	11.19	8.54	0.0004	0.0027	0.16
	XCR1	12.84	11.60	0.0001	0.0003	0.42
	CCR1	3.05	3.85	0.1206	0.0693	1.74
	CCR2	4.29	5.95	0.0510	0.0162	3.16
	CCR3	9.36	10.86	0.0015	0.0005	2.82
	CCR4	4.61	4.49	0.0409	0.0445	0.92
	CCR5	11.72	11.28	0.0003	0.0004	0.74
	CCR6	8.55	7.83	0.0027	0.0044	0.61
	CCR7	2.27	2.29	0.2070	0.2045	1.01
	CCR8	6.52	8.88	0.0109	0.0021	5.13
Patient OV1 (Short-Term Survivor)	CCR9	8.14	6.65	0.0035	0.0100	0.36
	CX3CR1	7.11	5.31	0.0072	0.0252	0.29
	IL8RA	8.02	8.98	0.0038	0.0020	1.94
	XCR1	12.23	11.28	0.0002	0.0004	0.52
	CCR1	3.16	3.80	0.1122	0.0717	1.56

Patient	Receptor Gene	AVG ΔC_t (Ct(GOI) - Ave Ct)		$2^{-\Delta\Delta C_t}$		Fold ^{**} Difference
		Post-Treatment (Day 120)	Pre-Treatment (Day 0)	Post-Treatment (Day 120)	Pre-Treatment (Day 0)	Post /Pre-Treatment
	CCR2	5.32	5.84	0.0250	0.0174	1.43
	CCR3	9.29	9.73	0.0016	0.0012	1.36
	CCR4	5.02	4.92	0.0309	0.0330	0.94
	CCR5	11.17	11.97	0.0004	0.0002	1.75
	CCR6	10.01	8.98	0.0010	0.0020	0.49
	CCR7	2.88	3.10	0.1362	0.1165	1.17
	CCR8	7.28	8.55	0.0065	0.0027	2.42
	CCR9	8.00	8.48	0.0039	0.0028	1.40
	CX3CR1	6.10	3.58	0.0146	0.0835	0.18
	IL8RA	8.80	8.13	0.0022	0.0036	0.63
	XCR1	11.17	11.97	0.0004	0.0002	1.75

* PBMC were obtained from patients prior to (Day 0) and one month following final treatment (Day 120) and restimulated with MUC1 peptide and IL-2 for 72 hours. Cells were harvested and total RNA was isolated and first strand cDNA were prepared as described in Materials and Methods. Template cDNA were 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 ΔC_t and/or Relative Gene Expression Level ($\Delta C_t = C_t$ (gene of interest) - Avg Ct (house keeping genes) by the $\Delta\Delta C_t$ method.

** Fold changes in gene expression between post (Day 120)- and pre (Day 0)-treatment 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-term surviving and representative expired patients.

Table 3
Chemokine Gene Expression Among Ovarian Cancer Patients Following Three Cycles of Adoptive T Cell Transfer*

Patient	Ligand Gene	AVG ΔC_t (Ct(GOI) - Ave Ct (HKG))		$2^{-\Delta\Delta C_t}$		Fold Difference**
		Post-Treatment (Day 120)	Pre-Treatment (Day 0)	Post-Treatment (Day 120)	Pre-Treatment (Day 0)	Post/Pre-Treatment
Patient OV2 (Disease-Free Long Term Survivor)	CCL2	1.35	11.60	0.3928	0.0003	1219.44
	CCL3	2.92	5.93	0.1323	0.0164	8.07
	CCL4	2.81	5.38	0.1428	0.0240	5.95
	CCL5	3.80	2.85	0.0719	0.1387	0.52
	CCL7	8.33	11.60	0.0031	0.0003	9.66
	CCL11	13.17	11.60	0.0001	0.0003	0.34
Patient OV7 Long-Term Survivor) with recurrence	CCL16	12.45	11.60	0.0002	0.0003	0.56
	CCL1	13.05	11.60	0.0001	0.0003	0.37
	CCL2	3.14	11.99	0.1133	0.0002	461.12
	CCL3	2.08	6.50	0.2362	0.0110	21.39
	CCL4	1.52	4.00	0.3482	0.0625	5.58
	CCL5	1.68	1.05	0.3117	0.4826	0.65
	CCL7	7.30	11.59	0.0063	0.0003	19.55
	CCL11	12.69	11.99	0.0002	0.0002	0.62
	CCL16	12.69	11.99	0.0002	0.0002	0.62
	CCL1	8.93	11.99	0.0020	0.0002	8.33
Patient OV1 (Short-Term Survivor)	CCL2	1.07	11.97	0.4757	0.0002	1910.85
	CCL3	2.29	4.69	0.2042	0.0387	5.28
	CCL4	2.24	2.29	0.2114	0.2042	1.04
	CCL5	2.29	0.81	0.2042	0.5696	0.36
	CCL7	7.54	11.16	0.0054	0.0004	12.30
	CCL11	13.57	11.97	0.0001	0.0002	0.33
	CCL16	10.66	11.97	0.0006	0.0002	2.08
	CCL1	11.88	11.97	0.0003	0.0002	1.06

* PBMC were obtained from patients prior to (Day 0) and one month following final treatment (Day 120) and restimulated with MUC1 peptide and IL-2 for 72 hours. Total RNA was obtained and template cDNA were processed and characterized as described in Table 2. The raw Ct (cycle threshold) values were calculated by the instrument and converted into the Avg Δ Ct and/or Relative Gene Expression Level (Δ Ct=Ct (gene of interest) – Avg Ct (house keeping genes) by the $\Delta\Delta$ Ct method.

** Fold changes in gene expression between post- (Day 120) and pre (Day 0)-treatment 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-term surviving and representative expired patients.