

Interleukin-2 Administration Alters the CD4⁺FOXP3⁺ T-Cell Pool and Tumor Trafficking in Patients with Ovarian Carcinoma

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Abstract

Interleukin (IL)-2 is used in the immunotherapy of patients with certain cancer and HIV infection. IL-2 treatment reliably results in 16% to 20% objective clinical response rate in cancer patients, with significant durability of responses in selected patients. However, the mechanisms of therapeutic activity in responding versus nonresponding patients remain poorly understood. CD4⁺CD25⁺FOXP3⁺ regulatory T (Treg) cells contribute to immunosuppressive networks in human tumors. We treated 31 ovarian cancer patients with IL-2. We show that administration of IL-2 induces the proliferation of existent Treg cells in patients with ovarian cancer. The potency of Treg cell proliferation is negatively determined by the initial prevalence of Treg cells, suggesting that Treg cells are a factor for self-controlling Treg cell proliferation. After IL-2 cessation, the number of Treg cells more efficiently dropped in clinical responders than nonresponders. Furthermore, IL-2 treatment stimulates chemokine receptor CXCR4 expression on Treg cells, enables Treg cell migration toward chemokine CXCL12 in the tumor microenvironment, and may enforce Treg cell tumor accumulation. Our findings support the concept that administration of IL-2 numerically and functionally affects the Treg cell compartment. These data provide an important insight in evaluating the clinical benefit and therapeutic prediction of IL-2 treatment in patients with cancer. [Cancer Res 2007;67(15):7487–94]

Introduction

Ovarian cancer is the most lethal of the gynecologic cancers, with 14,500 deaths in the United States each year. Patients who have disease persisting after primary platinum-based chemotherapy are not able to attain sustained disease free status by the standard therapy (1). Most second-line cytotoxic therapies in this setting produce responses in the range of 6% to 12% with progression-free survivals of 9 to 13 weeks (2). Thus, novel therapy including immune-based therapy is warranted in treating ovarian cancer.

Interleukin (IL)-2 is used to boost immunity in patients with HIV infection (3–6) and with cancer including ovarian cancer, metastatic melanoma, and renal cell carcinoma (7–12). IL-2 treatment reliably results in a 16% to 20% objective clinical response rate in

cancer patients, with significant durability of responses in selected patients (7, 8, 13, 14). However, the mechanisms of therapeutic activity in responding versus nonresponding patients remain poorly understood. CD4⁺CD25⁺FOXP3⁺ regulatory T (Treg) cells maintain peripheral T-cell homeostasis (15, 16). Compelling evidence shows that Treg cells play a crucial role in tumor immune pathogenesis and temper immune therapeutic efficacy (15–21). Strikingly, the *in vitro* and *in vivo* mouse studies show that IL-2 is crucial for the production and function of Treg cells (22–25). Recent reports have documented that administration of IL-2 increased Treg cell pool in patients with melanoma (26, 27), renal cell carcinoma (27), and pediatric sarcoma (28). To further understand the effects of IL-2 on Treg cells, we studied the kinetic changes of Treg cell phenotype and function in patients with ovarian cancer undergoing IL-2 treatment.

On the other hand, high levels of CCL22 are found in human ovarian tumor environment (18, 29, 30), and Treg cells migrate into tumor environment through CCL22 and suppress T-cell effector function (18, 31). We thus also studied the potential role of IL-2 in modulating Treg cell chemokine receptor expression and potential tumor trafficking in patients with ovarian cancer.

Materials and Methods

Human subjects and clinical responses. Thirty-one patients with ovarian cancers sequentially entered into an open-labeled phase II IL-2 study. Patients received peritoneal infusion of proleukin (aldesleukin) rIL-2 at a dose of 6×10^5 IU/m² weekly for 16 consecutive weeks. This clinical trial was based on our previous report in treating ovarian cancer patients with IL-2 peritoneal infusion (7). Eligibility criteria included six or more courses of prior first- or second-line platinum-based chemotherapy and laparotomy-confirmed persistent or recurrent ovarian cancer. Complete clinical responses were defined by the disappearance of all clinically detectable lesions and no detection of new malignant lesions and confirmed by open laparoscopy or laparotomy. Progressive disease was defined by a $\geq 25\%$ increase in the product of perpendicular cross diameters at laparotomy or two-dimensional areas by exam, computer-assisted tomography or magnetic resonance imaging. Stable disease was defined as disease for which dimensions failed to meet the criteria defined above for progression or regression. Patients who withdrew from the trial due to various reasons were included in the Treg cell assessments in different time points and considered as nonresponders. Patients were followed until the date of death or until the date the study closed. Survival time calculations were computed from the time of the first dose of IL-2 to the completion of their follow-up. The study was approved by the Institutional Review Boards of the University of Pittsburgh and the University of Michigan.

Human cells. Peripheral blood mononuclear cells were obtained from ovarian cancer patients treated with IL-2 at different time points and from HLA-A2⁺ and HLA-A2⁻ healthy donors. CD14⁺ cells [antigen-presenting cells (APC)], CD4⁺CD25⁻ T cells, and CD4⁺CD25^{high} T cells were sorted with FACSaria (Becton Dickinson Immunocytometry Systems) for phenotyping

Note: S. Wei and I. Kryczek contributed equally to this work.

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and functional assays. Cell purity was >98% as confirmed by flow cytometry (LSR II, Becton Dickinson).

Human FOXP3 detection. Real-time reverse transcription-PCR was carried out for FOXP3 (upstream, 5'-cagctgccacactgcacctag-3'; downstream, 5'-catttccagcagtgaggtag-3') and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Complementary DNA was normalized against and expressed as fold differences relative to GAPDH (32) as described (18). FOXP3 protein was detected by intracellular staining with rat anti-human FOXP3 antibody (clone PCH101, e-Bioscience).

Treg cell-mediated immunosuppressive assay. T-cell immunosuppression was tested in a coculture system. CD4⁺CD25⁻ T cells (2×10^5 /mL) were stimulated with 2.5 μ g/mL antihuman CD3 (clone, UCHT1), 1.2 μ g/mL antihuman CD28 (clone CD28.2; Becton Dickinson Biosciences), and fresh monocytes (2×10^5 /mL) in the presence of different concentrations of CD4⁺CD25^{high} Treg cells. Seventy-two hours after coculture, T-cell proliferation was evaluated by thymidine incorporation.

Treg cell *in vitro* proliferation assay. CD4⁺CD25^{high} Treg cells and CD4⁺CD25⁻ T cells were sorted by FACSaria (Becton Dickinson) with high purity (>98%). The two populations were mixed based on the FOXP3 expression determined by flow cytometry analysis and cocultured with recombinant IL-2 (50–200 ng/mL; R&D Systems) or CD14⁺ cells (APCs; 2×10^5 /mL) in the presence of anti-CD3 (2.5 μ g/mL) plus anti-CD28 (1.2 μ g/mL). In some cases, the T cells were labeled before coculture with 10- μ m 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) for 10 min in the dark at 37°C. Cell proliferation was determined by CFSE dilution or antihuman Ki67 staining and was analyzed by flow cytometry analysis.

Treg cell chemokine receptor expression and migration assay. Chemokine receptor expression, including CCR4, CCR5, CCR6, CCR7, CCR8, CXCR1, CXCR3, and CXCR4, was analyzed on human CD4⁺FOXP3⁺ T cells. These CD4⁺FOXP3⁺ T cells were either from normal peripheral CD4⁺CD25^{high} cells (1×10^7 /mL) cultured with IL-2 (100 ng/mL) for 1 to 3 days or from peripheral CD4⁺CD25^{high} cells in ovarian cancer patients treated with IL-2 for different weeks.

Treg cell migration was assessed as we previously described (29) using human CD4⁺CD25^{high} Treg cells (5×10^4 – 20×10^4). Treg cells were induced to migrate with recombinant human CXCL12, CCL22 (100 ng/mL; R&D Systems), or ovarian tumor ascites. In some cases, mouse anti-human CCL22 [57226.11, immunoglobulin G2b (IgG2b); 500 ng/mL] and mouse anti-human CXCR4 (44717, IgG2b; 500 ng/mL) were added 2 h before migration assay. Identity of migrating Treg cells was further confirmed by fluorescence-activated cell sorting (FACS) for CD4 and FOXP3 expression.

Statistical analysis. Differences in cell-surface molecule expression were determined by χ^2 test, and in other variables by unpaired *t* test, with *P* < 0.05 considered significant. Clinical response rates were calculated for all patients with a final clinical evaluation as well as on an intention-to-treat basis. Median survival was calculated for all eligible patients including those without a final clinical evaluation. A test based on the Cox proportional hazards with a time-dependent covariate of the difference between the two groups was used to test survival differences between responders and nonresponders. A test based on the log-rank test was used to compare Kaplan-Meier survival curves between different tumor dimensions.

Results

IL-2 and APCs induce the proliferation of FOXP3⁺ T cells.

Exogenous IL-2 may increase the Treg cell pool in patients with melanoma (26, 27) and pediatric sarcoma (28). We tested whether IL-2 can induce human FOXP3⁺ T-cell proliferation. Normal peripheral blood CD4⁺ T cells were labeled with CFSE and stimulated with IL-2 for 2 weeks. We observed that FOXP3⁺ T cells actively entered into cell division (Fig. 1A). To determine whether FOXP3⁺ T-cell proliferation is unique to IL-2 stimulation, we exposed CD4⁺ T cells to APCs. In line with our observation on T cells stimulated with exogenous IL-2 (Fig. 1A), we showed that APCs induced Ki67⁺ cells in FOXP3⁺ T cells (Fig. 1B). Not only the percent

but also the mean fluorescence intensity (MFI) of FOXP3 expression was increased, suggesting novel FOXP3 synthesis in each individual FOXP3⁺ T cell. We further cultured CD4⁺ T cells with different concentrations of IL-2. We observed that 50 to 200 ng/mL IL-2 increased FOXP3⁺ T cells in a dose-dependent manner. IL-2 at <50 ng/mL induced minimal increase of FOXP3⁺ T cells whereas 200 ng/mL IL-2 induced maximal increase of FOXP3⁺ T cells (Fig. 1C). We used 100 ng/mL IL-2 in the following *in vitro* experiments. Our data indicate that exogenous IL-2 and APCs stimulate FOXP3⁺ T-cell proliferation.

IL-2 stimulates the proliferation of existent FOXP3⁺ T cells. IL-2 promotes FOXP3⁺ T-cell proliferation (Fig. 1). It is possible that IL-2 may stimulate existent FOXP3⁺ T-cell proliferation and/or IL-2 may initially convert FOXP3⁻ T cells into FOXP3⁺ T cells and subsequently stimulate FOXP3⁺ T-cell proliferation. To examine these possibilities, HLA-A2⁻CD4⁺CD25^{high} T cells, HLA-A2⁻CD4⁺CD25⁻ T cells, and HLA-A2⁺CD4⁺CD25⁻ T cells were sorted by FACSaria to high purity. The sorted CD4⁺CD25^{high} T cells with >85% FOXP3 expression and CD4⁺CD25⁻ T cells with <0.6% FOXP3 expression were selected for further experiments (Fig. 2, left). We proportionally mixed these three populations to reach a physiologic concentration of Treg cells (9% FOXP3⁺ cells; Fig. 2, center). We cultured the mixed cells with IL-2. We observed that IL-2 significantly increased the FOXP3⁺ T-cell population. The increased FOXP3⁺ T-cell population was predominantly HLA-A2⁻. There were <1% HLA-A2⁺FOXP3⁺ T cells (Fig. 2, right). We obtained

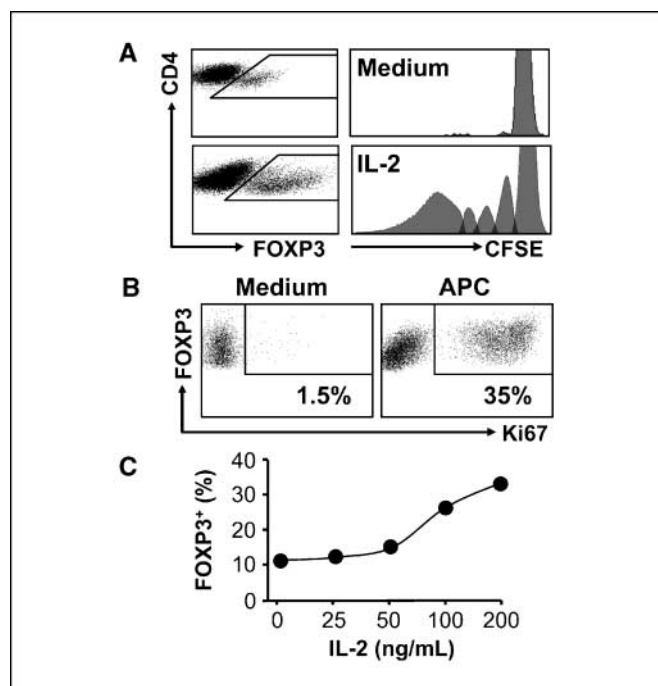
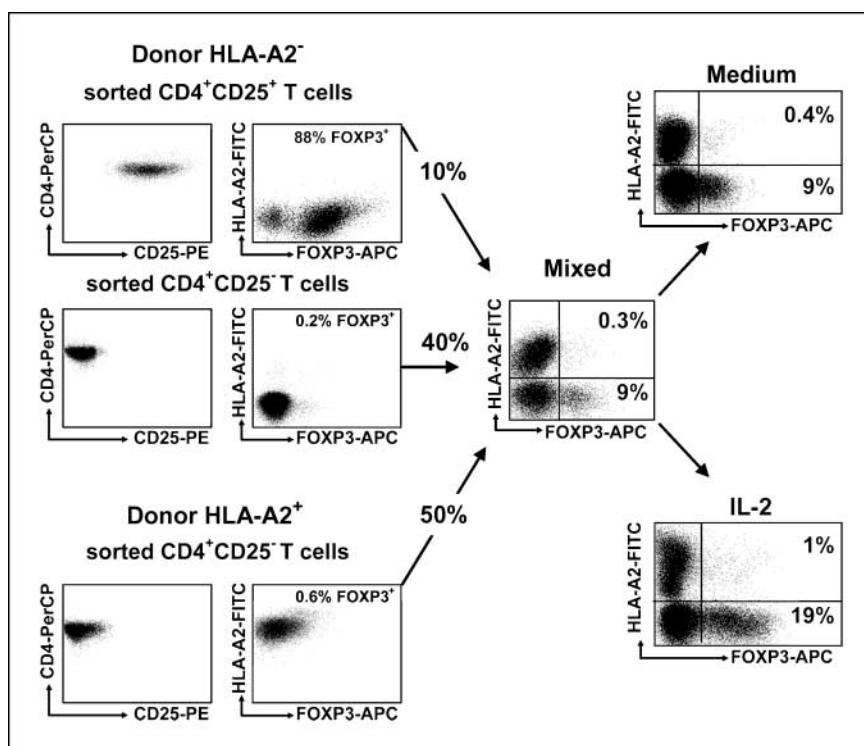


Figure 1. IL-2 stimulates the proliferation of FOXP3⁺ cells. A, IL-2 induces FOXP3⁺ T-cell division. Normal CD4⁺ T cells were labeled with CFSE and stimulated with 100 ng/mL IL-2 for 2 wk. CFSE dilution was analyzed on FOXP3⁺ T cells by FACS. Left, cells stained with anti-CD4 and FOXP3. Right, numbers of cell divisions of FOXP3⁺ T cells (gated on CD4⁺FOXP3⁺ T cells). Representative of eight experiments. B, APCs induce Ki67⁺FOXP3⁺ T cells. CD4⁺ T cells were stimulated with APCs as we described in Materials and Methods. Ki67 expression was analyzed on FOXP3⁺ T cells by FACS. Results were expressed as the percent of Ki67⁺ cells in FOXP3⁺ T cells by gating on CD4⁺FOXP3⁺ T cells. Representative of six independent experiments. C, IL-2 induces FOXP3⁺ T-cell expansion in a dose-dependent manner. CD4⁺ T cells were stimulated with different concentrations of IL-2 for 2 wk. The percentage of FOXP3⁺ T cells was determined by FACS.

Figure 2. IL-2 induces existent FOXP3⁺ T-cell proliferation. Sorted HLA-A2⁻ CD4⁺CD25^{high} cells were mixed with HLA-A2⁻ CD4⁺CD25⁻ cells and HLA-A2⁺CD4⁺CD25⁻ cells as we described and cocultured with IL-2. FOXP3 expression was analyzed by FACS. Representative of six experiments. Results were expressed as the percent of FOXP3⁺-expressing cells in CD4⁺ T cells.



similar results when HLA-A2⁻CD4⁺CD25^{high} T cells were replaced by HLA-A2⁺CD4⁺CD25^{high} T cells (not shown). The data suggest that IL-2 minimally converts CD4⁺CD25⁻FOXP3⁻ T cells into FOXP3⁺ T cells and that the increased FOXP3⁺ T cells were mainly from existent FOXP3⁺ T cells. Therefore, the increased FOXP3⁺ T-cell pool by IL-2 is largely due to the proliferation of existent FOXP3⁺ T cells.

Prevalence of Treg cells determines the efficacy of FOXP3⁺ T-cell expansion. Four percent to 10% of CD4⁺ T cells are Treg cells in peripheral blood and lymphoid organs in normal human beings. We examined the effects of the initial prevalence of existent FOXP3⁺ T cells on the efficacy of FOXP3⁺ T-cell proliferation induced by IL-2 and APCs. We sorted by FACS ARIA autologous CD4⁺CD25⁻ and CD4⁺CD25^{high} T cells to high purity. The sorted CD4⁺CD25⁻ T cells with negligible levels of FOXP3 expression (<0.6%) and CD4⁺CD25^{high} T cells with high levels of FOXP3 (>85%) were selected for further experiments. To experimentally alter the prevalence of FOXP3⁺ T cells in the culture system, we mixed autologous CD4⁺CD25⁻ T cells with different concentrations of CD4⁺CD25^{high} T cells based on the expression of FOXP3. The mixed cells expressed defined but variable levels of FOXP3 (Fig. 3A, left). The mixed cells were cultured with IL-2. When the percent of the initial FOXP3⁺ T cells was increased, the percent of Ki67⁺FOXP3⁺ T cells in FOXP3⁺ T cells was accordingly reduced (Fig. 3A and B). More strikingly, when the initial FOXP3⁺ T cells were <25%, IL-2 induced a profound Ki67⁺FOXP3⁺ T cells in FOXP3⁺ T cells, whereas when the initial FOXP3⁺ T cells were >25%, IL-2 induced limited Ki67⁺FOXP3⁺ T cells in FOXP3⁺ T cells (Fig. 3A and B). The data indicate that the prevalence of existent FOXP3⁺ T cells negatively determines the efficacy of FOXP3⁺ T-cell proliferation induced by IL-2.

The mixed cells were also stimulated with APCs (Fig. 3C and D). Similarly, when the percent of the initial FOXP3⁺ T cells was increased, the percent of yielded FOXP3⁺ T cells was accordingly

reduced (Fig. 3C and D). In addition, when the initial FOXP3⁺ T cells were <25%, APCs significantly increased the number of FOXP3⁺ T cells, whereas when the initial FOXP3⁺ T cells were >25%, APCs induced limited FOXP3⁺ T-cell expansion (Fig. 3C and D). The data indicate that the prevalence of existent FOXP3⁺ T cells negatively determines the efficacy of FOXP3⁺ T-cell proliferation induced by APCs.

Altogether, regardless of the nature of stimulation, our data suggest that FOXP3⁺ T cells may be the decisive factor for self-controlling FOXP3⁺ T-cell proliferation.

Administration of IL-2 increases CD4⁺CD25^{high} T cells in patients with ovarian cancer. After determining the role and characteristics of IL-2 treatment in human Treg cells *in vitro*, we further studied Treg cells in patients with ovarian cancer treated with IL-2. Thirty-one patients with ovarian cancer received i.p. IL-2 weekly infusion for 16 weeks (Table 1). We observed that the mean percentage of peripheral blood CD4⁺CD25^{high} T-cell was 7% at week 0 (before IL-2 treatment), 13% at weeks 4 to 6 (range, 4–17%), 24% at weeks 8 to 10 (range, 12–35%), and remained at high levels during the treatment at weeks 12 to 16. Three weeks after IL-2 cessation, the percentage of CD4⁺CD25^{high} cells dropped to 18% (range, 8–28%; Fig. 4A and B). In line with the percent changes of CD4⁺CD25^{high} T cells, the mean absolute number of CD4⁺CD25^{high} T cells increased significantly from 70/mm³ to 154/mm³ at weeks 4 to 6, 223/mm³ at weeks 8 to 10, and 278/mm³ at weeks 12 to 16 (Fig. 4C). The absolute number of CD4⁺CD25^{high} T cells was reduced to 173/mm³ three weeks after IL-2 cessation (Fig. 4C). Consistent with our *in vitro* observations (Fig. 1), IL-2 administration induced Ki67 expression in CD4⁺CD25^{high} T cells in patients with ovarian cancer (Fig. 4B). The data suggest that IL-2 induced Treg cell proliferation *in vivo*. Thus, administration of IL-2 increases CD4⁺CD25^{high} T cells and promotes CD4⁺CD25^{high} T-cell proliferation in patients with ovarian cancer.

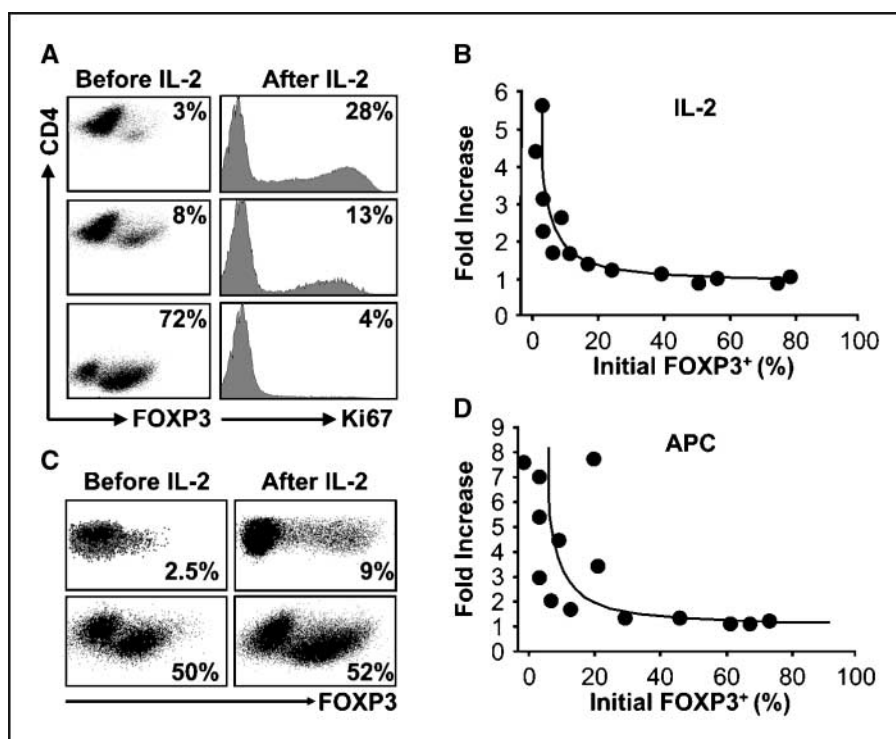


Figure 3. Prevalence of existing Treg cells determines the efficacy of Treg cell proliferation. *A and B*, CD4⁺CD25^{high} cells and CD4⁺CD25⁻ cells were sorted by FACSaria. CD4⁺CD25⁻ cells were cultured with different concentrations of CD4⁺CD25^{high} cells in the presence of IL-2 for 3 days. *A*, relationship between Ki67⁺FOXP3⁺ T cells and the initial FOXP3⁺ T cells. Ki67 expression was analyzed on FOXP3⁺ T cells by FACS. *Left*, the different percentages of CD4⁺FOXP3⁺ cells in CD4⁺ T cells before IL-2 treatment. *Right*, the different percentages of CD4⁺FOXP3⁺Ki67⁺ cells in CD4⁺FOXP3⁺ cells after IL-2 treatment. Representative FACS analysis of six individual experiments. *B*, relationship between the increased fold of CD4⁺FOXP3⁺ T cells after coculture with IL-2 and the initial percentages of CD4⁺FOXP3⁺ T cells. Each individual point represents one experiment. *C and D*, CD4⁺CD25^{high} cells and CD4⁺CD25⁻ cells were sorted by FACSaria. CD4⁺CD25⁻ cells were cultured with different concentrations of CD4⁺CD25^{high} cells in the presence of APCs for 3 d. *C*, relationship between the percentages of the initial CD4⁺FOXP3⁺ T cells and of the CD4⁺FOXP3⁺ T cells after coculture with APCs. *Left*, the different percentages of CD4⁺FOXP3⁺ cells in CD4⁺ T cells before APC coculture. *Right*, the different percentages of CD4⁺FOXP3⁺ cells in CD4⁺ cell APC coculture. Two representative FACS plots of eight individual experiments. *D*, relationship between the fold increase of CD4⁺FOXP3⁺ T cells after coculture with APCs and the initial percentages of CD4⁺FOXP3⁺ T cells. Each individual point represents one experiment. *B and D*, the fold increase of FOXP3⁺ T cells is expressed in Y axis and the initial percent of FOXP3⁺ T cells is expressed in X axis.

IL-2-induced CD4⁺CD25^{high} T cells express FOXP3 and suppress T-cell activation. Peripheral and tumor-associated CD4⁺CD25^{high} T cells express FOXP3 in ovarian cancers (18). We sorted CD3⁺CD4⁺CD25^{high} cells from IL-2-treated patients to quantify FOXP3 mRNA expression by real time-PCR (18). We found a potent FOXP3 mRNA expression in CD4⁺CD25^{high} T cells at different time points (Fig. 4D), indicating that IL-2-expanded CD4⁺CD25^{high} T cells are phenotypically Treg cells. Notably, FOXP3 mRNA expression was enhanced by IL-2 administration at weeks 4 to 10 (Fig. 4D), suggesting IL-2 induced novel FOXP3 synthesis on a per cell basis. However, 3 weeks after the cessation of IL-2 administration, FOXP3 mRNA expression was reduced by 40% as

compared with the peak values, suggesting that IL-2 may be needed to support FOXP3 mRNA expression *in vivo*.

We further sorted CD4⁺CD25^{high} T cells and tested their suppressive activities (18, 33). In a typical immune suppressive assay, we observed a dose-dependent suppression mediated by CD4⁺CD25^{high} T cells sorted from peripheral blood mononuclear cells at week 0 (before IL-2 infusion) and weeks 9 to 10 (Fig. 4E), indicating that IL-2-expanded CD4⁺CD25^{high} T cells are functional regulatory T cells, and hereafter referred to as Treg cells. Of note, although the levels of FOXP3 were higher in CD4⁺CD25^{high} T cells (Fig. 4E), the suppressive capacity was comparable in the IL-2-expanded Treg cells as compared with their counterparts before

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Table 1. Clinical characteristics of patients

Age (y), mean ± SE	53 ± 11	Residual diseases (n)	
Tumor histologic type (n)		Microscopic-0.5 cm	17
Serous, mucinous, or endometrioid	23	0.5-2 cm	11
Clear-cell or undifferentiated	8	> 2 cm	3
Stages (n)		Prior chemotherapy (n)	
II	2	Taxol	26
III	26	Carboplatinum or cisplatinum	9
IV	3	Others	4

IL-2 administration (Fig. 4E). The data indicate that IL-2-induced Treg cells are functionally suppressive.

IL-2 treatment induces CXCR4 expression on Treg cells. We recently showed that primary peripheral Treg cells constitutively express high levels of CCR4 and low levels of CXCR4 (18, 33). We then studied the effects of IL-2 on Treg cell chemokine receptor expression. Normal peripheral Treg cells were sorted and cultured *in vitro* with IL-2. Multiple chemokine receptors were analyzed. IL-2 treatment had no effects on the expression of CCR6, CCR7, CCR8, and CXCR1 (data not shown). Expression of CCR4, CCR5, and CXCR3 was slightly increased by IL-2. CXCR4 expression was dramatically increased by IL-2 starting on day 4 and peaked on day 8 on peripheral blood Treg cells (Fig. 5A). We sorted Treg cells from malignant ascites in patients with ovarian cancer and cultured with IL-2 and similar results were observed.

We next examined the *in vivo* effects of IL-2 administration in patients with ovarian cancer. We showed that CCR4 and CXCR4 expression on Treg cells was increased by IL-2 administration on week 6 and peaked on week 8. The levels of CCR4 and CXCR4 expression remained high during the IL-2 treatment and declined 3 to 4 weeks after IL-2 cessation (Fig. 5B). The data indicate that IL-2 up-regulated CCR4 and CXCR4 expression on Treg cells in patients with ovarian cancer.

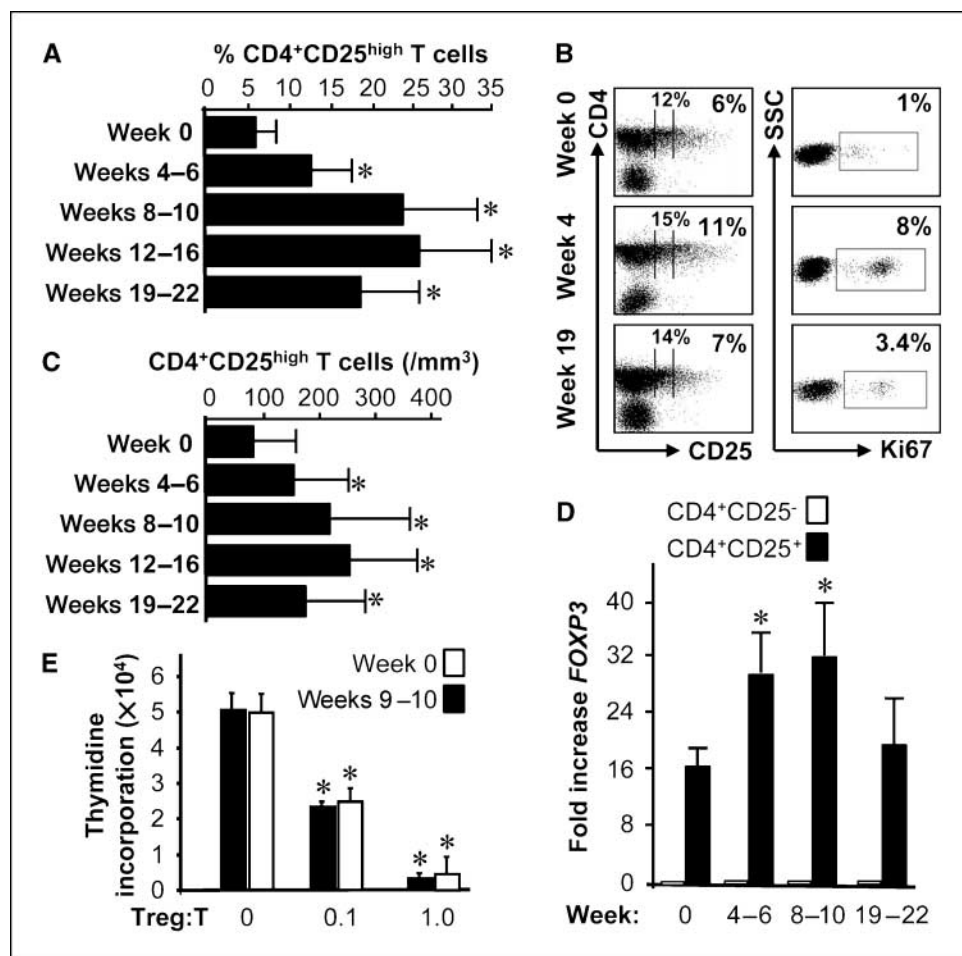
IL-2 treatment promotes Treg cell tumor trafficking. We further tested the function of CXCR4 and CCR4 on IL-2-treated Treg cells. We previously showed that homeostatic Treg cells

migrate in response to CCL22, the ligand for CCR4, but not to CXCL12, the ligand for CXCR4 (18). We confirmed this observation and further showed that IL-2-expanded Treg cells efficiently migrate with both CCL22 and CXCL12 (Fig. 5C). High levels of CXCL12 and CCL22 are found in the tumor environment (18, 30). As expected, IL-2-treated Treg cells efficiently migrated with ovarian tumor ascites (Fig. 5D). Antihuman CXCR4 and anti-CCL22 decreased Treg migration toward malignant ascites. Simultaneous blockade of CCR4 and CXCR4 completely inhibited Treg cell migration (Fig. 5D). The data suggest that IL-2 administration may trigger Treg cell tumor trafficking in response to CCL22 and CXCL12 in the tumor microenvironment.

Clinical responses and CD4⁺CD25^{high} T cells. Among 31 patients, there were 24 patients assessable for evaluating clinical response. Of the 24 patients, there were 6 clinical responses including 4 patients with complete responses and 2 partial responses as confirmed by laparotomy (Table 1; Fig. 6A). The overall clinical response rate was 25% [95% confidence interval (95% CI), 11–45%]. Mean time to progression in the four patients with a complete response was 22.5 months (range, 4–54 months). The median survival of the 31-patient cohort was 2.1 years (95% CI, 1.3–4.4 years; Table 1; Fig. 6A).

Tumor Treg cells suppressed tumor-associated antigen (TAA)-specific immunity and predicted patient survival (18). We predicted that IL-2 might induce less Treg cells in clinical responders. Unexpectedly, we failed to observe a significant difference on the

Figure 4. IL-2 administration increases functional Treg cells in patients with ovarian cancer. **A to C,** IL-2 administration increases peripheral CD4⁺CD25^{high} cells in patients with ovarian cancer. Peripheral blood CD4⁺CD25^{high} cells and Ki67⁺ cells were analyzed by FACS. Results were from all patients enrolled in the trial from whom data are available in each time point and were expressed as the mean of the percent of CD4⁺CD25^{high} cells in CD4⁺ T cells (**A** and **B, left**), the Ki67⁺ cells in CD4⁺CD25^{high} cells (gated on CD4⁺CD25^{high} cells; **B, right**), and the mean of the absolute number of CD4⁺CD25^{high} cells per microliter of blood (**C**). **D,** CD4⁺CD25^{high} cells induced by IL-2 administration expressed FOXP3 mRNA. CD4⁺CD25^{high} cells and CD4⁺CD25^{low} cells were sorted by FACSaria before and after IL-2 administration. FOXP3 was detected by real-time PCR on the indicated cells. There were 8 to 21 cases at each time point. *, *P* < 0.001, compared with CD4⁺CD25^{low} cells. **E,** CD4⁺CD25^{high} cells induced by IL-2 administration suppressed conventional T-cell proliferation. Normal CD4⁺CD25^{low} T cells were stimulated with APCs in the presence of anti-CD3 and anti-CD28 for 3 d with or without different numbers of CD4⁺CD25^{high} cells sorted from ovarian cancer patients treated with IL-2. T-cell proliferation was detected by [³H]thymidine incorporation. *n* = 6 to 9. *, *P* < 0.01, for all.



percent and absolute numbers of Treg cells between responders and nonresponders at weeks 0, 8, and 14 (Fig. 4 and not shown). Strikingly, more than 3 weeks after IL-2 treatment cessation, the percent and absolute number of Treg cells were significantly lower in patients with clinical responses than without clinical response (Fig. 6B and C). It remains to be defined how and why Treg cells were more efficiently reduced in patients with clinical responses. The data suggest that Treg cell dynamics after IL-2 treatment may predict clinical response.

Discussion

In this study, we report that administration of IL-2 promotes FOXP3⁺ T-cell proliferation and potential tumor trafficking in patients with ovarian cancer.

IL-2 has been used to treat patients with HIV infection and patients with various tumors (8, 12, 34, 35). Studies of the mechanisms of IL-2 action have revealed that IL-2 restores the ability of the immune system to produce CD4⁺ T cells from T-cell precursors, stimulate CD4⁺ T-cell expansion, and activate natural killer (NK) cells and CD8⁺ T cells (6, 11, 36, 37). Recent studies have shown that IL-2 signals during priming (38) are required for secondary expansion of CD8⁺ memory T cells (39). Prior studies have shown that IL-2 contributes to Treg cell differentiation, expansion, maintenance, and suppressor function in mice (23, 25, 34, 40, 41). We now report for the first time that in patients with ovarian cancer, IL-2 administration increases CD4⁺CD25^{high} T cells. These CD4⁺CD25^{high} T cells highly express FOXP3 and efficiently suppress T-cell activation, suggesting that the IL-2-induced CD4⁺CD25^{high} T cells are predominantly suppressive Treg cells. The data are consistent with recent studies in patients with melanoma (26), pediatric sarcoma (28), and HIV infection (42), and reveal a critical role of IL-2 in maintaining the Treg cell pool in humans.

There are three potential sources for the expanded Treg cell pool in patients treated with IL-2: Treg cell differentiation, conventional T-cell conversion, and existent Treg cell proliferation. Recent studies in patients with HIV infection (42), melanoma (26, 27), and pediatric sarcoma (28) indicate that the IL-2-expanded Treg cells were largely from periphery rather than thymus. We observed an increased FOXP3⁺ T-cell proliferation in patients treated with IL-2. We further provide direct evidence that IL-2 and APCs stimulate existent FOXP3⁺ T-cell proliferation. In the absence of transforming growth factor β , CD4⁺CD25⁻ T cells are minimally converted to FOXP3⁺ T cells in our setting. The data suggest that the proliferation of existent Treg cells is the principal mechanism responsible for IL-2-induced Treg cell pool expansion.

We observed another important characteristic in the expansion of FOXP3⁺ T-cell pool induced by IL-2 or APCs: FOXP3⁺ T-cell proliferation is proportionally controlled by FOXP3⁺ T cells themselves. When we experimentally altered the prevalence of FOXP3⁺ T cells in our culture system, we found that the lower percentage of FOXP3⁺ T cells resulted in more efficient FOXP3⁺ T-cell proliferation when exposed to IL-2 or APCs. When the FOXP3⁺ T-cell percentage is >25%, the proliferative response to IL-2 or APCs is significantly reduced. The cutoff point seems to be ~25% FOXP3⁺ T cells. The data suggest several possibilities: (a) Treg cells may suppress Treg cell proliferation. (b) Depletion of Treg cells is suggested to be a novel strategy to treat human cancer (19, 43–45). It is a technical challenge to completely deplete Treg cells in the human body. Strategies resulting in partial reduction of Treg cells and following IL-2 or APC vaccination may promote Treg cell proliferation and comprise the effects of immunotherapy and vaccination.

In addition to stimulating Treg cell expansion, we have further shown that IL-2 may enforce Treg cell tumor trafficking. This

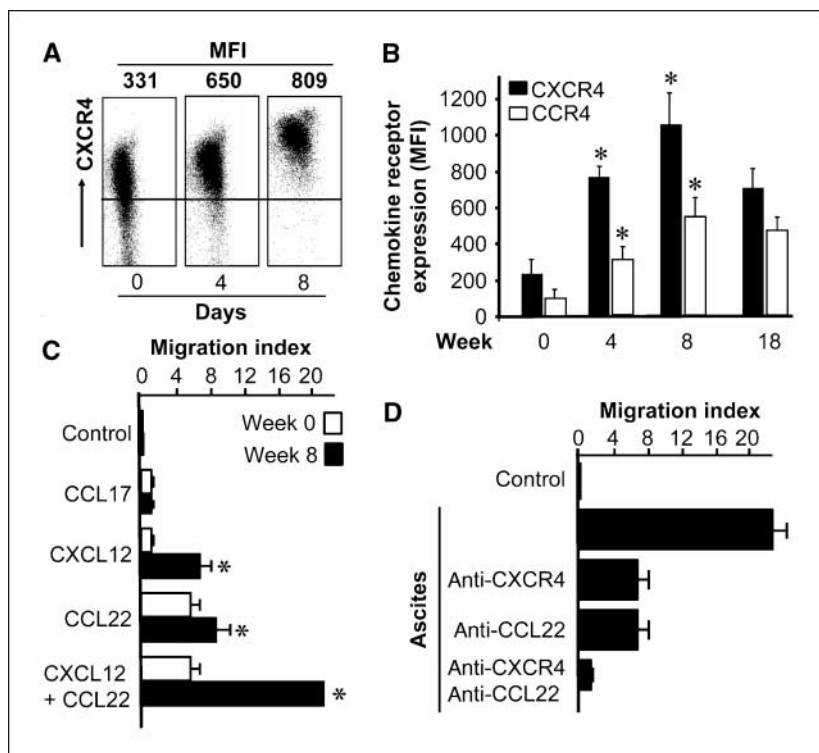


Figure 5. IL-2 treatment induces CXCR4 and CCR4 expression on Treg cells and promotes Treg cell migration toward tumor environmental factors. **A**, IL-2 treatment stimulates CXCR4 expression on Treg cells. Normal CD4⁺CD25^{high} cells were sorted by FACS and cultured with IL-2. CXCR4 expression was analyzed on FOXP3⁺ cells by FACS. The MFI of CXCR4 expression was determined by gating on CD4⁺FOXP3⁺ T cells. Representative FACS histogram of eight experiments. **B**, IL-2 treatment increased CXCR4 and CCR4 expression on Treg cells in patients with ovarian cancer. CXCR4 and CCR4 expression was detected by FACS. The mean MFI of CXCR4 and CCR4 expression was determined by gating on CD4⁺FOXP3⁺ T cells. **Columns**, mean ($n = 6-12$ at each point); **bars**, SD. *, $P < 0.01$. **C** and **D**, IL-2 treatment promotes Treg cell migration toward tumor environmental factors. Treg cells from IL-2-treated ovarian cancer patients were subject to migration with recombinant chemokines (**C**) or ovarian cancer ascites fluid in the presence of different blocking antibodies (**D**). **Columns**, mean; **bars**, SD. *, $P < 0.01$.

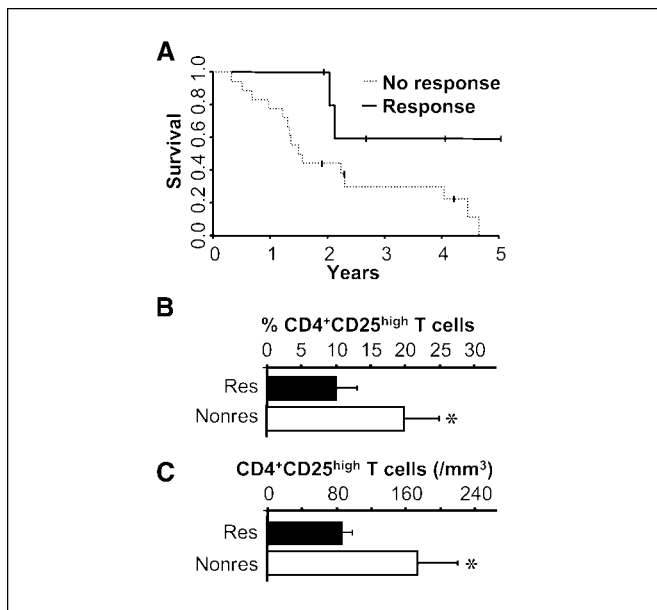


Figure 6. Clinical responses and IL-2-induced Treg cells. **A**, clinical responses and patient survival. Six patients treated with IL-2 observed clinical responses. A test based on the Cox proportional hazards with a time-dependent covariate of the difference between the two groups was used to test survival differences between responders ($n = 6$) and nonresponders ($n = 25$). **B** and **C**, clinical responses and Treg cells. The percent (**B**) and absolute number (**C**) of CD4⁺CD25^{high} T cells were analyzed by FACS at 3 wk after IL-2 cessation. Columns, mean; bars, SD. *, $P < 0.01$. Res, responders; Nonres, nonresponders.

concept is supported by two lines of evidence. First, IL-2 stimulates CXCR4 and CCR4 expression on Treg cells *in vitro* and *in vivo*. Second, after IL-2 treatment, Treg cells efficiently migrate with recombinant and tumor-derived CXCL12 and CCL22 in the tumor

microenvironment. Altogether, our study suggests a previously unappreciated role of IL-2 in Treg cell trafficking and migration. Enhanced Treg cell tumor trafficking may possibly contribute to tumor relapse.

Treg cells suppress TAA-specific T-cell immunity and negatively affect patient survival (18). It is predicted that the increased Treg cells may affect clinical responses in patients treated with IL-2. Unexpectedly, although our sample size is limited, we observed no significant difference for the percent and absolute number of Treg cells before and during IL-2 treatment between responders and nonresponders. Although our data suggest that initial Treg cells do not predict clinical responses in IL-2 immunotherapy among responders and nonresponders, we observed that after IL-2 cessation, the reduction of Treg cells is significantly faster in responders than nonresponders. It is possible that IL-2 efficiently activates CD8 and NK cells, and these cells may possibly induce Treg cell killing and subsequently result in Treg cell reduction, or Treg cells traffic into other compartment such as lymph nodes and bone marrow (31, 33). It is essential to confirm and extend this observation in a larger patient pool with a substantial amount of responders. Nonetheless, our data suggest that Treg cell dynamics may be an important determinant of IL-2-induced tumor immunity.

In summary, our studies show that IL-2 administration stimulates existent FOXP3⁺ T-cell proliferation and may promote Treg cell tumor trafficking in patients with ovarian cancer.

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