Inability to Mediate Prolonged Reduction of Regulatory T Cells After Transfer of Autologous CD25-depleted PBMC and Interleukin-2 After Lymphodepleting Chemotherapy

Daniel J. Powell, Jr, Christiaan R. de Vries, Tamika Allen, Mojgan Ahmadzadeh, and Steven A. Rosenberg

Summary: $CD25^+CD4^+$ regulatory T cells (Treg) regulate peripheral self-tolerance and possess the ability to suppress antitumor responses, which may explain the poor clinical response of cancer patients undergoing active immunization protocols, and provides the rationale for neutralizing Treg cells in vivo to strengthen local antitumor immune responses. Because interleukin-2 (IL-2) mediates tumor regression in about 15% of treated patients but simultaneously increases Treg cells, we hypothesized that transient elimination of Treg cells will enhance the clinical effectiveness of IL-2 therapy. In the current study, 5 patients with metastatic melanoma who were refractory to prior IL-2 received a lymphodepleting preparative regimen followed by the adoptive transfer of autologous lymphocytes depleted of $CD25^+$ Treg cells and high-dose IL-2 administration. $CD25^+$ cells were eliminated from patient leukapheresis samples using a clinical-grade, large-scale immunomagnetic system, leaving $CD8^+$ and $CD25^-CD4^+$ T cells intact. In the early aftermath of $CD25^+$ Treg cell-depleted cell infusion, $CD25^+FOXP3+$ $CD4^+$ Treg cells rapidly repopulated the peripheral blood of treated patients with 18% to 63% of $CD4^+$ T cells expressing FOXP3. Recovering $CD25^+CD4^+$ T cells exhibited suppressive activity against $CD25^-CD4^+$ effector T-cell proliferation in vitro. No patient experienced objective tumor regression or autoimmunity. Our results indicate that in vivo transfer of autologous CD25-depleted mononuclear populations to lymphopenic patients in combination with high-dose IL-2 is not sufficient to mediate prolonged reduction of Treg cells after IL-2 administration.

Key Words: immunotherapy, ACT, IL-2, human, CD25, regulatory T cells, depletion

(*J Immunother* 2007;30:438–447)

Received for publication October 23, 2006; accepted December 5, 2006.

From the Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD.

Financial Disclosure: The authors declare there are no financial conflicts of interest related to this work.

Supported (in part) by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

Reprints: Daniel J. Powell Jr, Surgery Branch, National Cancer Institute, National Institutes of Health, Clinical Research Center, Room 3W-3888, 10 Center Drive, MSC 1201, Bethesda, MD 20892-1201 (e-mail: Daniel_Powell@nih.gov).

Interleukin-2 (IL-2) is a cytokine primarily produced by human T-helper lymphocytes that mediates multiple immune regulatory effects, including the expansion of lymphocytes after the activation by specific antigen. Administration of high-dose recombinant IL-2 to humans can mediate the regression of bulky, invasive tumors in a subset of patients with metastatic melanoma and renal cancer.[1–3] In IL-2 clinical studies, 15% to 20% of patients with these metastatic cancers experienced an objective cancer regression, nearly half of which resulted in complete regression.[4] The mechanism of IL-2–mediated cancer regression has not been fully elucidated, however, IL-2 has no direct impact on cancer cells, which can grow unimpeded in vitro in high concentrations of IL-2. In mice, radiation induced lymphoablation before IL-2 administration abrogates the antitumor effects of IL-2 seen in immunocompetent hosts, suggesting that the impact of IL-2 on cancers in vivo is immune-mediated and may derive from its ability to expand lymphocytes with antitumor activity.[5] Cancer patients often possess circulating melanoma antigen-specific T cells and tumor-infiltrating lymphocytes (TIL) with antitumor reactivity can be isolated and expanded from progressive melanoma lesions, however, their role in IL-2–mediated cancer regression remains unclear.[6–8]

One factor that might inhibit the potential for antitumor responses in IL-2–treated patients is the recognized ability of IL-2 to mediate the in vivo expansion of a subset of $CD4^+$ T cells known as regulatory T (Treg) cells in both humans and mice.[9–11] Naturally occurring Treg cells possess the ability to maintain tolerance to self-proteins and can inhibit immune reactions.[12] Treg cells are phenotypically characterized by their constitutive expression of activation-associated proteins such as IL-2 receptor-α (CD25), cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), and the transcription factor Forkhead box P3 (FOXP3).[12] In humans, the importance of Treg cell-mediated maintenance of peripheral self-tolerance is noted in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), an early-onset, recessive and frequently fatal disorder that results from loss of function mutations of *FOXP3* and a consequential lack of functional Treg cells.[13–16] Because

many cancer antigens are derivatives of aberrantly expressed self-tissue proteins,[17] IL-2 induction of Treg cells may represent an impediment to the success of cancer immunotherapies which use IL-2.[18] The generation of Treg cells may also explain why melanoma patients who experience an objective response and then recur do not rerespond to a second IL-2 treatment.[19] In mice, $CD25^+$ Treg cells inhibit the antitumor activity of melanoma-reactive $CD8^+$ effector cells in the presence of exogenous IL-2 in vivo, whereas CD25-depleted CD4 T (T helper) cells augment this activity.[20] Furthermore, the survival and suppressive mechanism of mouse Treg cells is IL-2 signal-dependent as $CD25^{+/+}$ Foxp3+ Treg cells, but not $CD25^{-/-}$ Foxp3+ T cells, can suppress T-helper cells, prevent effective expansion of $CD8^+$ T cells and inhibit tumor treatment in vivo.[9] These observations provide the basis for developing novel immunotherapeutic strategies which neutralize Treg cells in vivo to bolster local antitumor immune responses.

Because melanoma patients have naturally circulating antimelanoma effector cells, we hypothesized that transient elimination of Treg cells will enhance the clinical effectiveness of IL-2 administration. We have recently reported on the ability to selectively deplete Treg cells from patient peripheral blood mononuclear cells (PBMC) by removing CD25-expressing cells using a clinical-grade, large-scale immunomagnetic system.[21] CD25-depleted cell populations were comprised of monocytes and lymphocytes, including $CD8^+$ T cells and $CD4^+CD25^-$ T-helper cells, but devoid of Treg cells as demonstrated by *FOXP3* RNA and CD25 protein expression analyses. Since nonmyeloablative but lymphodepleting chemotherapy transiently purges lymphocytes in vivo including Treg cells, we hypothesized that the transfer of CD25-depleted PBMC to lymphodepleted hosts might result in the restoration of a Treg cell-deficient immune compartment in vivo. In the current clinical trial, 5 patients with IL-2 refractory metastatic melanoma were administered a nonmyeloablative but lymphodepleting chemotherapy followed by the autologous transfer of Treg cell-depleted PBMC and high-dose IL-2. This regimen was not capable of mediating prolonged elimination of Treg cells in vivo.

MATERIALS AND METHODS

Treatment Regimen

All patients in this study had metastatic melanoma and were entered on institutional review board-approved protocols in the Surgery Branch of the National Cancer Institute. Informed consent was obtained from all subjects. All enrolled patients underwent apheresis (≥ 20 L) and subsequent nonmyeloablative lymphodepleting conditioning with 2 days of cyclophosphamide (60 mg/kg) followed by 5 days of fludarabine (25 mg/m^2). Over this time, leukapheresis cell products were depleted of CD25-expressing cells as described previously,[21] measured for a $>80\%$ reduction in CD25 expression by cytometric analysis and sufficient cell number (3×10^9 to 3×10^{10}) and cryopreserved until day of transfer. Two days after the final dose of fludarabine (day 0), cryopreserved CD25-depleted cells were thawed, measured for total cell number and viability, and administered intravenously to autologous patients. Beginning ≥ 6 hours after cell infusion, infused patients received the first cycle of high-dose IL-2 (720,000 IU/kg; Chiron Corporation, Emeryville, CA) by bolus intravenous infusion every 8 hours to tolerance. Ten to 14 days after the first cycle of IL-2, patients received a second cycle of high-dose IL-2. Four to 6 weeks later, patients were evaluated for tumor response and toxicity.

Cell Preparation and Depletion of $CD25^+$ T Cells Ex Vivo

Depletion of $CD25^+$ T cells was performed as described[21] using the CliniMACS^{PLUS} Instrument (Miltenyi Biotech, Auburn, CA) according to standard user protocol. Donor PBMC were obtained by leukapheresis. In some cases, PBMC were purified on Ficoll-Hypaque step gradients (LSM Lymphocyte Separation Medium; ICN Biochemicals, Aurora, OH). In brief, the leukapheresis product was washed and resuspended in CliniMACS PBS/EDTA (Miltenyi Biotech) supplemented with human serum albumin (ZLB Bioplasma Inc, Glendale, CA) in a cell preparation bag to which one vial of CliniMACS CD25 Reagent (Miltenyi Biotech) was added. Incubation was performed for 30 minutes at room temperature on an orbital shaker. Cells were washed, resuspended, and applied to the CliniMACS^{PLUS} Instrument with the depletion 2.1 program selected. Upon completion of the depletion program, CD25-depleted cells and the $CD25^+$ cell fraction were collected in separate collection bags. CD25-depleted cells were cryopreserved in 40% Plasmalyte A (Baxter, Deerfield, IL), 10% DMSO (Edwards Lifesciences, Irvine, CA), human serum (Valley Biomedical, Winchester, VA), 15 USP units/mL heparin sodium (American Pharmaceutical Partners, Inc, Schaumburg. IL), 25 U/mL DNase (Genentech, Inc, San Francisco, CA) in a controlled-rate freezer (Planer plc, Middlesex, UK). On the day of cell transfer, CD25-depleted cells were thawed, counted, and assessed for viability before infusion.

Monoclonal Antibodies and Flow Cytometric Immunofluorescence Analysis

Fluorescein isothiocyanate-conjugated antihuman CD4, CD8, CD20, and CD16 antibodies and APC-labeled CD3 antibody were all obtained from BD Biosciences (San Jose, CA). For CD25 detection, PE-conjugated CD25 antibodies (4E3 clone; Miltenyi Biotech) were used. FOXP3 (clone PCH101) and control rat IgG2a antibodies were obtained from eBiosciences. Fresh or thawed cells were resuspended in FACS buffer consisting of PBS with 2% FBS (Gemini Bioproducts, Woodland, CA) at 10^7 cells/mL and blocked with 10% normal mouse Ig (Caltag Labs, Burlingame, CA) for 10 minutes on ice. Cells (10^6) in 100 μL were stained with fluorochrome-conjugated mABs at 4°C for 40 minutes in the dark. Cells were washed twice, briefly stained with

propidium iodide for nonviable cell exclusion and subsequently analyzed in a FACSCalibur (BD Biosciences). FOXP3 staining was performed according to manufacturer's instructions (eBiosciences).

Culture Media

Complete media consisted of RPMI 1640 (Invitrogen Corp, Carlsbad, CA) supplemented with 2 mM of glutamine (Biofluids, Rockville, MD), 25 mM of HEPES buffer (Biofluids), 100 U/mL of penicillin (Biofluids), 100 μg/mL of streptomycin (Biofluids), 50 μM of 2-mercaptoethanol (Invitrogen), and 10% heat-inactivated human AB sera (Gemini Bioproducts). $CD4^+$ T-cell enrichments were performed using an indirect magnetic labeling system, the $CD4^+$ T-Cell Isolation Kit II (Miltenyi Biotech).

Suppression Assays

Cryopreserved cell samples were thawed, washed, stained with specific antibodies and sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations using a FACSVantage SE flow cytometry system (BD Biosciences). Cocultures containing 2.5×10^4 $CD4^+CD25$ negative effector T cells, equal numbers of $CD4^+CD25^+$ Treg cells and 2.5×10^5 autologous CD3-depleted feeder cells (4000 rad) were established in complete media in 96-well U-bottom plates, in triplicate. $CD3^+$ cells were removed from feeder populations by immunomagnetic depletion (Dynal ASA, Oslo, Norway). Independent cultures of $CD4^+CD25^+$ Treg or $CD4^+CD25$ negative effector T cells were cultured as controls required for determination of percent suppression. Cell cultures were stimulated with 2.5 μg/mL soluble anti-CD3 (OKT3) antibody, incubated at 37°C for 2 days and pulsed with 1uCi [^{3}H]-thymidine per well for the final 18 hours of incubation. Plates were harvested onto nylon filters using the Betaplate system and radioactivity quantified using a Betaplate counter. Results are expressed in counts per minute as the mean of triplicate cultures ± SEM. Percent suppression was calculated as $1 - [(CD25^+{:}CD25^-$ mixed culture proliferation $- CD25^+$ cell proliferation)/ $CD25^-$ cell proliferation$] \times 100$.

RNA Isolation and cDNA Synthesis

Total RNA was isolated and cDNA synthesized as previously reported.[21]

Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) for quantitative mRNA expression analysis, as previously described.[21] Primers and probes for β-actin[22]: forward 5′-GCGAGA-AGATGACCCAGGATC-3′, reverse 5′-CCAGTGGTA-CGGCCAGAGG-3′, TaqMan probe 5′(FAM)-CCAG-CCATGTACGTTGCTATCCAGGC-(TAMRA)3′ were synthesized by Applied Biosystems. For analysis of Foxp3, Assay on Demand (Applied Biosystems) primers and probes were used.

RESULTS

Patient Selection and Treatment

Six HLA-A*0201-negative patients with progressive metastatic melanoma were selected for CD25-depleted PBMC transfer and IL-2 therapy (Table 1). All patients were refractory to prior treatment with high-dose IL-2. Patients underwent apheresis (≥ 20 L) and subsequent nonmyeloablative lymphodepleting conditioning with 2 days of cyclophosphamide (60 mg/kg) followed by 5 days of fludarabine (25 mg/m^2). Apheresed PBMC were depleted of CD25-expressing cells by positive magnetic separation and cryopreserved until day of infusion.[21] Patient 3 had 2 pheresis samples (referred to hereafter as

TABLE 1. Demographics, Prior Therapies, and Treatment of Patients Receiving CD25-depleted PBMC and IL-2 Therapy

				Treatment		
Patient No.	Age/Sex	Prior Therapies	Sites of Disease	$CD25^-$ Cell No.	IL-2 Doses	Side Effects
1	53/M	IFN, ONTAK, HD IL-2	Lungs, mediastinum, perinephric mass, LN, SQ, small bowel	5.5E + 09	14, 15	None
2	55/F	HD IL-2	Lungs, breast, buttock, paraspinal, adrenal, kidney	3.3E + 09	10, 0	Somnolence, pleural effusion, SVT, Clostridium difficile colitis
3	36/M	S, IFN, HD IL-2	Axilla, liver, spleen	8.4E + 09	5, 0	Typhlitis
4	50/M	HD IL-2	Lung, axilla, calf, liver	N/A	N/A	Not treated
5	49/M	S, IFN, HD IL-2	Adrenal, flank, chest wall, scapula	1.19E + 10	14, 7	Tachycardia
6*	51/M	S, HD IL-2, ONTAK, MDX	Hilum, lung	1.34E + 10	15, 7	None

Non-HLA-A2 patients with metastatic melanoma refractory to high-dose IL-2 therapy were treated with CD25-depleted PBMC transfer and IL-2 therapy.

*Patient 6 had recurrent disease after previously experiencing a complete response to IL-2 therapy. The number of transferred CD25-depleted PBMC ($CD25^-$ cell no.) and number of IL-2 doses administered (first cycle, second cycle) are shown. All patients received high-dose (HD; 720,000IU/kg) exogenous IL-2 administration every 8 hours to tolerance subsequent to cell transfer.

HD IL-2 indicates high-dose IL-2; IFN, interferon; LN, lymph node; MDX, anti-CTLA-4 antibody; ONTAK, denileukin diftitox; S, surgery; SQ, subcutaneous; N/A, not applicable.

samples 3.1 and 3.2) that were independently depleted of $CD25^+$ cells and combined for therapy. Two days after lymphodepleting chemotherapy, cryopreserved CD25-depleted cells were thawed, measured for total cell number and viability, and administered intravenously to autologous patients. Numbers of infused CD25-depleted PBMC ranged from 3.3×10^9 to 1.3×10^{10} cells. One patient, patient 4, was taken off protocol after the first dose of chemotherapy and before CD25-depleted PBMC infusion after developing severe fevers and hypotension related to gram-positive infection. Six hours after cell infusion, patients received the first cycle of high-dose IL-2 by bolus intravenous infusion every 8 hours to tolerance. About 2 weeks after the first cycle of IL-2, patients received a second cycle of high-dose IL-2. Patients 2 and 3 did not receive a second cycle of IL-2 owing to the development of significant side effects consistent with those associated with IL-2 administration.[23] No patient developed adverse events distinctly attributable to CD25-depleted cell infusion. Observed side effects were consistent with those related to chemotherapy preparative regimen, IL-2 administration or metastatic melanoma. Six to 8 weeks after CD25-depleted PBMC infusion (4 to 6 wk after the second cycle of IL-2), patients were evaluated for tumor response and toxicity. No patient treated on this study experienced objective cancer regression; all patients had progressive disease.

Selective Depletion of CD25-expressing Cells From PBMC for Patient Infusion

Flow cytometric analysis was performed on predepletion and postdepletion cell products to evaluate efficiency of the CD25 depletion after magnetic selection. Predepletion pheresis samples and CD25-depleted cells showed normal forward and side scatter profiles with lymphocyte and monocyte populations evident (not shown). Compared with predepletion pheresis samples, which contained a subset of $CD4^+$ T cells expressing high levels of CD25, little to no CD25 was expressed by $CD4^+$ or $CD4^-$ ($CD8^+$) T lymphocytes in postdepletion samples (Fig. 1A). The efficiency of $CD4^+CD25^{HI}$ T-cell depletion was $\geq 99.5\%$ for all CD25-depleted cell samples (mean; $99.8 \pm 0.0\%$). Quadrant gating based on isotype antibody staining, which can be used to discriminate intermediate and high CD25-expressing cells from CD25-negative T cells, produced similar results with a range of depletion efficiency between 82.2% and 99.9% (mean; $93.7 \pm 2.7\%$). Selective elimination of CD25-expressing cells resulted in a small reduction in the overall percentage of $CD3^+$ T cells compared with predepletion pheresis samples (Fig. 1B). Within the $CD3^+$ cell population, CD25-depletion resulted in a selective reduction in $CD4^+$ T-cell frequency counterbalanced by an increase in $CD8^+$ T-cell percentage. These patterns of change within the T lymphocyte population were consistent among all postdepletion PBMC samples.

The goal of CD25-depletion was to selectively remove Treg cells, which are the only cells in the resting peripheral blood that express high levels of CD25. To assess whether CD25-depletion had successfully eliminated Treg cells from patient leukapheresis products, predepletion and postdepletion cell products were measured for the expression of *FOXP3* mRNA by TaqMan RT-PCR (Fig. 1C). Compared with the starting pheresis products, *FOXP3* mRNA expression was reduced by 77% to 99% in the total PBMC population after CD25-depletion (mean; $94.1 \pm 2.9\%$). To evaluate the impact of separation on FOXP3 protein expression by the $CD4^+$ T-cell population, intracellular FOXP3 antibody staining was performed. Before CD25-depletion, 3.9% to 13.5% of $CD4^+$ T cells expressed FOXP3 protein (Fig. 1D; mean, $8.1 \pm 1.5\%$). FOXP3 protein was limited to $CD4^+$ T cells and not expressed by $CD8^+$ T cells or B cells (not shown). $CD4^+$ T cells from CD25-depleted PBMC expressed FOXP3 protein at levels 51.1% to 88.0% lower than predepletion samples with a mean reduction of $73.9 \pm 6.1\%$. The overall frequency of $CD4^+$ T cells expressing FOXP3 in PBMC after depletion was 0.5% to 3.7% with a mean frequency of $2.0 \pm 0.5\%$. Remnant FOXP3-expressing cells appeared largely confined to CD4 cells expressing low to intermediate levels of CD25 expression (not shown).

After separation and phenotypic characterization, CD25-depleted PBMC samples were cryopreserved until the day of cell infusion. At the time of PBMC cryopreservation, the mean number of CD25-depleted PBMC available for infusion was $13.5 \pm 1.9 \times 10^9$, containing $3.8 \pm 0.7 \times 10^9$ T lymphocytes, $2.1 \pm 0.4 \times 10^9$ $CD8^+$ T cells, and $1.5 \pm 0.4 \times 10^9$ $CD4^+$ $CD25^-$ T cells. Cell viability after thawing was $81.5 \pm 2.2\%$ on average.

Effects of CD25-depleted PBMC Therapy on Peripheral Blood Lymphocytes

Treated patients experienced hematologic toxicities consistent with the lymphodepleting chemotherapy and IL-2 regimen including nadir absolute neutrophil counts in the range of 0.003 to 0.013×10^3 cells/μL which resolved to normal levels (1.32 to 7.50×10^3 cells/μL) 1 week after cell infusion, and low platelet counts of 6 to 21×10^3/μL that returned to normal numbers (154 to 345×10^3/μL) beginning about 10 days after cell infusion. Administration of cyclophosphamide and fludarabine with subsequent IL-2 also caused a severe but transient lymphopenia that lasted approximately 1 week after the last dose of fludarabine (Fig. 2A). At the time of CD25-depleted PBMC transfer, absolute lymphocyte counts were below the normal range (460 to 4700 cells/μL) at nadir values between 6 and 25 cells/μL. The recovery of absolute lymphocyte counts for the first four treated patients was unremarkable, returning to normal levels 5 to 11 days after cell infusion but remaining lower than pretreatment counts at day 10. Patient 6, who received the largest number of transferred CD25-depleted PBMC, experienced a lymphocytosis (> 4700 cells/μL) 9 days after infusion. About 3 weeks after cell infusion, when all patients' cell counts had returned to the normal range, patients 1, 5, and 6 received a second cycle of high-dose

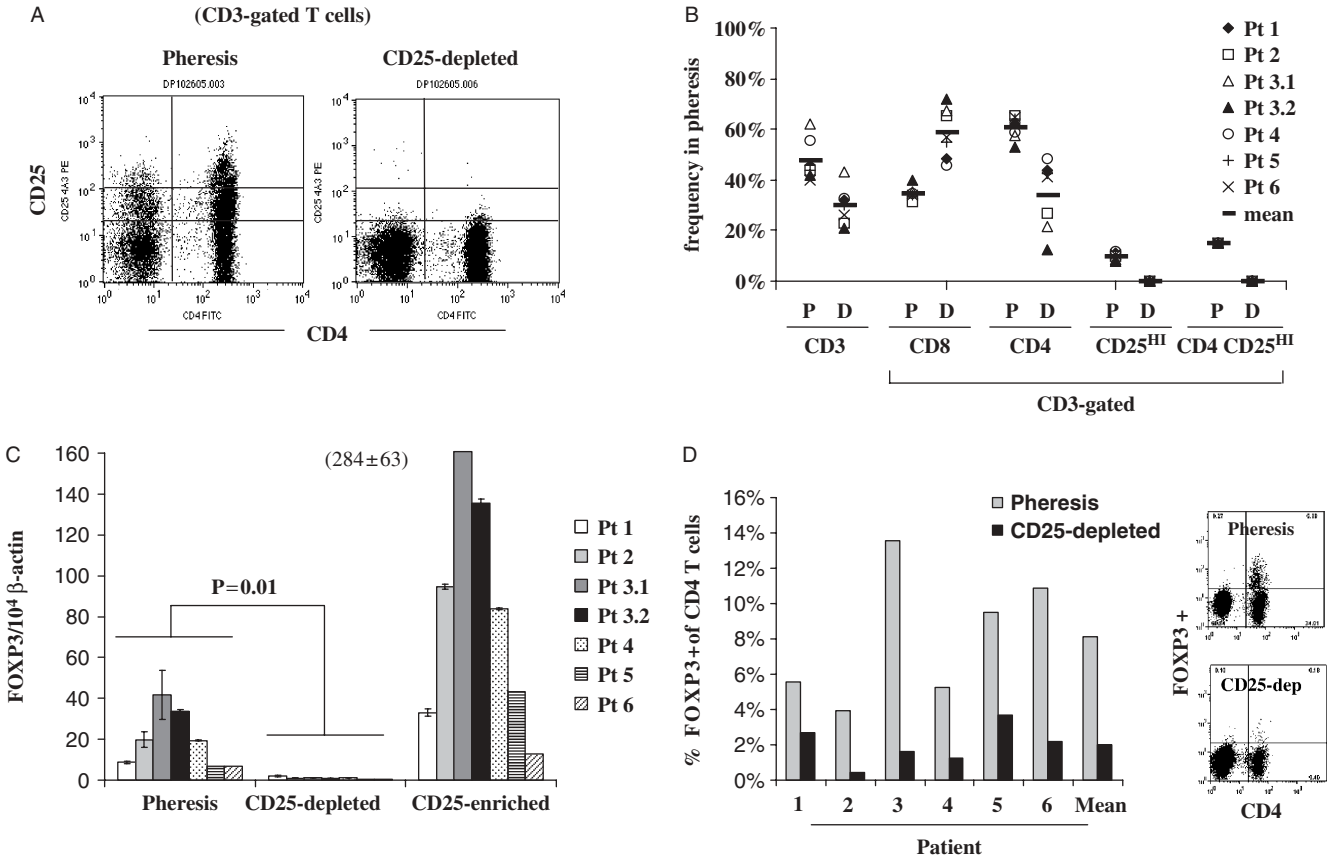

FIGURE 1. $CD4^+CD25^+$ Treg cells are selectively depleted from leukapheresis samples by CD25-depletion ex vivo. A, CD25-depletion by immunomagnetic separation results in successful elimination of CD25-expressing $CD4^+$ T cells from patient pheresis cell products. Representative dot plots of CD3-gated T cells from a pre (pheresis, left) and post-CD25-depletion leukapheresis product (right) from patient 2 are shown. Quadrant gating for CD25-high or CD25-intermediate (via isotype-gating) expression is shown. B, Changes in T-cell subset frequencies following CD25-depletion. The frequencies of $CD3^+$ T cells and $CD3^+$ T-cell subsets were measured in the starting pheresis sample (P) and the same pheresis sample after CD25-depletion (D) for all patients. Patient 3 had 2 pheresis products (Patients 3.1 and 3.2) which were independently depleted of CD25-expressing cells before being combined for therapy. C, *FOXP3* RNA expression is reduced in all pheresis cell products after CD25-depletion. The *y*-axis shows the mean number of *FOXP3* RNA copies per 10^4 β-actin copies expressed by the starting, CD25-depleted and CD25-enriched pheresis cell products from each patient as measured in triplicate by TaqMan RT-PCR. D, Immunomagnetic depletion of CD25-expressing cells also results in the selective reduction in $CD4^+$ T cells that express FOXP3 protein. The frequency of $CD4^+CD3^+$ T cells that express FOXP3 protein in the starting pheresis and CD25-depleted pheresis products are shown (left). CD4 and FOXP3 staining is shown in representative dot plots of CD3-gated T cells from a pre (pheresis, right top) and post-CD25-depletion leukapheresis product (right bottom) from patient 3.

IL-2 that resulted in transient lymphopenia, a subsequent lymphocyte rebound and ultimate restoration of normal cell counts. Patient 6 experienced a substantial lymphocytosis of nearly 16,000 cells/μL after the second cycle of IL-2.

Before the treatment, lymphocyte subset counts were at or near normal levels, with CD4 > CD8 > NK cells (Fig. 2B). Within the first 3 weeks after adoptive transfer of CD25-depleted PBMC, $CD8^+$ T-cell counts had returned to the normal range (194 to 836 cells/μL) in the blood of all patients. $CD4^+$ T cells were evident at numbers below normal (358 to 1259 cells/μL) in patients 1, 2, and 3, but were at normal numbers in patients 5 and 6, whom had received the greatest number of transferred cells. After receiving a second cycle of IL-2, patients 1, 5, and 6 experienced a lymphocytic burst comprised of $CD8^+$ T cells, $CD4^+$ T cells, and NK cells. Interestingly, the majority of circulating lymphocytes in patients 5 and 6 at this time were NK cells, which were present at or above the normal cell numbers (87 to 505 cells/μL), respectively.

Rapid Recovery of $CD4^+CD25^+$ Regulatory T Cells In Vivo

As $CD4^+$ T cells were present in the lymphocyte population emerging after CD25-depleted PBMC transfer, posttransfer PBMC were evaluated for the presence of Treg cells. While CD25 was not detectable on the

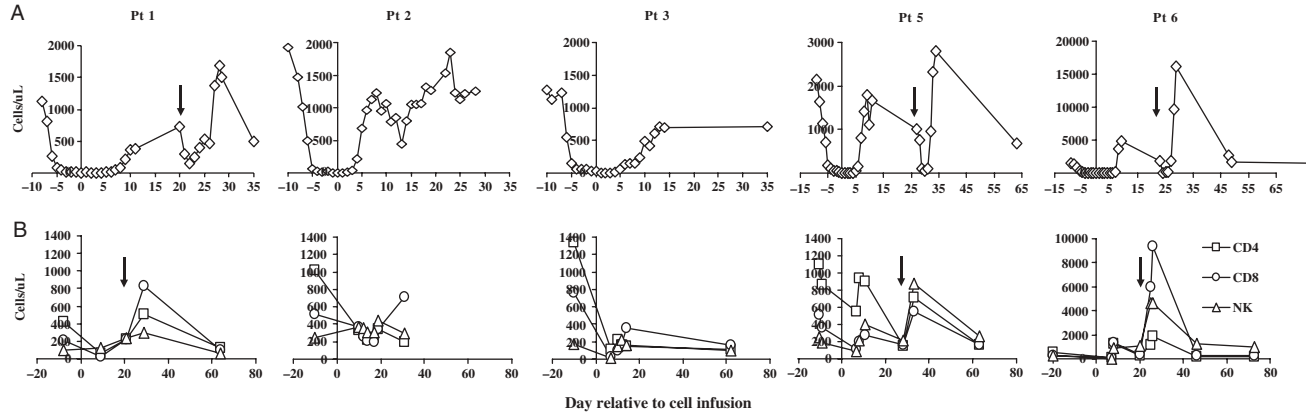

FIGURE 2. Lymphocyte recovery after lymphodepletion and subsequent CD25-depleted PBMC transfer. A, Absolute lymphocyte counts (cells/μL of blood) following nonmyeloablative but lymphodepleting chemotherapy, subsequent infusion of CD25-depleted PBMC and administration of high-dose IL-2 to tolerance. B, Cell subset analysis shows the composition of lymphocytes emerging after CD25-depleted cell infusion and IL-2 administration. The absolute number of $CD4^+$ T cells (squares), $CD8^+$ T cells (circles), and NK cells (triangles) in the peripheral blood are shown for each patient. Arrows indicate the timing of the second IL-2 cycle administration for patients 1, 5, and 6.

surface of PBMC used for transfer, $CD4^+$ T cells emerging in the blood in the early aftermath of cell infusion expressed high levels of CD25 (Figs. 3A, B). CD25 expression by $CD4^+$ T cells peaked 7 to 12 days after cell infusion with peak $CD25^{HI}$ frequencies ranging between 49.6% and 78.3% of circulating $CD4^+$ T cells in 4/5 patients. Reemerging $CD4^+$ T cells from patient 1, who had received the maximum allotted dose of IL-2, expressed CD25 (14.3%) at levels similar to that observed before treatment (15%). Three weeks after infusion, CD25-expressing $CD4^+$ T-cell frequencies remained elevated over pretreatment levels in 4/5 patients. Administration of a second cycle of high-dose IL-2 resulted in a subsequent spike in CD25 expression levels in patients 5 and 6 (from 33.4% to 76.9% and from 56.1% to 74.5%, respectively), whereas $CD4^+$ T cells from patient 1 appeared unaffected (11.0% to 9.9%). At the latest time point measured, $CD25^{HI}CD4^+$ T-cell frequencies remained elevated compared with pretreatment in levels in all patients except patient 1.

$CD25^{HI}CD4^+$ T cells emerging in the blood after CD25-depleted PBMC transfer were evaluated for their phenotypic characteristics. Representative results from the phenotypic analysis of PBMC from patient 2 are shown (Fig. 3B). Before separation, a small portion of $CD25^+CD4^+$ T cells were shown to express Treg cell-associated proteins including FOXP3, GITR, and CTLA-4. CD27 costimulatory molecule and CD45RO expression was also detectable on most $CD25^+CD4^+$ T cells while expression of the lymph node-homing molecule CD62L was mixed and HLA-DR expression low. CD25-depleted $CD4^+$ T cells for transfer expressed little FOXP3, GITR, CTLA-4, and HLA-DR and possessed both high and low CD27, CD62L, and CD45RO subpopulations. After autologous transfer of CD25-depleted PBMC and IL-2 administration to lymphodepleted recipients, emerging $CD25^+CD4^+$ T cells expressed high levels of Treg cell-associated molecules FOXP3, GITR, and CTLA-4, as well as CD27, CD45RO, and HLA-DR suggestive of an antigen experienced population.

Although human $CD4^+$ T cells can be induced to express FOXP3 by in vitro activation, the only cells known to express FOXP3 protein in the resting peripheral blood are Treg cells.[15] To determine the contribution of Treg cells to the emerging CD4 population after transfer, FOXP3 protein expression analysis was performed on all available patient PBMC samples. Although FOXP3 expression by $CD4^+$ T cells was low in the CD25-depleted PBMC used for infusion (0.5% to 3.7%), the frequency of $FOXP3^+$ CD4 T cells emerging in the blood after transfer ranged from 18.3% to 63% of circulating $CD4^+$ T cells (Fig. 3C). This represented a significant increase in frequency compared with levels detected in pretreatment samples of all patients ($P < 0.006$). FOXP3+ $CD4^+$ T cells from the blood of patient 1 were present at a lower overall frequency compared with the remaining patients, yet was elevated compared with pretreatment levels. FOXP3 expression by $CD8^+$ T cells was not substantially detectable at any time point. Secondary IL-2 dosing caused a resultant increased in FOXP3 expression by $CD4^+$ T cells, similar to that seen for CD25 expression. The pattern of longitudinal FOXP3 expression by $CD4^+$ T cells nearly mirrored that of the $CD25^{HI}CD4^+$ T-cell population at all time points and regression analysis revealed a significant correlation between the frequency of $CD25^{HI}$ and FOXP3+$CD4^+$ T cells ($P < 0.0001$; $R^2 = 0.831$).

Because frequency analysis does not adequately address cell numbers, circulating FOXP3+ $CD4^+$ T-cell count was enumerated. FOXP3+ $CD4^+$ T-cell numbers ranged from 23 to 180 cell/μL of blood in patients before therapy (Fig. 3D). In comparison, the number of

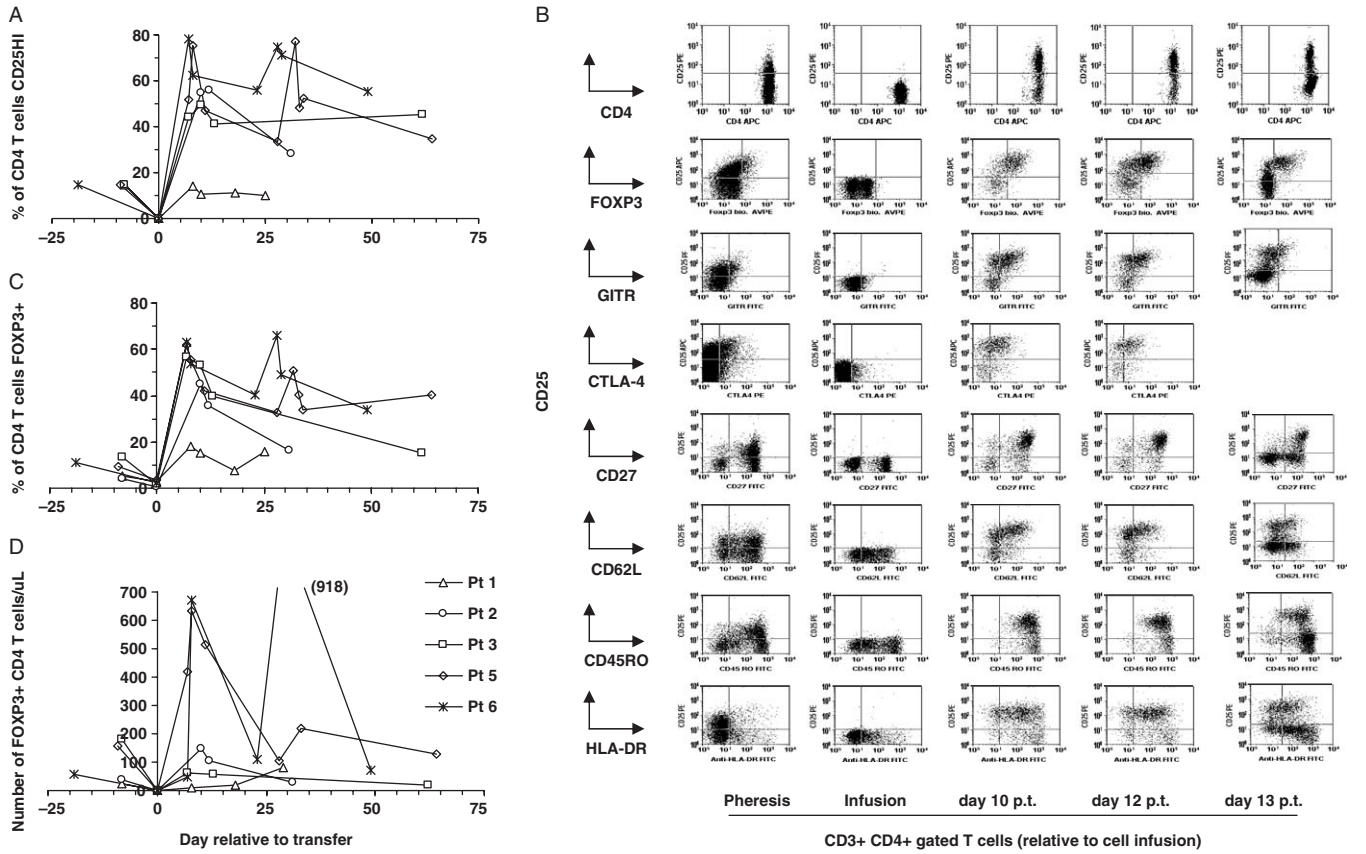

FIGURE 3. Vigorous emergence of Treg cells in lymphodepleted patients after CD25-depleted PBMC transfer and IL-2 dosing. A, The frequency of $CD4^+$ T cells that express high levels of CD25 is elevated in the peripheral blood of patients posttherapy and augmented by subsequent IL-2 administration. All $CD4^+$ T cells were gated for $CD4^+$ $CD3^+$ events. B, Longitudinal phenotypic analysis of $CD25^+$ $CD4^+$ T cells in the pretreatment pheresis, the CD25-depleted PBMC sample (infusion), and in the peripheral blood 10, 12, and 31 days posttransfer (p.t.). Representative staining of patient 2 cell samples is shown. All plots were pregated on $CD4^+CD3^+$ events. CD25 expression is shown on the *y*-axis of each dot plot whereas the expression of the indicated protein is shown on the *x*-axis. C, FOXP3+ $CD4^+$ T-cell frequency is elevated in the peripheral blood of patients after transfer of CD25-depleted PBMC and IL-2 administration to lymphodepleted patients. All $CD4^+$ T cells were gated for $CD4^+CD3^+$ events. D, The number of $CD4^+$ T cells that express FOXP3 protein is transiently elevated in the posttherapy peripheral blood of select patients and augmented by subsequent IL-2 administration. $FOXP3^+$ $CD4^+$ T-cell number was calculated as the number of $CD4^+CD3^+$ cells/μL of blood multiplied by the percentage of $CD4^+$ T cells that express FOXP3 at the indicated time point. The *x*-axis for graphs A, C, and D indicates time in days relative to cell infusion.

FOXP3+ $CD4^+$ T cells was transiently elevated in 3/5 patients (patients 2, 5, and 6) 7 to 10 days after CD25-depleted PBMC transfer and IL-2 administration. This represented a 3.7-fold, 4.1-fold, and 12.0-fold increase in total number, respectively. FOXP3+ $CD4^+$ T cells peaked at nearly 650 cell/μL of blood from patients 5 and 6. The absolute number of FOXP3+ $CD4^+$ T cells in the blood of patients 1 and 3 one week after transfer were reduced from 23 to 10 cells/μL (56.5% reduction) and from 180 to 60 cells/μL (66.6% reduction), respectively, perhaps reflecting the indolent reconstitution of lymphocytes in these patients. A secondary spike in FOXP3+ $CD4^+$ T-cell numbers was induced in all patients receiving a second cycle of IL-2. Patients 1, 5, and 6 experienced 4.8-fold, 2.1-fold, and 8.6 fold increases, in that order. The difference in absolute number of FOXP3+ $CD4^+$ T cells in the blood of all patients before therapy compared with the latest time point measured was not statistically significant ($P = 0.538$).

$CD25^{HI}CD4^+$ T Cells Emerging After Cell Infusion Possess Suppressive Activity

To evaluate the suppressive capacity of $CD25^{HI}CD4^+$ T cells emerging in the blood after CD25-depleted PBMC transfer, PBMC from patient 2 collected 12 days after cell therapy were purified into $CD25^{HI}$ and CD25-negative $CD4^+$ T-cell populations by FACS, with $CD4^+$ T-cell subpopulation purities of $\geq 98\%$ (Fig. 4). Compared with CD25-negative $CD4^+$ effector T cells, $CD25^{HI}CD4^+$ T cells were hypoproliferative after 72-hour stimulation with anti-CD3 antibody and irradiated autologous CD3-depleted feeder cells, a

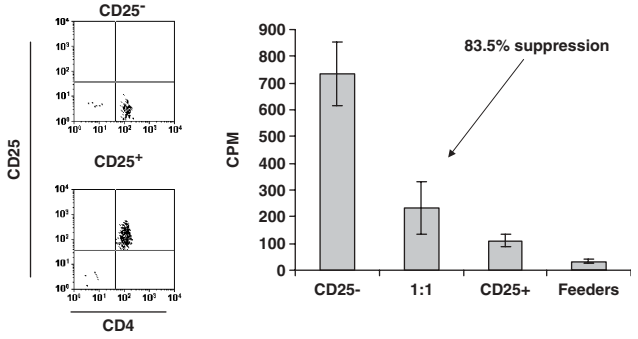

FIGURE 4. Functional suppression by $CD4^+CD25^+$ Treg cells emerging after CD25-depleted PBMC infusion. PBMC from patient 2 collected 12 days after cell infusion were sorted into $CD25^+$ and CD25-negative $CD4^+$ T-cell populations (left) and used in a standard 72 hour ^{3}H-thymidine incorporation assay (right). Dot plots on the left show the purity of $CD25^+$ and CD25-negative $CD4^+$ T-cell populations used. The graph on the right shows proliferation of cultures containing 2.5 μg/mL anti-CD3 antibody and irradiated autologous CD3-depleted PBMC (feeders) alone or with CD25-negative $CD4^+$ T cells, $CD25^+CD4^+$ T cells, or $CD25^+$ and CD25-negative $CD4^+$ T cells at a 1 to 1 ratio. Values represent counts per minute ± SEM.

common characteristic of Treg cells in vitro.[12] Coculture of $CD25^{HI}CD4^+$ Treg cells and CD25-negative $CD4^+$ effector T cells at equal numbers resulted in an 83.5% suppression of effector cell proliferation. Independent analysis of $CD25^{HI}CD4^+$ Treg cells from another patient, patient 5, collected 8 days postinfusion yielded the same result, with 89.6% suppression of CD25-negative $CD4^+$ T-cell proliferation (not shown).

DISCUSSION

Animal models have demonstrated that selective depletion of Treg cells in lymphopenic mice can result in the development of severe autoimmunity and augment antitumor responses in vivo; however, the role of Treg cells in human tumor immunity is less well understood.[24–26] Higher than normal frequencies of Treg cells have been reported in the peripheral blood of patients with some solid and hematologic cancers.[26] In ovarian carcinoma, tumor-infiltrating Treg cells were reported to contribute to growth of human tumors in vivo by suppressing tumor-specific T-cell immunity and were reportedly associated with a high death hazard and reduced survival,[27] although these findings were not reproduced in another study.[28] Patients with melanoma often harbor TIL which are reactive with their own tumor cells yet, even in their presence, disease progression occurs.[29] Since self proteins, including the melanocyte differentiation antigens MART-1, gp100, and tyrosinase, represent common targets of human melanoma-reactive T cells,[17] Treg cell-mediated suppression has been proposed as a potential mechanism to explain the lack of antitumor responses in these patients. Indeed, patients with metastatic melanoma harbor circulating Treg cells with suppressive activity[30] and melanoma antigen-specific Treg cells have been described.[31,32] In the current study, $8.1 \pm 0.5\%$ of $CD4^+$ T cells in nonseparated patient leukapheresis samples were Treg cells by FOXP3 protein expression analysis, with a range of 3.9% to 13.5%. In addition, Treg cells are reportedly overrepresented in human metastatic melanoma lymph nodes and can inhibit the function of infiltrating T cells.[33] Thus, the negative immunoregulatory effects of Treg cells may explain the poor clinical response rates reported in cancer patients receiving nonimmunodepleting immunotherapies. Accordingly, novel immunotherapeutic approaches designed to neutralize the suppressive effects of human Treg cells in vivo and bolster antitumor immune responses are warranted.

In the current study, a combinatorial approach of lymphodepleting preconditioning and subsequent infusion of Treg cell-depleted PBMC was administered to patients with metastatic melanoma to assess whether this treatment regimen, by analogy to animal studies of $CD25^+$ cell-depletion,[20,34–36] might result in restoration of a Treg cell-deficient immune system and the induction of self/tumor antigen-directed immune responses in vivo. As we were giving IL-2 and Treg cell-depleted PBMC in the lymphodepleted setting, we chose to treat patients whom were refractory to high-dose IL-2 therapy. It is unknown whether this selection criterion compromised the result. Because Treg cells are the only lymphocytes that express high levels of CD25 in the resting peripheral blood, CD25 was chosen as a selectable protein target for their elimination ex vivo. In accordance with our previous findings, selective depletion of CD25-expressing cells from patient leukapheresis samples using a clinical-grade immunomagnetic system resulted in a $CD25^+$ Treg cell-depleted product of sufficient selection, recovery, and viability for use in adoptive immunotherapy.[21] Flow cytometry results showed the efficiency of $CD4^+CD25^{HI}$ T cell-depletion was nearly complete in all CD25-depleted cell products. By RNA analysis, the average reduction of *FOXP3* by CD25-depletion was 94%. FOXP3 protein analysis showed a 74% mean reduction in protein expression by $CD4^+$ T cells after depletion. Overall, the frequency of FOXP3 protein expressing $CD4^+$ T cells in the transferred cell product was low (0.5% to 3.7%) compared with nondepleted samples (3.9% to 13.5%). These data indicate that small numbers of human $CD4^+$ T cells that do not express high levels of CD25, express FOXP3 protein and RNA, a finding reported by others,[15] and demonstrates both the limitation of the current approach for some patients and the necessity for improved methods of Treg cell-depletion. It is not known whether human FOXP3+ CD25-depleted $CD4^+$ T cells possess suppressive activity. Although Foxp3+ $CD4^+$ T cells from $CD25^{-/-}$ mice can exhibit suppressive activity in vitro,[37] $CD25^{-/-}$ and IL-$2^{-/-}$ mice accumulate activated $CD4^+$ T cells, produce autoantibodies and develop autoimmune disease despite having Foxp3+ T cells, suggesting a lack functional suppression by these

cells in vivo.[38,39] This is further supported by the observation that $CD25^{-/-}$ Foxp3+ T cells, unlike $CD25^{+/+}$ Foxp3+ Treg cells, do not suppress the expansion or antitumor function of $CD8^+$ T effector cells in vivo.[40]

Recent reports suggest that low-dose cyclophosphamide therapy alone can decrease Treg cell numbers and functionality to enhance immune responses in vivo.[41–43] Further, preparative lymphoconditioning is thought to improve the host environment for cell transfer by removing endogenous suppressors cells, including Treg cells, and also cells which compete for APC interaction and homeostatic cytokines such as IL-7 and IL-15.[44] Patients on the current protocol were rendered lymphopenic by nonmyeloablative but lymphodepleting chemotherapy, and then administered CD25-depleted PBMC and high-dose IL-2. In rodent studies, transfer of Treg cell-depleted $CD4^+$ T cells to syngeneic lymphopenic recipients results in the spontaneous induction of organ-specific autoimmune diseases, including thyroiditis and gastritis and systemic wasting disease within a few months.[45,46] However, under the current clinical conditions, no patient (0/5) experienced an objective response or autoimmunity uniquely associated with CD25-depleted PBMC transfer. Patient accrual was closed after the fifth patient treatment when it was revealed that Treg cells were consistently and, in some cases, vigorously reconstituted after therapy. Recent studies suggested that lymphopenia coupled with IL-2 promotes robust homeostatic peripheral expansion of human $CD4^+CD25^{HI}$ T cells.[11] Early expansion and restoration of $CD25^+$ FOXP3+ $CD4^+$ Treg cells with suppressive function in the peripheral blood of CD25-depleted PBMC recipients suggests a role for these cells in the continued inhibition of self/tumor antigen-specific immune responses in vivo. Further, these findings indicate that the mere transfer of CD25-depleted cells alone into lymphopenic patients may not confer immunity. Indeed, autoimmunity is rarely induced in mice by depletion of $CD25^+$ cells alone but may be initiated by a second signal such as homeostatic proliferation, T cell receptor activation or inflammation.[47] By analogy, induction of antitumor responses and autoimmunity in patients receiving CD25-depleted cell transfer may require prolonged suppression of Treg cells, specific vaccination, and/or transfer of self/tumor antigen-reactive T cells, such as TIL. Accordingly, transfer of CD25-depleted PBMC to lymphopenic patients in the absence of IL-2 may help foster the generation of a Treg cell-depleted immune compartment in vivo, as reconstituting $CD4^+CD25^{HI}$ cell numbers are reduced for a longer period of time in patients after lymphodepleting chemotherapy alone, compared with those also receiving IL-2.[11]

$CD25^+$FOXP3+ Treg cells that reconstitute patients after cell transfer may represent the conversion of transferred CD25-depleted $CD4^+$ T cells, the vigorous expansion of a small number of Treg cells that survived lymphodepleting preconditioning and/or recent thymopoiesis. Support for the first 2 possibilities exists. Mouse $CD4^+CD25^-$ T cells have previously been shown to spontaneously convert into $CD4^+CD25^+$ Treg cells in vivo after transfer into syngeneic hosts,[48] although these findings conflict with the recent observation that mouse Foxp3-negative CD4 T cells do not convert into Foxp3+ T cells after transfer to lymphopenic hosts.[49] Human T cells may, however, be more susceptible than mouse cells to FOXP3 protein up-regulation after activation[15] or lymphopenia-induced homeostatic proliferation. Both murine and human $CD25^+CD4^+$ T cells preferentially expand in the presence of IL-2 and lymphopenia, thus chemotherapy-surviving Treg cells may expand under these favorable homeostatic conditions.[10,11,40] Treg cell reconstitution resulting from thymopoiesis is less likely since treated patients were generally older (mean age of 49 y) with presumably little thymic output and the restoration of Treg cells after infusion was vigorous and rapid. In other another study, reconstituting $CD25^+CD4^+$ T cells appeared to expand during lymphopenia and in response to IL-2 primarily as a result of thymic-independent homeostatic peripheral expansion.[11] In the absence of distinguishing markers for the transferred CD25-depleted PBMC population, it is not possible to determine the origin of the circulating Treg cell population emerging in the early aftermath of adoptive immunotherapy.

The results shown here demonstrate that Treg cell are rapidly restored after infusion of $CD25^+$ Treg cell-depleted PBMC into lymphopenic cancer patients receiving high-dose IL-2 and suggest that alternative and/or combinatorial approaches including cotransfer of tumor antigen-specific T cells and/or cancer vaccination may be necessary to render patients Treg cell-deficient and induce potent antitumor response in vivo.

REFERENCES

1. Fyfe G, Fisher RI, Rosenberg SA, et al. Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. *J Clin Oncol*. 1995;13:688–696.
2. Rosenberg SA, Lotze MT, Muul LM, et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med*. 1985;313:1485–1492.
3. Rosenberg SA, Yang JC, White DE, et al. Durability of complete responses in patients with metastatic cancer treated with high-dose interleukin-2: identification of the antigens mediating response. *Ann Surg*. 1998;228:307–319.
4. Rosenberg SA, Yang JC, Topalian SL, et al. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *JAMA*. 1994;271:907–913.
5. Rosenberg SA, Mule JJ, Spiess PJ, et al. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. *J Exp Med*. 1985;161:1169–1188.
6. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*. 2002;298:850–854.
7. Dudley ME, Wunderlich JR, Yang JC, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol*. 2005;23:2346–2357.
8. Pittet MJ, Valmori D, Dunbar PR, et al. High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large proportion of

human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J Exp Med*. 1999;190:705–715.
9. Antony PA, Paulos CM, Ahmadzadeh M, et al. Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. *J Immunol*. 2006;176:5255–5266.
10. Ahmadzadeh M, Rosenberg SA. IL-2 administration increases CD4+ CD25(hi) Foxp3+ regulatory T cells in cancer patients. *Blood*. 2006;107:2409–2414.
11. Zhang H, Chua KS, Guimond M, et al. Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells. *Nat Med*. 2005;11:1238–1243.
12. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*. 2004;22:531–562.
13. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001;27:20–21.
14. Chatila TA, Blaeser F, Ho N, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest*. 2000;106:R75–R81.
15. Gavin MA, Torgerson TR, Houston E, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci USA*. 2006;103:6659–6664.
16. Wildin RS, Ramsdell F, Peake J, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. 2001;27:18–20.
17. Rosenberg SA. Progress in human tumour immunology and immunotherapy. *Nature*. 2001;411:380–384.
18. Gattinoni L, Powell DJ Jr, Rosenberg SA, et al. Adoptive immunotherapy for cancer: building on success. *Nat Rev Immunol*. 2006;6:383–393.
19. Sherry RM, Rosenberg SA, Yang JC. Relapse after response to interleukin-2-based immunotherapy: patterns of progression and response to retreatment. *J Immunother*. 1991;10:371–375.
20. Antony PA, Piccirillo CA, Akpinarli A, et al. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol*. 2005;174:2591–2601.
21. Powell DJ Jr, Parker LL, Rosenberg SA. Large-scale depletion of CD25+ regulatory T cells from patient leukapheresis samples. *J Immunother*. 2005;28:403–411.
22. Schoof E, Girstl M, Frobenius W, et al. Decreased gene expression of 11beta-hydroxysteroid dehydrogenase type 2 and 15-hydroxyprostaglandin dehydrogenase in human placenta of patients with preeclampsia. *J Clin Endocrinol Metab*. 2001;86:1313–1317.
23. Parkinson DR, Abrams JS, Wiernik PH, et al. Interleukin-2 therapy in patients with metastatic malignant melanoma: a phase II study. *J Clin Oncol*. 1990;8:1650–1656.
24. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol*. 2002;2:389–400.
25. Yamaguchi T, Sakaguchi S. Regulatory T cells in immune surveillance and treatment of cancer. *Semin Cancer Biol*. 2006;16:115–123.
26. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol*. 2006;6:295–307.
27. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10:942–949.
28. Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA*. 2005;102:18538–18543.
29. Yannelli JR, Hyatt C, McConnell S, et al. Growth of tumor-infiltrating lymphocytes from human solid cancers: summary of a 5-year experience. *Int J Cancer*. 1996;65:413–421.
30. Javia LR, Rosenberg SA. CD4+CD25+ suppressor lymphocytes in the circulation of patients immunized against melanoma antigens. *J Immunother*. 2003;26:85–93.
31. Wang HY, Lee DA, Peng G, et al. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. *Immunity*. 2004;20:107–118.
32. Wang HY, Peng G, Guo Z, et al. Recognition of a new ARTC1 peptide ligand uniquely expressed in tumor cells by antigen-specific CD4+ regulatory T cells. *J Immunol*. 2005;174:2661–2670.
33. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol*. 2004;173:1444–1453.
34. Onizuka S, Tawara I, Shimizu J, et al. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res*. 1999;59:3128–3133.
35. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol*. 1999;163:5211–5218.
36. Sutmuller RP, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med*. 2001;194:823–832.
37. Fontenot JD, Rasmussen JP, Gavin MA, et al. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005;6:1142–1151.
38. Suzuki H, Kundig TM, Furlonger C, et al. Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. *Science*. 1995;268:1472–1476.
39. Willerford DM, Chen J, Ferry JA, et al. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*. 1995;3:521–530.
40. Antony PA, Restifo NP. CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2. *J Immunother*. 2005;28:120–128.
41. Ghiringhelli F, Larmonier N, Schmitt E, et al. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol*. 2004;34:336–344.
42. Glaser M. Augmentation of specific immune response against a syngeneic SV40-induced sarcoma in mice by depletion of suppressor T cells with cyclophosphamide. *Cell Immunol*. 1979;48:339–345.
43. Lutsiak ME, Semnani RT, De Pascalis R, et al. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood*. 2005;105:2862–2868.
44. Klebanoff CA, Khong HT, Antony PA, et al. Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends Immunol*. 2005;26:111–117.
45. Itoh M, Takahashi T, Sakaguchi N, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol*. 1999;162:5317–5326.
46. Sakaguchi S, Fukuma K, Kuribayashi K, et al. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance: deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med*. 1985;161:72–87.
47. McHugh RS, Shevach EM. Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J Immunol*. 2002;168:5979–5983.
48. Liang S, Alard P, Zhao Y, et al. Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T cells in vivo requires B7 stimulation, but not the thymus. *J Exp Med*. 2005;201:127–137.
49. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci USA*. 2005;102:5126–5131.

