Regulatory CD4\(^+\)CD25\(^+\) T Cells in Tumors from Patients with Early-Stage Non-Small Cell Lung Cancer and Late-Stage Ovarian Cancer\(^1\)

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ABSTRACT

Immunosuppression may contribute to the progression of cancer. In this study we assessed the structural and functional status of T cells from tumor specimens obtained from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Although some groups have described structural alterations in the TCR in patients with other malignancies, we did not observe decreased expression of the CD3\(\gamma\) subunit in the tumor-associated T cells. However, increased percentages of CD4\(^+\)CD25\(^+\) T cells were observed in the non-small cell lung cancer tumor-infiltrating lymphocytes and ovarian cancer tumor-associated lymphocytes. Furthermore, these CD4\(^+\)CD25\(^+\) T cells were found to secrete transforming growth factor-\(\beta\), consistent with the phenotype of regulatory T cells. Despite a generalized expression of lymphocyte activation markers on the tumor-associated T-cell populations, the CD8\(^+\) T cells expressed low levels of CD25. To determine whether expression of CD25 could be restored on the CD8 cells, tumor-associated T cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies. After stimulation, nearly all of the CD8 T cells expressed CD25. Furthermore, despite the low levels of interleukin 2, IFN-\(\gamma\), and tumor necrosis factor-\(\alpha\) secretion by the tumor-associated and peripheral blood T cells at baseline, stimulation with anti-CD3 and anti-CD28 monoclonal antibodies significantly increased the fraction of cells producing these cytokines. Thus, tumor-associated T cells from patients with early and late-stage epithelial tumors contain increased proportions of CD4\(^+\)CD25\(^+\) T cells that secrete the immunosuppressive cytokine transforming growth factor-\(\beta\). Furthermore, our results are consistent with previous reports showing impaired expression of CD25 on CD8\(^+\) T cells in cancer patients. Finally, increased lymphocyte costimulation provided by triggering the CD28 receptor is able to increase CD25 expression and cytokine secretion in tumor-associated T cells. These observations provide evidence for the contribution of regulatory T cells to immune dysfunction in cancer patients.

INTRODUCTION

Lung cancer is the second most common malignancy in the United States, and it is the leading cause of cancer-related deaths among men and women. Given the high recurrence rate in early stage disease and the absence of a definitive therapy for late-stage disease, the overall 5-year survival rate remains low at \(\sim 15\%\). Ovarian cancer is responsible for the majority of gynecological cancer deaths. Although first-line agents have a 70–80% response rate, most patients eventually die of recurrent chemotherapy-resistant disease, leaving the overall 5-year survival at \(\sim 30\%\).

Because patients with NSCLC\(^4\) and OVC have high recurrence rates and poor long-term survival, there is interest in developing adjunctive therapies, including immunotherapy. Whereas successful immunotherapy requires a functional immune system, a defect in the immune response may contribute to tumor growth. Such defects include active suppression by the tumor as well as T-cell dysfunction, such as loss of signal-transducing proteins (1–3). Suppressor cells have also long been thought to play a part in the progression of cancer (4). Recent studies indicate that B cells of the myeloid lineage found in tumor-bearing mice are able to significantly reduce the T-cell proliferative response (3, 5). Furthermore, CD4\(^+\)CD25\(^+\) regulatory cells in mice have also been shown to inhibit T-cell proliferation (6). In fact, low levels of CD4\(^+\)CD25\(^+\) regulatory T cells may contribute to autoimmune diabetes in NOD mice, and the addition of these cells can prevent loss of self tolerance (7). The mechanism of regulatory cell-induced immunosuppression is not entirely clear, but secretion of immunosuppressive cytokines, such as TGF-\(\beta\), may play a role (8). The presence of these regulatory cells in cancer patients could be important in inducing T-cell suppression, thus allowing tumor growth. In addition, the T cells themselves may have intrinsic defects. For example, structural defects in the TCR complex may exist. Decreased levels of CD3\(\gamma\) expression have been observed in T cells from patients with renal cell carcinoma as well as colon carcinoma (9, 10). Because the CD3\(\gamma\) chain is an integral component of T-lymphocyte signaling, loss of the chain can lead to T-cell dysfunction (11).

In the present study we have tested the hypothesis that lymphocytes with the phenotype of regulatory T cells might contribute to immune dysfunction in cancer patients. Therefore, we compared lymphocyte subsets present in tumors and PBMCs from NSCLC patients and OVC patients with lymphocytes from normal donors. In lung cancer patients, T cells derived from the tumor and peripheral blood were examined, whereas, in OVC patients T cells from tumor or ascites as well as the peripheral blood were studied. Examination of T-cell subsets revealed significantly increased proportions of CD4\(^+\)CD25\(^+\) T cells in cancer patients. Furthermore, these cells produced TGF-\(\beta\) at baseline. In contrast, only a minimal number of CD8\(^+\) T cells expressed CD25 at baseline. Stimulation with anti-CD3 and anti-CD28 was able to significantly up-regulate the levels of CD25 on CD8\(^+\) T cells. Thus, we demonstrate that although an increased percentage of CD4\(^+\)CD25\(^+\) T-regulatory cells exist in patients with early and late-stage solid tumors and may be contributing to T-cell immune suppression, appropriate stimulation can induce expression of CD25 on CD8\(^+\) T cells as well as increase cytokine production.

MATERIALS AND METHODS

Patients. Peripheral blood, tumors, and/or ascites were collected from patients with NSCLC and patients with OVC after obtaining appropriate informed consent under Institutional Review Board-approved protocols. Review of pathology reports confirmed the diagnosis. Information regarding patient history was gathered by chart review.

Cell Isolation. Tumor specimens were collected at the time of surgery, processed by sterile mechanical dissection, and the tissue was stirred overnight at room temperature in an enzymatic bath containing RPMI 1640 (BioWhittaker, Walkersville, MD), HEPES buffer (5 mM; Mediatech, Herndon, VA),
penicillin/streptomycin (50 IU/ml–50 μg/ml; Mediatech), fungizone (0.25 μg/ml; BioWhittaker), gentamicin (0.05 mg/ml; Life Technologies, Inc., Grand Island, NY), collagenase type IV (Sigma Chemical Co., St. Louis, MO), DNase, type IV (30 units/ml; Sigma Chemical Co.), and hyaluronidase, type V (0.01%; Sigma Chemical Co.). The tumor suspension was then filtered through a wire grid, and the cells were washed 3 times with HBSS (Mediatech). Cells were separated on a Percoll (Pharmacia Biotech AB, Uppsala, Sweden) density gradient for 30 min at 1500 × g at room temperature. The dense layer, enriched for lymphocytes, was collected, washed, and cryopreserved in RPMI 1640 (containing 20% FCS and 10% DMSO) for future studies.

Ascites was collected at the time of surgery or office paracentesis. The fluid was centrifuged at 300 × g at room temperature for 10 min, and the resulting cell pellet was separated on a Percoll gradient, as described previously. The dense layer, enriched for lymphocytes, was collected, washed, and cryopreserved for future studies.

Peripheral blood was obtained at the time of tumor collection. Blood was drawn into heparin containing vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), diluted 2:1 with Dulbecco’s phosphate buffered salt solution 1 × without calcium or magnesium (Mediatech) and then separated by centrifugation over a ficoll (Pharmacia Biotech AB) density gradient for 20 min at 1000 × g at room temperature. PBMCs were collected, washed, and cryopreserved in RPMI 1640 (containing 20% FCS and 10% DMSO) for future use.

Cell Culture. PBMCs were thawed and cultured in RPMI 1640 (containing 10% human AB sera; BioWhittaker). Cells were cultured in multiwell plates or flasks depending upon cell number at 1 × 10^6/ml. To determine baseline levels of cytokine production, cells were incubated with Brefeldin A (10 μg/ml; Sigma Chemical Co.) for 4 h at 37°C 5% CO2 without any stimulation. Cells were cultured in RPMI 1640 containing 10% human AB sera for 4 h at 37°C 5% CO2. Cells stimulated by anti-CD3/anti-CD28-coated beads (12) were first depleted of monocytes by a 2-h adherence in tissue culture flasks. Nonadherent cells were removed and cultured at a 3:1 bead:cell ratio at a final concentration of 1 × 10^6/ml in RPMI 1640 containing 10% human AB sera for 2 days at 37°C 5% CO2. Brefeldin A (10 μg/ml) was added 4 h prior to analysis. Cells were harvested, and beads were removed by magnetic separation.

Flow Cytometric Analysis. Four-color flow cytometry was performed to determine cell type, activation marker expression, and intracellular cytokine production. Immunophenotype was determined by using anti-CD3-PerCP, anti-CD4-APC, or anti-CD8-FITC. Activation marker expression was assessed using anti-HLA-DR-PE, anti-CD25-PE, anti-CD54-PE, anti-CD69-PE, and anti-CD49b-PE (PharMingen, San Diego, CA). Isotype controls included mouse IgG1-FITC, PE, PerCP, APC, and mouse IgG2-PE (Becton Dickinson, San Jose, CA). Briefly, cells were incubated in the dark at room temperature for 30 min, washed once in FACS buffer (PBS 0.05%, FCS 2 mM, EDTA, and 0.01% sodium azide), and fixed with 2% formaldehyde (Tousimis, Rockville, MD). Flow cytometry was performed on a Becton Dickinson FACSCalibur.

Intracellular cytokine production was measured by flow cytometry. After cell surface staining, cells were permeabilized with FACS permeabilizing solution as per the manufacturer’s guidelines. After a 10-min incubation, cells were washed with the FACS buffer. Cells were then incubated with anti-IL-2 PE, anti-IFN-γ PE, or anti-TNF-α PE for 30 min in the dark at room temperature. Cells were washed once and then fixed with 2% formaldehyde.

The ratio of CD3ε and CD3ζ chain expression in T cells was determined by flow cytometry (13). Cell type was determined by adding anti-CD3ε-PerCP, anti-CD4-APC, or anti-CD8-PE. Cells were then washed and subsequently permeabilized. Anti-CD3ζ-FITC (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the cells and incubated for 30 min in the dark at room temperature. Cells were washed and fixed with 2% formaldehyde.

Analysis was performed using CellQuest software. Lymphocytes were gated by plotting forward scatter versus side scatter. Cell surface antigen expression and intracellular cytokine production was assessed by plotting CD3 versus the given molecule or cytokine. Relative CD3ζ chain expression was determined by comparing CD3ζ versus CD3ε expression as a ratio (11). To discriminate between CD4+ and CD8+ cells, CD3 was plotted against side scatter, and a gate was drawn on CD3 cells. CD4+ cells were then measured for CD4+ or CD8+ expression versus the given variable.

Cytokine Production. Tumor-infiltrating lymphocytes were isolated from freshly resected lung cancer specimens and stained with anti-CD3 FITC, anti-CD4 APC, and anti-CD25 PE. Enrichment of CD3+CD4−CD25+ cells was performed on a Cytomation MoFlo Cell Sorter by gating on lymphocytes, CD3+CD4+ T cells, and the respective CD25 population. Sorted cells were placed into culture for 2 days. Supernatants were then harvested and tested for cytokine production on Quantikine human TGF-β, IL-2, and IL-10 ELISA kit (R&D Systems, Minneapolis, MN).

Statistical Methods. Statistical analyses were performed using the Student t test.

RESULTS

CD3ζ Expression Is Not Down-Regulated in T Cells from Lung and OVC Patients. Structural alterations of the TCR have been reported in patients with lymphoma and renal cell carcinoma (2). To determine whether similar modifications were present in patients with early stage NSCLC and late-stage OVC, we performed intracellular staining for both the ζ and ε chains of the CD3 receptor. To quantify the results we compared the mean percentage of T cells expressing the CD3ζ versus the CD3ε chain as a ratio (13). Furthermore, we compared the mean fluorescent intensity of the CD3ζ and the CD3ε chains independently with controls. The ratios present in TILs from patients and controls, as well as flow cytometry data from a representative normal donor and patient are shown in Fig. 1. CD3ζ expression in NSCLC patient TIL cells did not differ from normal donor peripheral blood T cells. In contrast, there was a slight decrease in the CD3ζ expression in the tumor-associated lymphocytes from OVC patients, however this did not reach statistical significance. Furthermore, peripheral blood T cells from NSCLC and OVC patients had a mean CD3ζ to ε chain ratio of ~0.85, which was the same as normal donor T cells (data not shown). Finally, when comparing the mean fluorescent intensity of the CD3ζ and the CD3ε chains independently, we found no differences between patients and controls (data not shown). Thus, this group of cancer patients did not have evidence of down-regulation of CD3ζ chain expression.

Tumor-associated T Cells Contain Increased Proportions of CD4+CD25+ T Cells. We examined the expression of several activation and adhesion molecules on CD4+ T cells by flow cytometry.

![Flow Cytometry Data Staining for CD3ζ and CD3ε Expression](image)

**Fig. 1.** A, flow cytometry data staining for CD3ζ and CD3ε expression representative of one normal donor and one patient. B, ratio of CD3ζ to CD3ε expression was determined in unstimulated tumor-associated T cells by flow cytometry; bars, ± SE. Comparisons were made between NSCLC tumor infiltrating T cells (n = 8, LC) or OVC tumor associated T cells (n = 8, OV) to healthy donor PBL T cells. There were no statistically significant differences.

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The baseline surface expression of HLA-DR, MHC class II, CD25, CD49b, CD54, and CD69 was assessed (14-17). T cells derived from the peripheral blood of healthy donors served as controls (n = 9).

Expression of all of the activation markers examined was significantly higher in CD4^+ T cells from both early stage lung cancer TILs (n = 8) and late stage OVC lymphocytes (n = 9) as compared with control CD4^+ T cells from healthy donor peripheral blood (Fig. 2A). Peripheral blood T cells derived from NSCLC and OVC PBLs also had significantly increased expression of activation molecules, although to a lesser degree (data not shown). To ensure that the increase in activation molecule expression was not an artifact of the tumor processing and enzymatic digestion, control T cells were processed in the same manner. The expression of activation markers by control cells was not affected by the processing (data not shown). Thus, tumor-associated T cells, as well as the peripheral blood T cells, demonstrate a significant state of activation.

Interestingly, among the CD4^+ tumor-associated T cells from both NSCLC and OVC there was a striking increase in the proportion of CD4^+CD25^+ T cells (Fig. 3). In other settings, lymphocytes with this immunophenotype have been shown to have regulatory functions (8). Furthermore, in the peripheral blood from OVC patients there was a significant increase in the proportion of CD4^+CD25^+ T cells as well (data not shown). In contrast, the peripheral blood of NSCLC patients did not demonstrate a significant increase in the CD4^+CD25^+ T-cell population (data not shown). Thus, in the immediate tumor environment, as well as the peripheral blood of OVC patients, there is a significant increase in the proportion of CD4^+CD25^+ T cells, which in other settings has been shown to have suppressive effects.

**TGF-β Secretion by Tumor-associated CD4^+CD25^+ T Cells.**

Having observed that increased portions of CD4^+CD25^+ T cells exist in the cancer patients, we were interested in determining whether these cells displayed immunosuppressive functions. Whereas the mechanisms used by regulatory cells are probably complex and remain a subject of active investigation, in other settings the cells have been shown to secrete immunosuppressive cytokines such as TGF-β and IL-10. To assess this possibility, tumor-associated lymphocytes from patients with early stage NSCLC were stained for CD3, CD4, and CD25 expression and then sorted to isolate the CD4^+CD25^+ T-cell subpopulation. The isolated TIL subpopulations were placed in short-term culture without stimulation to assess for TGF-β production (Fig. 4). In six of six patients with NSCLC, we found that TGF-β was indeed produced by CD4^+CD25^+ TIL cells. These cells did not produce IL-2 or IL-10 at baseline (Table 1). CD3^+CD4^+CD25^+ T cells produced significantly more TGF-β at baseline than...
CD3⁺CD4⁺CD25⁻ T cells (Table 1, P = 0.01). It is possible that anti-CD3 and anti-CD4 staining may have led to increased cytokine production. However, it is unlikely that the CD3⁺CD4⁺CD25⁻ cells, which also bear these antibodies, have minimal TGF-β production, and none of the sorted cells produce IL-2 or IL-10. Furthermore, consistent with previous reports, unstimulated peripheral blood T cells from normal donors did not secrete detectable amounts of TGF-β (data not shown). It is possible that the minimal amounts of TGF-β produced by the CD3⁺CD4⁺CD25⁻ TIL cells is attributable to contaminating regulatory T cells. The production of TGF-β and other soluble factors by tumor-associated T cells may have immunoregulatory effects.

**CD8⁺ T Cells from Tumor-associated T Cells Have Decreased Expression of CD25.** Given that >30% of tumor-associated CD8⁺ T cells from patients with NSCLC and OVC coexpressed CD25, we next assessed CD25 expression on CD8⁺ T cells. In contrast, whereas CD8⁺ T cells from NSCLC TILs and OVC TALs also demonstrated significantly increased expression of HLA-DR, CD49b, CD54, and CD69 as compared with controls (Fig. 2B), they had minimal expression of CD25 (Fig. 3). This suggested that there might be impaired CD25 expression by the CD8⁺ cells, given the up-regulation of the other activation markers. Alternatively, it was possible that there was shedding of the CD25 receptor. To investigate this additionally, CD8⁺ T cells were stained for intracellular CD25 expression. We found that there was minimal internal CD25 expression suggesting a lack of production of this protein (data not shown).

CD8⁺ T cells from the peripheral blood of OVC patients (n = 8) were similar to tumor-associated T cells from NSCLC and OVC in that they expressed CD25 at a level statistically similar to controls (6.9% versus 3.5%). In contrast, peripheral blood CD8⁺ T cells from lung cancer patients (n = 9) demonstrated significantly higher mean levels of CD25 (16.5%) when compared with controls (3.5%). Thus, CD8⁺ T cells in the tumor environment, as well as the peripheral blood of OVC patients, demonstrated decreased expression of CD25.

**CD8⁺ T Cells Express CD25 after Stimulation.** To determine whether there was a block to CD25 expression in the tumor-derived T cells, we used anti-CD3 and anti-CD28-coated beads to stimulate the T cells. Stimulation effectively up-regulated the expression of CD25 on tumor-associated CD8⁺ T cells. The mean percentage of TILs from lung cancer patients expressing CD25 increased from 9.1% to 87.8% after 2 days of stimulation (Fig. 5). In OVC patients, the mean percentage of tumor-associated T cells expressing CD25 increased from 9.9% to 89%. Peripheral blood CD8⁺ T cells from lung cancer and OVC patients also had a significant increase in the mean expression of CD25 from 16.6% to 89.3% and from 6.9% to 83.1%, respectively (data not shown). Experiments with T cells stained with CFSE indicated that only 10% of cells had divided by day 2 of stimulation (data not shown). Thus, the decreased expression of CD25 can be reversed by anti-CD3 and anti-CD28 stimulation and cannot be explained by outgrowth of normal T cells.

**Baseline Production of IL-2, IFN-γ, and TNF-α Is Minimal in Tumor-associated T Cells.** Given the activated immunophenotype of the tumor-associated T cells (Fig. 2), we next asked if the cells had spontaneous cytokine secretion. We assessed the baseline cytokine production of T cells by intracellular staining for IL-2, IFN-γ, and TNF-α. Tumor-associated T cells from lung cancer and OVC patients demonstrated a low level of baseline cytokine production (Fig. 6). Similarly, at baseline, PBL T cells from both lung cancer and OVC patients showed only minimal IL-2, IFN-γ, and TNF-α production. We also measured baseline levels of the Th2 cytokine, IL-4, in the tumor-associated T cells and found them to be at the similar low levels of controls (data not shown). Thus, there was no significant production of Th1 or Th2 cytokines at baseline in the tumor-associated T cells.

**Th1 Cytokine Production Is Increased in Tumor-associated T Cells after Costimulation.** To assess whether the tumor-associated lymphocytes were functional, the fraction of T cells secreting cytokines after anti-CD3 and anti-CD28 stimulation was measured. In the lung cancer patients, there were significant increases in intracellular cytokine production in the tumor-associated T cells and in the peripheral blood T cells (Fig. 7). After stimulation, the percentage of TILs producing IL-2, IFN-γ, and TNF-α was similar to the peripheral blood T cells. Tumor-associated T cells from OVC patients also showed statistically significant increases in cytokine production after stimulation (Fig. 8A). However, peripheral blood T cells from OVC patients tended to have a somewhat diminished response when compared with the tumor-associated T cells (Fig. 8B). Thus, anti-CD3/anti-CD28-coated beads are able to induce the production of IL-2, IFN-γ, and TNF-α in both NSCLC and OVC T lymphocytes. This demonstrates that their capacity to respond is intact, and that these T cells may be

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**Table 1** Constitutive cytokine production by T-cell subsets from patients with early stage NSCLC

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CD4⁺CD25⁺</th>
<th>CD3⁺CD25⁻</th>
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<tr>
<td>TGFβ (pg/mL)</td>
<td>279 ± 35</td>
<td>101 ± 52</td>
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<tr>
<td>IL-2 (pg/mL)</td>
<td>&lt;30⁰⁺</td>
<td>&lt;30</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>&lt;7⁰⁺</td>
<td>&lt;7</td>
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⁰⁺ Lower limit of detection by ELISA.

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**Fig. 5.** Tumor-associated CD8⁺ T cells express CD25 after costimulation. CD8⁺ T cells from NSCLC (n = 8) or OVC patients (n = 9) were stimulated with anti-CD3 and anti-CD28-coated beads for 2 days. CD25 expression was determined at rest or after stimulation by flow cytometry.

**Fig. 6.** Low level constitutive cytokine secretion by tumor-associated T cells. The mean percentage of T cells producing IL-2, IFN-γ, and TNF-α at baseline was determined by intracellular cytokine staining and flow cytometry. Bars, + SE. Tumor-associated T cells were obtained from NSCLC (n = 8) and OVC (n = 9), as well as the peripheral blood of NSCLC, OVC, and healthy donors.
able to mount a response against a tumor after appropriate activation stimuli.

**DISCUSSION**

In the hope of identifying common mechanisms of immune dysfunction, we focused on the tumor-associated lymphocytes from patients with two epithelial cancers. T cells derived from patients with early stage NSCLC and late-stage OVC were studied. We found that there are increased proportions of tumor-associated CD4\(^+\)CD25\(^+\) T cells in patients with early stage NSCLC. It is possible that an increase in regulatory T cells at the tumor site may promote local tumor growth, whereas the increase in regulatory cells in the peripheral blood may be relevant to the progression of systemic disease. Our results may have implications for strategies that use therapeutic vaccination.

The level of expression of CD3\(\zeta\) is controversial in tumor-bearing patients. Although CD3\(\zeta\) chain down-regulation has been observed in patients with many types of cancer (3, 9, 10), including OVC (11), we did not observe this. Our findings are in agreement with recent studies where patients with early stage breast and colon cancer had normal CD3\(\zeta\) expression (18, 19). Underexpression of CD3\(\zeta\) is observed in patients with advanced but not early stage breast and head and neck tumors (19–21). Consistent with this, our cohort of patients with early stage NSCLC had normal CD3\(\zeta\) expression, and the patients with advanced OVC had lower levels of expression.

We were interested in whether cancer patients may harbor regulatory T cells. Recent results suggest that the CD28/B7 costimulatory pathway is essential for the development and homeostasis of the regulatory T cells that control spontaneous autoimmune diseases (6, 7, 22, 23). Because tumor-associated antigens recognized by autologous T cells in cancer patients are often normal self-constituents, the induction of tumor immunity is, in part, the induction of autoimmunity (24). In the tumor-associated T cells of patients with NSCLC and OVC, it is likely that there are increased proportions of these regulatory T cells, given the increased percentage of CD4\(^+\)CD25\(^+\) cells in these populations and the spontaneous TGF-\(\beta\) secretion in NSCLC TILs. Whereas the mechanisms leading to the biological effects of regulatory T cells remain the subject of investigation, recent studies have revealed that a part of the suppressive phenomena can be attributed to secretion of immunosuppressive cytokines (8, 22, 24). TGF-\(\beta\) has been implicated as an important immunosuppressive cytokine, which may play a role in cancer progression (25). In mice, regulatory T cells have been shown to express CTLA-4 (7), and triggering of CTLA-4 has been shown to induce TGF-\(\beta\) secretion (26). Thus, in the tumor-associated lymphocytes, these regulatory T cells may be preventing appropriate antitumor immune responses. Manipulation
of these regulatory T cells could lead to new strategies for the treatment of cancer by facilitating the loss of tolerance to self-antigens.

The CD28 costimulatory pathway plays an important role in antitumor responses. Immunodeficient mice have been cured of established human and rodent tumors when the mice were treated with anti-CD28 antibodies (27, 28). To determine the functional capacity of the patient T cells and their potential utility for immunotherapy, we measured intracellular cytokine production after stimulation with anti-CD3 and anti-CD28 antibodies. The low level of baseline cytokine production despite an activated immune phenotype suggests a dysfunctional T-cell state (29, 30). The levels of Th1 cytokines have been shown to be decreased or absent in T cells of both lung and OVC patients (29, 31, 32). Furthermore, T cells cultured in IL-2 from patients with OVC have been shown to produce IL-2 and IFN-γ (33). We found that culturing the tumor-associated T cells with anti-CD3 and anti-CD28 antibodies induced significant production of IL-2, IFN-γ, and TNF-α. Increased production of these cytokines may be helpful in inducing cytolytic cell function, recruiting and expanding antigen-presenting cells, and mediating direct antitumor effects. Collectively, the present results in conjunction with previous studies suggest that the dysfunction of T cells may be a reversible phenomenon, dependent on the tumor-bearing environment of the patient.

The tumor-associated T cells from NSCLC and OVC patients had activated phenotypes at baseline. However, despite the activated phenotype of these CD8+ T cells, they did not express the IL-2 receptor. It is unclear why the CD8+ T cells lacked expression of the IL-2 receptor, even though this has been reported in other malignancies (34, 35). Previous studies of IL-2-receptor expression on mononuclear cells from OVC patients have reported low levels of CD25+ cells (36). Furthermore, lymphocytes from patients with intracranial tumors have decreased expression of the high-affinity IL-2 receptor after mitogen activation (37). Anergic T cells do not fully express the IL-2 receptor (38, 39). Thus, increased levels of regulatory cells in the tumor environment of NSCLC and OVC patients, as well as the peripheral blood of OVC patients, may contribute to an immunosuppressive state resulting in the abnormal expression of CD25 by CD8 cells. Up-regulating the IL-2 receptor may be important for potential therapeutic strategies.

In summary, tumor-associated T cells derived from both lung and OVC patients demonstrate increased proportions of CD4+CD25+ T cells. In patients with early stage NSCLC, secretion of TGF-β is largely confined to TILs with this immunophenotype, which may contribute to immune dysfunction. Furthermore, whereas CD8+ T cells displayed an activated immunophenotype, they have minimal expression of the IL-2 receptor, which may render them unresponsive to IL-2. Nevertheless, stimulation via anti-CD3 and anti-CD28 is not only able to induce cytokine production but also able to up-regulate the IL-2 receptor, which may prove useful to restoring an appropriate immune response for immunotherapy.

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