A Unique Subset of $CD4^+CD25^{high}Foxp3^+$ T Cells Secreting Interleukin-10 and Transforming Growth Factor-β1 Mediates Suppression in the Tumor Microenvironment

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Abstract

Purpose: Immunosuppression, including that mediated by $CD4^+CD25^{high}Foxp3^+$ regulatory T cells (Treg), is a characteristic feature of head and neck squamous cell carcinoma (HNSCC). Tregs with a distinct phenotype in tumor-infiltrating lymphocytes (TIL) contribute to local immune suppression.

Experimental Design: The frequency and phenotype of Treg in TIL and/or peripheral blood mononuclear cells (PBMC) in 15 HNSCC patients and PBMC in 15 normal controls were compared. Single-cell sorted $CD4^+CD25^{high}$ T cells were tested for regulatory function by coculture with carboxyfluorescein diacetate succinimidyl ester–labeled and activated autologous $CD4^+CD25^-$ responder T cells. Transwell inserts separating Treg from responders and neutralizing interleukin-10 (IL-10) or transforming growth factor-β1 (TGF-β1) antibodies were used to evaluate the mechanisms used by Treg to suppress responder cell proliferation.

Results: In TIL, $CD25^+$ cells were enriched in the $CD3^+CD4^+$ subset (13 ± 3%) relative to circulating $CD3^+CD4^+$ T cells (3 ± 0.7%) in HNSCC patients ($P \leq 0.01$) or normal controls (2 ± 1.5%; $P \leq 0.001$). Among the $CD3^+CD4^+$ subset, $CD25^{high}$ Treg represented 3 ± 0.5% in TIL, 1 ± 0.3% in PBMC, and 0.4 ± 0.2% in normal controls. Tregs in TIL were $GITR^+$, $IL\text{-}10^+$, and $TGF\text{-}\beta1^+$, although circulating Treg up-regulated CD62L and CCR7 but not GITR, IL-10, or TGF-β1. Treg in TIL mediated stronger suppression ($P \leq 0.001$) than Treg in PBMC of HNSCC patients. The addition of neutralizing IL-10 and TGF-β antibodies almost completely abrogated suppression (5 ± 2.51%). Transwell inserts partly prevented suppression (60 ± 5% versus 95 ± 5%).

Conclusions: Suppression in the tumor microenvironment is mediated by a unique subset of Treg, which produce IL-10 and TGF-β1 and do not require cell-to-cell contact between Treg and responder cells for inhibition.

The role of tumor-infiltrating lymphocytes (TIL) in patients with cancer has been debated for many years. A considerable body of evidence suggests that TIL contribute to tumor escape from the immune system (1–3). In many cases, however, TIL accumulations have been linked to improved patient outcome and TIL-T recognized as effectors of antitumor immune responses (4–8). Recent studies have revealed that a subset of $CD4^+$ T cells, referred to as $CD4^+CD25^{high}Foxp3^+$ naturally occurring regulatory T cells (nTreg), may accumulate in the tumor environment and suppress tumor-specific T-cell responses, thereby hindering tumor rejection. In patients with cancer, accumulations of Treg cells in the tumor were associated with a significant reduction in survival (9, 10).

To date, three types of $CD4^+$ Treg cells have been partly characterized in humans: (*a*) $CD4^+CD25^-IL\text{-}10^+Foxp3^{low}$ type 1 T regulatory (Tr1) cells, which arise in the periphery on encountering antigen in a tolerogenic environment via a process that is interleukin (IL)-10 dependent (11, 12); (*b*) naturally occurring $CD4^+CD25^{high}Foxp3^+$ T cells (nTreg), which arise directly in the thymus and have the ability to suppress responses of both $CD4^+CD25^-$ and $CD8^+CD25^-$ T cells in a contact-dependent, cytokine-independent, and antigen-nonspecific manner (13–15); and (*c*) Th3 cells, which are dependent on IL-4 for functional differentiation (16). We have previously described an enrichment of $CD4^+CD25^{high}$ T cells (Treg) among tumor-infiltrating (17) as well as circulating lymphocytes in patients with head and neck squamous cell carcinoma (HNSCC; refs. 18, 19). The presence of Treg at the tumor site may be detrimental to the host defense against the tumor. It could inhibit antitumor immune responses and interfere with immunotherapeutic strategies.

The inconsistent reports in the literature about the role of TIL in the control of cancer progression in humans seem to

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result from the fact that phenotypic rather than functional attributes of these cells are used for their identification and characterization. Predictions of tumor behavior solely based on numbers and tumoral or peritumoral localization of TIL are likely to be inaccurate, as it is the functional potential of immune cells that determines their effectiveness as antitumor effectors *in situ*. Functions of TIL are difficult to assess *in vitro* because of the necessity for their isolation from tissues and purification, which are labor intensive and require large tumor specimens. For these reasons, studies of TIL, including Treg subpopulations, have been largely confined to *in situ* examinations generally involving immunocytochemistry (4, 20).

Treg contributions to shaping of the tumor microenvironment and to regulation of antitumor responses *in situ* can only be evaluated after their isolation from tumors. To this end, we describe here phenotypic and functional characteristics of Treg isolated from TIL in HNSCC. We show that Treg accumulating in tumors express a unique phenotype distinct from that of Treg in peripheral blood mononuclear cells (PBMC), and they are more suppressive. Furthermore, their presence in the tumor is linked to a poor prognosis in subjects with HNSCC.

Materials and Methods

Patients and tumor tissues. Patients with HNSCC included in this study (n = 24) were initially seen at the Outpatient Otolaryngology Clinic at the University of Pittsburgh Medical Center. The patients who underwent surgery signed the Institutional Review Board–approved informed consent releasing the use of specimens (tissues and blood) for research purposes. Tumors were obtained from 15 patients undergoing surgery for treatment of primary disease between September 2000 and March 2001. The tissues were processed and banked at the University of Pittsburgh Cancer Institute Tissue Procurement Facility. Samples of PBMCs were obtained before surgery from five of these patients. In addition, PBMCs were donated by nine patients seen between January and October 2006 who had active disease (six had primary and three had recurrent disease). The patients who donated PBMC were males (n = 6) and females (n = 3) with a mean age of 60 years (range, 23-82). The oncologic therapy, if given, was terminated from 3 weeks to 2 months at the time of phlebotomy for this study. In addition, samples of PBMC were obtained from 15 age-matched normal controls. The age, sex, and clinicopathologic characteristics of the patients whose TILs were studied are listed in Table 1.

Collection of PBMC. Peripheral venous blood (20-30 mL) was drawn into heparinized tubes. The samples were hand carried to the laboratory and immediately centrifuged on Ficoll-Hypaque gradients. PBMCs were recovered, washed in AIM-V medium (Invitrogen), counted in the presence of a trypan blue dye to evaluate viability, and either immediately used for experiments or cryopreserved.

Isolation of TIL. TIL were isolated at the University of Pittsburgh Cancer Institute Tissue Procurement Facility according to a standard operating procedure as described previously (21). Briefly, after removal of fat, blood, or necrotic areas, tumor tissues were washed in RPMI 1640 containing 50 μg/mL gentamicin (Invitrogen) and cut into 1 mm^3 pieces in a Petri dish while covered with RPMI 1640 plus antibiotics. Tumor fragments were transferred to flasks and dissociated using 0.05% collagenase (type IV; Sigma) and 0.02% DNase (type I; Sigma) in the same medium supplemented with 5% (v/v) fetal bovine serum (Invitrogen). The digest was passed through 90-μm and 50-μm nylon mesh to remove clumps, and the filtrate was washed two to three times in medium followed by centrifugation at 350 × g for 10 min. To separate tumor cells from lymphocytes, the cell suspension was layered onto a discontinuous Ficoll-Paque gradient of 75% over 100% Ficoll-Paque in medium and centrifuged for 800 × g for 20 min at room temperature (21). Lymphocytes were collected from the interface between 75% and 100% Ficoll-Paque and washed twice before further use.

Table 1. Clinicopathologic characteristics of patients with HNSCC whose TILs were examined in this study

	n
Age (y)	
Mean range	76-42
Sex	
Male	10
Female	5
Total	15
Tumor site	
Oral cavity	10
Oropharynx	2
Hypopharynx	0
Larynx	3
Tumor differentiation	
Poor	4
Moderate	8
Well	2
Not determined	1
Tumor stage	
T_1	1
T_2	2
T_3	5
T_4	7
Nodal status	
N_0	6
N_1	2
N_2	5
N_3	2
Active disease	15
No previous therapy	15

Cryopreservation of TIL and PBMC. TIL and autologous or allogeneic PBMCs were cryopreserved at a cell concentration of up to 20 × 10^6 cells/mL in a freezing medium consisting of RPMI 1640 supplemented with 20% (v/v) human AB serum (Nabi) and 10% (v/v) DMSO (Fisher Scientific). Cells were cooled at a rate of 1°C per min to -80°C (Cryomed) and stored in liquid nitrogen. Before use, cells were thawed at 37°C and immediately resuspended in an excess of warm AIM-V medium. Cells were washed twice in the same medium equilibrated for 2 to 4 h, adjusted to the concentration of 5 × 10^6/mL, and used for experiments.

Antibodies. The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD3-ECD, anti-CD4-PC5, anti-CD25-FITC, anti-GITR-FITC, anti-CD62L-FITC, anti-CD127-FITC, anti-CCR7-FITC, anti-TGF-β1-FITC, anti-Foxp3-FITC, anti-CD122-FITC (IL-2Rβ), anti-CD45RA-FITC, anti-CD45RO-FITC, anti-Fas-FITC, anti-FasL-PE (NOK-1, 42 kDa), anti-CD132-PE (IL-2Rγ), anti-CCR4-PE, anti-CD25-PE, anti-CD152-PE (CTLA-4), and anti-IL-10-PE. Antibodies and their respective isotypes, used as negative controls for surface and intracellular staining, were all purchased from Beckman Coulter, except for anti-IL-10-PE, anti-CD45RA-FITC, and anti-FasL-PE (BD PharMingen); anti-h/TNFRSF18GITR (clone FAB689F), anti-CCR7-FITC, and anti-CCR4-PE (R&D Systems, Inc.); anti-TGF-β1-FITC (Biocompare, Inc.); and anti-Foxp3-FITC and anti-CD127-FITC (eBioscience). Before use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions.

Surface and intracellular staining. To determine the frequency of $CD4^+CD25^{high}$ T cells and the "Treg" surface marker profile, TIL or PBMC (at least 0.2 × 10^6 cells/tube) were stained with mAbs in the above-described panel for 15 min at 4°C. Appropriate isotype antibody controls were used for each sample. Cells were washed and examined by four-color flow cytometry.

Intracellular staining for Foxp3, CD152 (CTLA-4), and GITR was done as described previously (22). Intracytoplasmic expression of transforming growth factor-β1 (TGF-β1) and IL-10 was assessed before and after stimulation of PBMC for 4 h with phorbol 12-myristate 13-acetate (20 μg/mL) and ionomycin (1 μmol/L/mL). Briefly, samples were first incubated with mAbs against surface markers CD4, CD3, and CD25. After extensive washing, cells were fixed with 4% (v/v) formaldehyde in PBS for 20 min at room temperature, washed once with PBS containing 0.5% (v/v) bovine serum albumin and 2 nmol/L EDTA, permeabilized with PBS containing 0.5% bovine serum albumin and 0.1% (v/v) saponin, and stained with anti-CTLA-4-PE, anti-Foxp3-FITC, anti-TGF-β1-FITC, or anti-IL-10-PE mAb for 30 min at room temperature. Cells were further washed twice with PBS containing 0.5% bovine serum albumin and 0.2% (v/v) saponin, resuspended in fluorescence-activated cell sorting flow solution, and immediately analyzed by flow cytometry. Appropriate isotype controls were included for each sample.

Flow cytometry. Flow cytometry was done using a FACScan flow cytometer (Beckman Coulter) equipped with Expo32 software (Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate as determined by characteristic forward and side scatter properties of lymphocytes (Fig. 1A). Forward and side scatters were set in a linear scale. For analysis, 1×10^5 lymphocytes were acquired. The analysis gates were restricted to the $CD3^+CD4^+$ (e.g., Fig. 1B), $CD4^+CD25^+$, and $CD4^+CD25^{high}$ T-cell subsets (e.g., Fig. 1C), as appropriate. Cells expressing Treg markers were acquired and analyzed in the FL1 or FL2 logarithmic scale using the set gates.

Suppression experiments. Single-cell sorting of $CD4^+CD25^{high}$ T-cell populations was done as described previously (22). Treg obtained from TIL or PBMC were tested for regulatory function by coculture analysis at the suppressor to responder ratios of 1:2 or 1:5 with at least 0.5×10^5 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled autologous $CD4^+CD25^-$ T responder cells per well. Soluble OKT3 (10 μg/mL; American Type Culture Collection) and soluble anti-CD28 mAb (1 μg/mL) were used for stimulation in the presence of 150 IU/mL IL-2 for 5 days.

CFSE labeling of responder T cells was done as described previously (22). Briefly, $CD4^+CD25^-$ T cells separated by single-cell sorting were stained with 1.5 μmol/L CFSE (Molecular Probes/Invitrogen) for 10 min at room temperature. The CFSE label was quenched by the addition of an equal volume of FCS (Invitrogen), and then cells were washed extensively with PBS.

T-cell populations were classified as suppressive if they inhibited proliferation of autologous $CD4^+CD25^-$ responder cells in the coculture assay and if decreasing the number of $CD4^+CD25^+$ T cells relative to the number of $CD4^+CD25^-$ responder cells in coculture restored proliferation. $CD4^+CD25^+$ T-cell populations that satisfied both of these criteria were classified as suppressor T cells. These criteria were applied to all populations tested.

All CFSE data were analyzed using the ModFit software provided by the Verity Software House. The percentages of suppression were calculated based on the proliferation index of responder cells alone compared with the proliferation index of cultures containing responder plus Treg. The program determines the percentage of proliferating cells within each peak, and the sum of all peaks in the control culture (no Treg) is taken as 100% of proliferation and 0% of suppression.

Transwell assays. To assess whether cell-to-cell contact was necessary for Treg to mediate suppression, polycarbonate 24-well Transwell inserts (0.4 μmol/L; Corning Costar Corp.) were used. At least 5×10^4 CFSE-labeled $CD4^+CD25^-$ T cells were stimulated with soluble OKT3 and soluble anti-CD28 mAb (each at 1 μg/mL) in the presence of 150 IU/mL IL-2 in the lower chambers of the plates. Autologous Treg or control cells ($CD4^+CD25^-$) were added to the upper chambers at the suppressor to responder ratio of 1:2. To analyze whether TGF-β1 and IL-10 are required for Treg-mediated suppression, neutralizing IL-10 antibody (2 μg/mL; R&D Systems) and/or TGF-β1 antibody (20 μg/mL; R&D Systems) or nonblocking IgG anti-mouse control antibody were added to Treg in the upper chambers. Cells were cultured for 5 days and tested for suppression of responder cell proliferation.

Immunofluorescence of tumor tissue sections. Tissue samples were embedded in OCT, and 5-μm frozen sections were cut in a cryostat, dried, fixed for 10 min in acetone and ethanol (1:1) or 4% (w/v) paraformaldehyde, and again dried in air. The following antibodies were used for staining: monoclonal anti-human CD25-PE (diluted 1:50 in PBS) and anti-human IL-10-PE (1:25) purchased from BD PharMingen; unlabeled anti-human IL-10 (1:100) purchased from Santa Cruz Biotechnology; anti-CD25-FITC (1:50), anti-CD4-FITC

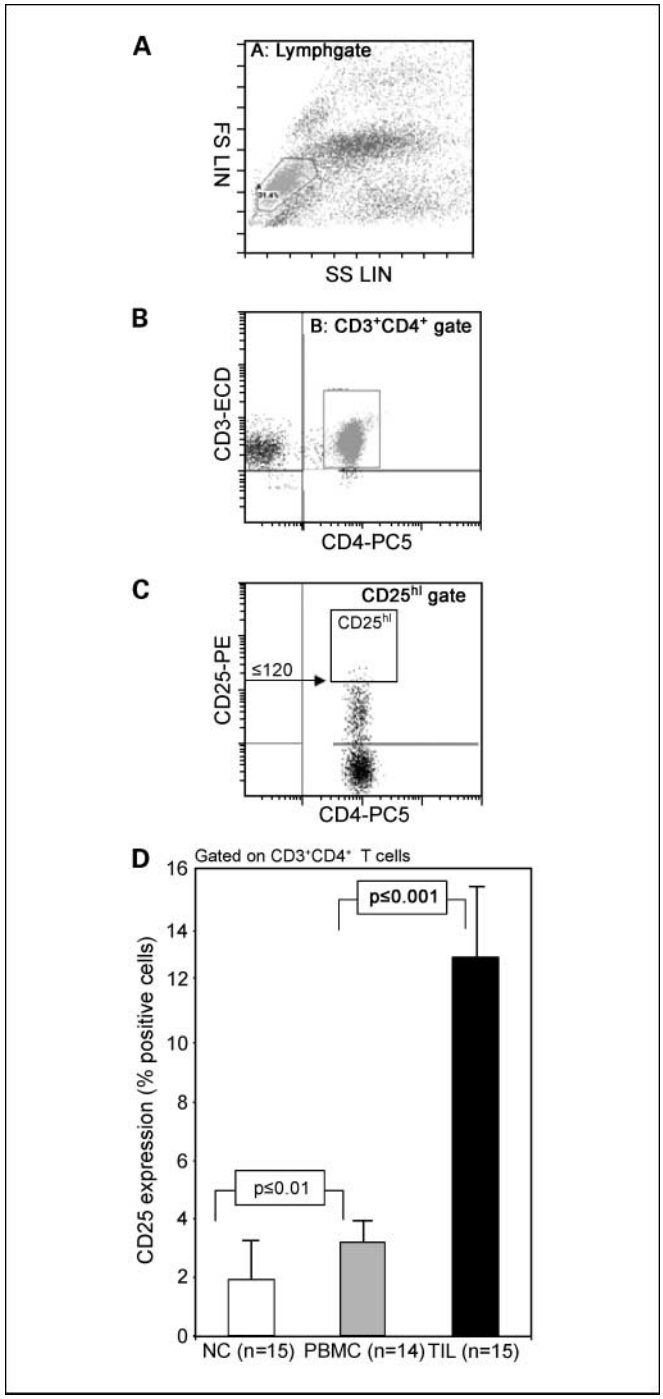

Fig. 1. Gating strategy (*A-C*) and percentages of $CD25^+$ T cells in $CD3^+CD4^+$ populations isolated from PBMC and TIL of HNSCC patients and PBMC of normal controls (*NC*; *D*).

(1:50), and anti-CTLA-4-FITC (1:25) purchased from Beckman Coulter; anti-GITR (1:25) purchased from R&D Systems; polyclonal anti-human TGF-β1 (1:100) purchased from Santa Cruz Biotechnology; and biotinylated anti-Foxp3 (1:50) purchased from eBioscience. As a secondary antibody for Foxp3 detection, mouse anti-biotin FITC-labeled antibody (1:200) purchased from Jackson ImmunoResearch was used. Secondary antibodies for detection of IL-10 and TGF-β1 were Cy5-labeled goat anti-rat Ig and Cy5-labeled donkey anti-goat Ig, respectively (1:1,000), both purchased form Molecular Probes/Invitrogen. To eliminate nonspecific binding of secondary antibodies, tissue sections were initially incubated with 10% normal mouse serum for 20 min or with bovine serum albumin (2%) for 45 min. Sections were incubated with antibodies for 1 h at room temperature in a moist chamber and in the dark. Next, slides were extensively washed in PBS. In the case of Foxp3, IL-10, or TGF-β1, sections were incubated with the secondary antibodies under the same conditions. Primary antibodies were omitted in all negative controls. Sections were incubated in a medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) to trace cell nuclei. Slides were evaluated in a Nikon Eclipse E-800 fluorescence microscope under ×200 magnification. For digital image analysis, the software Adobe Photoshop 6.0 version was used.

Statistical analysis. Differences between groups were assessed using Wilcoxon test with *P* values adjusted by resampling (2,000 permutations). Two-tailed adjusted *P* values of <0.05 were considered to be significant.

Results

Phenotypic characteristics of TIL and PBMC in HNSCC patients. Flow cytometry analysis of TIL isolated by enzymatic digestion from tumor tissues showed that the $CD3^+CD4^+$ T-cell subset accounted for 44 ± 15% (mean ± SD) of all T cells, with a range of 30% to 60%. The gating strategy used to identify this cell subset is shown in Fig. 1A to C. Among these $CD4^+$ T cells, an average of 13 ± 3% was $CD25^+$ (Fig. 1D). Immunohistochemistry of HNSCC tissues (n = 15) confirmed the prominent presence of $CD4^+CD25^+$ T cells in tumor infiltrates (Fig. 2). This enrichment in $CD4^+CD25^+$ T cells was significant ($P \leq 0.001$) compared with circulating $CD3^+CD4^+$ T cells (3 ± 0.7%) in HNSCC patients or in normal controls (2 ± 1.5%). We also determined the proportions of $CD25^{high}$ cells (Treg) present in the $CD4^+CD25^+$ subset in TIL (3 ± 0.5%; n = 15), and in PBMC (1 ± 0.3%; n = 14) of HNSCC patients as well as normal controls (0.4 ± 0.2%; n = 15), as illustrated in Fig. 3. Our data showed that the mean percentages of $CD4^+CD25^+$ as well as $CD4^+CD25^{high}$ T cells were significantly higher in TIL and in PBMC of HNSCC patients ($P \leq 0.001$ and 0.01, respectively) compared with the mean percentage of these cells in PBMC of normal controls.

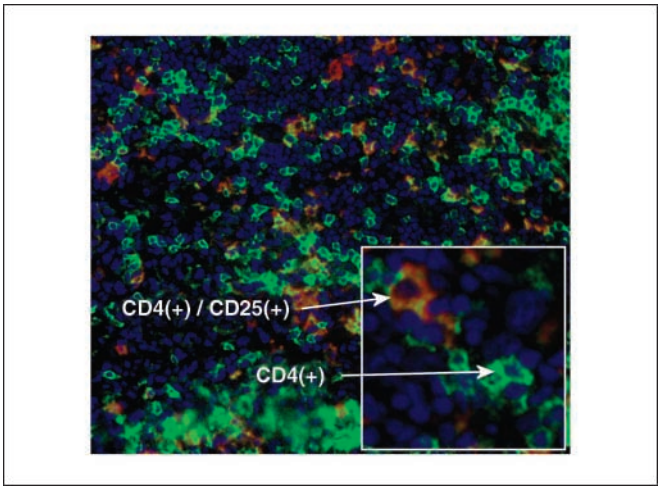

Fig. 2. Immunofluorescence for $CD4^+$ and $CD25^+$ cells among autologous T lymphocytes in tumor infiltrates. $CD4^+$ and $CD25^+$ TILs in sections obtained from a representative HNSCC of 15 examined. $CD25^+$ (*red*) and $CD4^+$ (*green*) cells with DAPI; note coexpression of CD25 and CD4 in many TIL (*yellow*). Magnification, ×500.

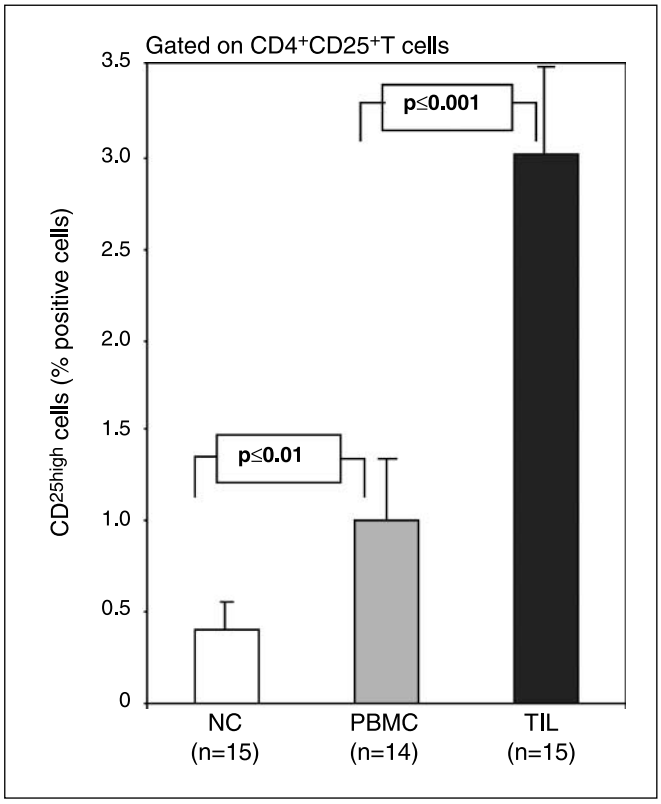

Fig. 3. Percentages of $CD25^{high}$ T cells within the $CD4^+CD25^+$ T-cell subset in PBMC and TIL of HNSCC patients and PBMC of normal controls as determined by flow cytometry.

For this analysis, $CD4^+$ T cells with a mean fluorescence intensity (MFI) of CD25 expression ≥120 were classified as $CD25^{high}$ (Fig. 1C). This cutoff point was selected based on our earlier observation that only $CD4^+CD25^+$ T cells with MFI ≥120 had suppressor activity after single-cell sorting of normal control PBMC. $CD4^+CD25^+$ T cells with MFI <120 for CD25 did not suppress autologous or allogeneic responder populations and probably consisted of activated T effector cells. An average MFI for $CD4^+CD25^{high}$ cells in TIL was 160 ± 25, whereas that for $CD4^+CD25^{high}$ cells in PBMC was 125 ± 12. $CD4^+$ TIL expressed higher levels of CD25 than did circulating $CD4^+$ T cells.

Phenotypic characteristics of $CD4^+CD25^{high}$ T lymphocytes in TIL and PBMC. We next analyzed T-cell activation and Treg markers as well as IL-10 and TGF-β1 (membrane bound and intracellular) on gated $CD4^+CD25^{high}$ T cells in isolated TIL and PBMC of HNSCC subjects or normal controls by flow cytometry. No significant differences were observed in percentages of $CD4^+CD25^{high}$ T cells positive for various Treg markers between the PBMC autologous with five TIL samples and allogeneic PBMC (n = 9) in HNSCC patients with active disease

($P \geq 0.05$; data not shown). Therefore, we were able to compare the Treg phenotype in TIL (n = 15) with that in normal controls (Fig. 4) or in combined autologous and allogeneic PBMC (n = 14) of HNSCC patients (Fig. 5).

Backgating on $CD4^+CD25^{high}$ T cells, we observed that these cells in TIL and PBMC of HNSCC patients were largely $Foxp3^+$ and CTLA-4^+, with the majority expressing Fas (CD95; Fig. 5A). Expression of Foxp3 on $CD4^+CD25^+$ TIL was also determined on frozen sections of tumors by immunofluorescence (Fig. 6). Staining *in situ* confirmed the presence of numerous $Foxp3^+CD25^+$ T cells in the tumor (Fig. 6A). Several other T-cell markers were differentially expressed on $CD4^+$ $CD25^{high}$ T cells in TIL compared with this subset of T cells in PBMC of HNSCC patients. For example, most $CD4^+CD25^{high}$ T cells in TIL were $GITR^+$, $FasL^+$, IL-10^+, and TGF-$\beta1^+$ (Figs. 5A and C and 6) relative to $CD4^+CD25^{high}$ T cells in PBMC ($P \leq 0.0015$), only few of which were positive for GITR and all of which were negative for FasL, IL-10, and membrane-bound or intracellular TGF-$\beta1$ (Fig. 5). Intracytoplasmic expression of IL-10 in $CD25^+$ TIL (Fig. 6A) or TGF-$\beta1$ in $CD4^+CD25^+$ TIL (Fig. 6B) *in situ* was clearly shown by immunofluorescence. Tumor sections were also stained for CD11c, CD4, or CD25 and IL-10 to determine whether IL-10 was expressed by tumor-associated macrophages/dendritic cells or Tregs. We observed that most IL-10^+ cells were also $CD4^+CD25^+$ but not $CD11^+$ (data not shown). Further, as shown in Fig. 6B, TGF-$\beta1^+$ TILs

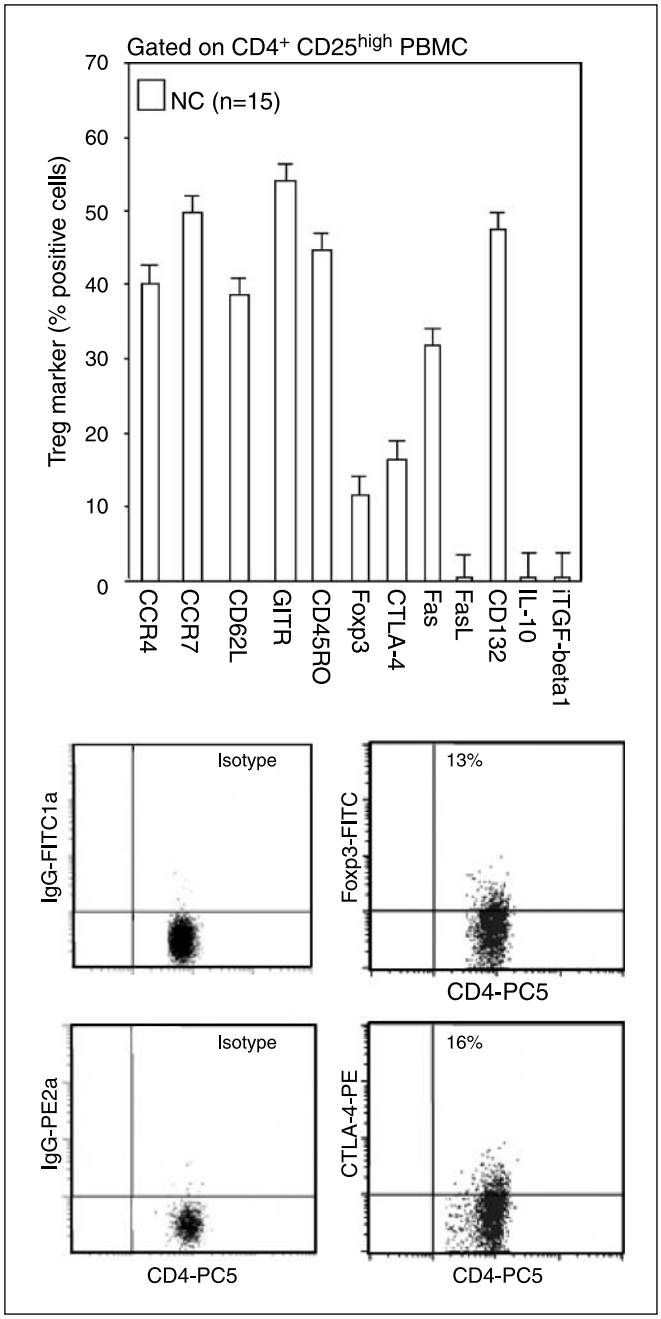

Fig. 4. Phenotypic characteristics of $CD4^+CD25^{high}$ T cells in PBMC of normal controls. Columns, mean percentages obtained by flow cytometry; bars, SD. Representative flow cytometry dot plots from one normal controls show Foxp3, CTLA-4 expression, and the isotype controls (IgG1a-FITC and IgG2a-PE).

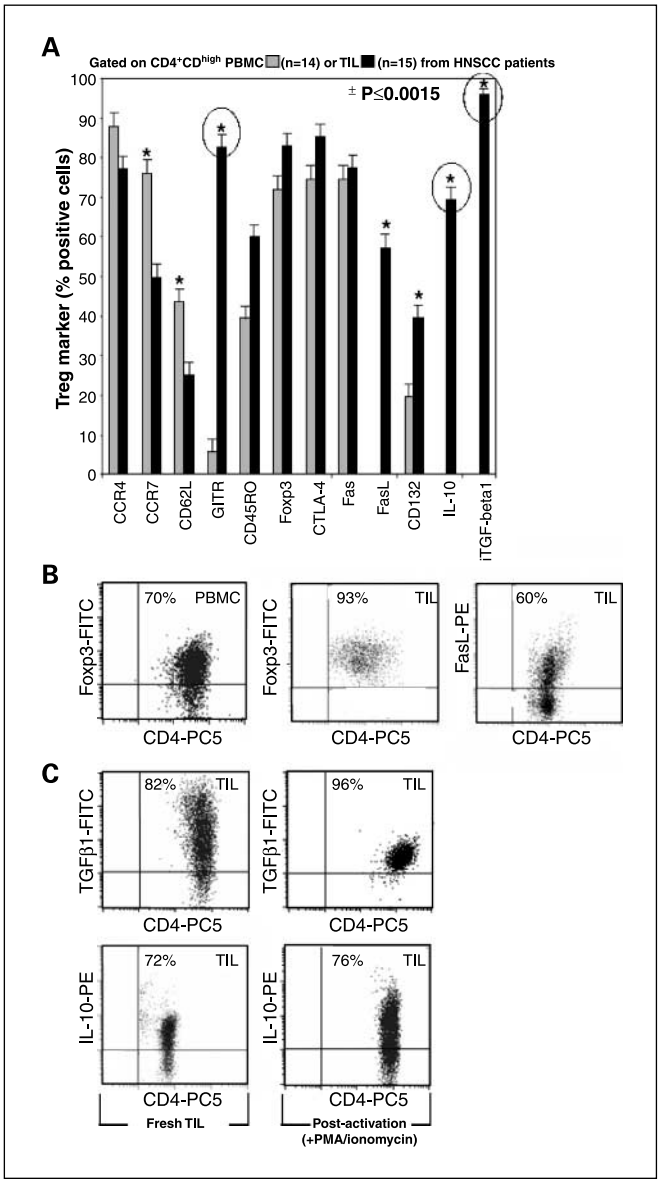

Fig. 5. *A,* phenotypic characteristics of $CD4^+CD25^{high}$ T cells in TIL or PBMC of HNSCC patients. Columns, mean percentages; bars, SD. Representative flow cytometry dot plots were obtained from one subject with HNSCC for Foxp3 and FasL (*B*) and for TGF-$\beta1$ and IL-10 before and after *ex vivo* stimulation (*C*).

were also $CD4^+CD25^+$. These *in situ* data corroborate flow cytometry results with isolated TIL indicating that 80% to 100% of $CD4^+CD25^{high}$ cells were positive for intracytoplasmic IL-10 and TGF-β1. As shown in Fig. 5C, TIL expressed IL-10 and TGF-β1 without *in vitro* activation, although, following stimulation with phorbol 12-myristate 13-acetate and ionomycin, expression of these two cytokines intensified.

A significantly higher percentage of $CD4^+CD25^{high}$ T cells was positive for the migration and lymph node homing receptors CCR7 and CD62L in PBMC of HNSCC patients than in TIL ($P \leq 0.0015$; Fig. 5A). Another chemokine receptor, CCR4, was found on nearly all $CD4^+CD25^{high}$ T cells in both PBMC of HNSCC patients and TIL (Fig. 5A) but on only ~40% of $CD4^+CD25^{high}$ T cells in normal controls (Fig. 4). $CD4^+CD25^{high}$ T cells were $CD45RO^+$ in TIL and PBMC of HNSCC patients (Fig. 5A) and normal controls (Fig. 4), indicating that Treg in humans largely belong to the memory T-cell compartment as suggested previously (23). Few $CD4^+CD25^{high}$ T cells were positive for CD45RA (5.7 ± 2.3%) or CD127 (12 ± 15%; data not shown).

Because only 60% of $CD25^{high}$ Treg were positive for CD45RO, and the remaining 40% cells did not express

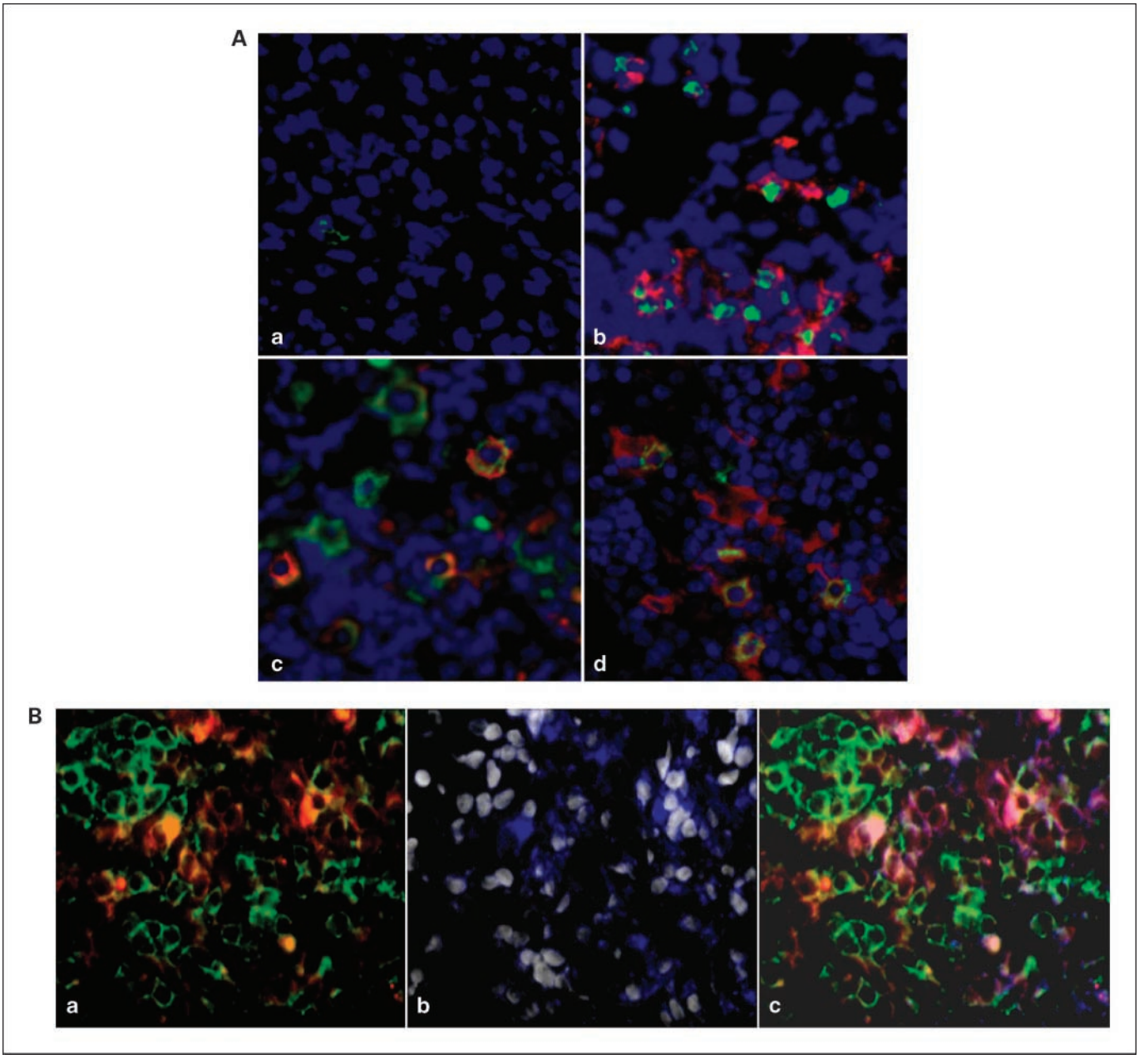

Fig. 6. Immunofluorescence for CD25, Foxp3, IL-10, GITR, or TGF-β1 in sections of HNSCC. *A,* TIL positive for CD25 and Foxp3, IL-10, GITR, or TGF-β1 on cryostat sections of a representative tumor (out of 15 examined). *a*, negative control using DAPI and secondary mouse anti-biotin FITC-labeled antibody alone. Nuclei are stained with DAPI. *b*, $CD25^+$ (*red*) and $Foxp3^+$ (*green*) cells with DAPI; note Foxp3 expression in $CD25^+$ TIL. *c*, $CD25^+$ (*green*) and $IL\text{-}10^+$ (*red*) cells with DAPI; note coexpression of CD25 and IL-10 in numerous TIL (*yellowish*). *d*, $CD25^+$ (*red*) and $GITR^+$ (*green*) cells with DAPI. Coexpression of CD25 and GITR in many TIL (*yellow*). Magnification, ×500. *B, a*, TIL positive for $CD4^+$ (*green*) and $CD4^+CD25^+$ (*yellow*); *b*, same tumor area stained for TGF-β1. Nuclei are stained with DAPI. *c*, an overlay of a + b with $CD4^+CD25^+$ TGF-$\beta1^+$ cells stained pink. Magnification, ×400.

CD45RA, we suspected that $CD25^{high}$ T cells (Treg) might selectively down-regulate CD45RA. Therefore, gating on $CD4^+CD45RO^{low}$, $CD4^+CD45RO^{high}$, $CD4^+CD45RA^{low}$, and $CD4^+CD45RO^{high}$ T-cell subsets, we analyzed CD25 expression in these subsets. Interestingly, all $CD4^+CD45RO^{low}$ T cells were also $CD45RA^{low}$ (MFI ≤ 50) and $CD25^{low/interm}$ (MFI ≤ 50). In contrast, most cells in the $CD4^+CD45RO^{high}$ subset were $CD25^{high}$ (MFI ≤ 165) but expressed no CD45RA. All of the $CD25^{high}$ T cells (Treg) were negative for CD45RA, whereas the $CD25^{low/interm}$ T cells were CD45RA low (MFI ≤ 80). These results suggest that (*a*) activated ($CD25^+$) non-Treg gain CD45RO expression and down-regulate CD45RA and (*b*) close to half of the $CD25^{high}$ Treg loose CD45RO expression but do not gain CD45RA. Whether this loss of CD45RO expression by Treg is related to the state of their differentiation or activation at the tumor site or to their sensitivity to apoptosis is presently unknown.

Compared with $CD4^+CD25^{high}$ Treg present in the peripheral circulation of normal controls, those in TIL and PBMC of HNSCC patients had significantly increased percentages of Foxp3, CTLA-4, and Fas ($P \leq 0.001$; Figs. 4 and 5). FasL was absent from the surface of PBMC in patients (Fig. 5) and normal controls (Fig. 4). Although GITR was expressed on ~80% of $CD4^+CD25^{high}$ cells in TIL, in PBMC of HNSCC patients, $CD4^+CD25^{high}$ T cells expressed little GITR (Fig. 5). In addition, in TIL (Fig. 5), close to half of $CD4^+CD25^{high}$ Treg up-regulated CD132 (IL-2Rγ). However, CD132 was not up-regulated on $CD4^+CD25^{high}$ T cells in PBMC of HNSCC patients (Fig. 5).

These data indicate that considerable phenotypic differences exist between the $CD4^+CD25^{high}$ populations in TIL and PBMC of HNSCC patients. Only TILs express GITR, secrete IL-10 and TGF-β1, and up-regulate CD132. Likewise, circulating $CD4^+CD25^{high}$ Treg in PBMC of normal controls are phenotypically distinct from those in PBMC as well as TIL in patients with cancer.

Suppressor activity of $CD4^+CD25^{high}$ T cells isolated from TIL or PBMC of HNSCC patients. To evaluate suppressor activity of $CD4^+CD25^{high}$ T cells in TIL or PBMC, single-cell sorting was used for their isolation followed by cocultures with CFSE-labeled autologous $CD4^+CD25^-$ T cells as responders. The cocultures were set up at the suppressor to responder ratios of 1:2 or 1:5 using responder proliferating in response to OKT3 and anti-CD28 antibodies in the presence of IL-2. To analyze proliferation of responder cells, gating was set to include $CD4^+CFSE^+$ T cells (Fig. 7A). The percentage of inhibition measured varied depending on the suppressor to responder ratios used and the Treg origin. Strong suppression of proliferation ($P \leq 0.001$) was observed using suppressor to responder ratios of 1:2 for Treg isolated from TIL or PBMC of HNSCC patients (Fig. 7B). At the suppressor to responder ratio of 1:5, only Treg isolated from TILs were suppressive (Fig. 7B). In contrast, $CD4^+CD25^{high}$ T cells isolated from the circulation of normal controls only minimally suppressed proliferation (Fig. 7B). This result confirms that the $CD25^+$ T-cell subset in normal controls is mainly composed of activated $CD25^+$ non-Treg as previously reported by us (22). On the other hand, $CD4^+CD25^{high}$ TILs are highly suppressive, and suppression mediated by Treg present in TIL is significantly higher ($P \leq 0.044$) than that mediated by the $CD4^+CD25^{high}$ subset in PBMC of patients with HNSCC.

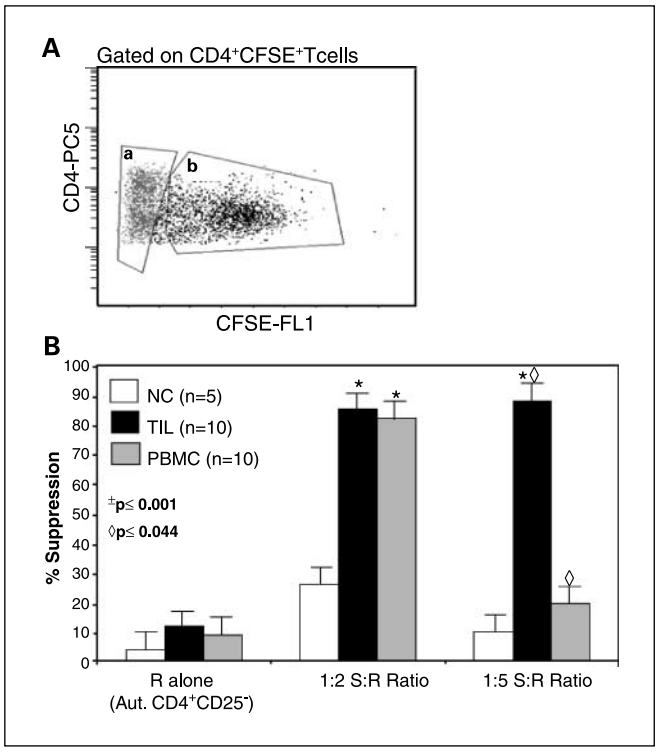

Fig. 7. Suppression of proliferation by $CD4^+CD25^{high}$ T cells single-cell sorted from TIL or PBMC of HNSCC patients and PBMC of normal controls. Following sorting, autologous $CD4^+CD25^-$ T cells were labeled with CFSE and stimulated with OKT3 and anti-CD28 antibody in the presence of IL-2 as described in Materials and Methods. *A,* the acquisition gates were restricted to the lymphocyte gate as determined by characteristic forward and side scatter properties of lymphocytes. Analysis gates on the ModFit program were restricted to the $CD3^+CD4^+$ and $CD4^+CFSE^+$ T-cell subsets. Gate "a" indicates the $CD4^+CD25^{high}$ non – CFSE-labeled T-cell fraction and gate "b" indicates the $CD4^+CD25^-$ CFSE-labeled T-cell fraction. *B,* $CD4^+CD25^{high}$ T cells obtained from PBMC or TIL were added to autologous responder cells at the start of the culture [suppressor (*S*) to responder (*R*) ratios of 1:2 or 1:5]. Flow cytometry was done on day 5. Cell division as represented by the dilution of CFSE was analyzed using the ModFit software. Average percentages of proliferation inhibition in responder cells.

Treg in TIL mediate suppression of autologous T cells via IL-10 and TGF-β1. A large majority (75-96%) of $CD4^+CD25^{high}Foxp3^+$ T cells in TIL isolated from HNSCC patients were positive for intracytoplasmic IL-10 and TGF-β1 by flow cytometry and *in situ* by immunofluorescence (Figs. 5 and 6). As described above, these cells mediated stronger suppression relative to Treg isolated from PBMCs that were negative for IL-10 and TGF-β1. Therefore, we hypothesized that Treg function in TIL was mediated via IL-10 and TGF-β1 secretion and did not require cell-to-cell contact. To test this hypothesis, $CD4^+CD25^+$ Tregs isolated from TIL were coincubated at the suppressor to responder ratio of 1:2 with CFSE-labeled autologous $CD4^+CD25^-$ T cells responding to OKT3 and anti-CD28 antibodies in the presence or absence of Transwell inserts that permit diffusion of soluble factors but prevent cell-to-cell contact.

Coincubation in the presence of Transwell inserts resulted in significant reduction of suppression ($P \leq 0.03$) as shown in Fig. 8A. Nevertheless, in the presence of Transwell inserts, suppression levels remained at 60%, suggesting that cell-to-cell contact was only partly necessary. When, however, Treg in the upper compartment were treated with both neutralizing antibodies to IL-10 and TGF-β1, suppression was completely abrogated ($P \leq 0.0001$; Fig. 8A). Further, the addition of

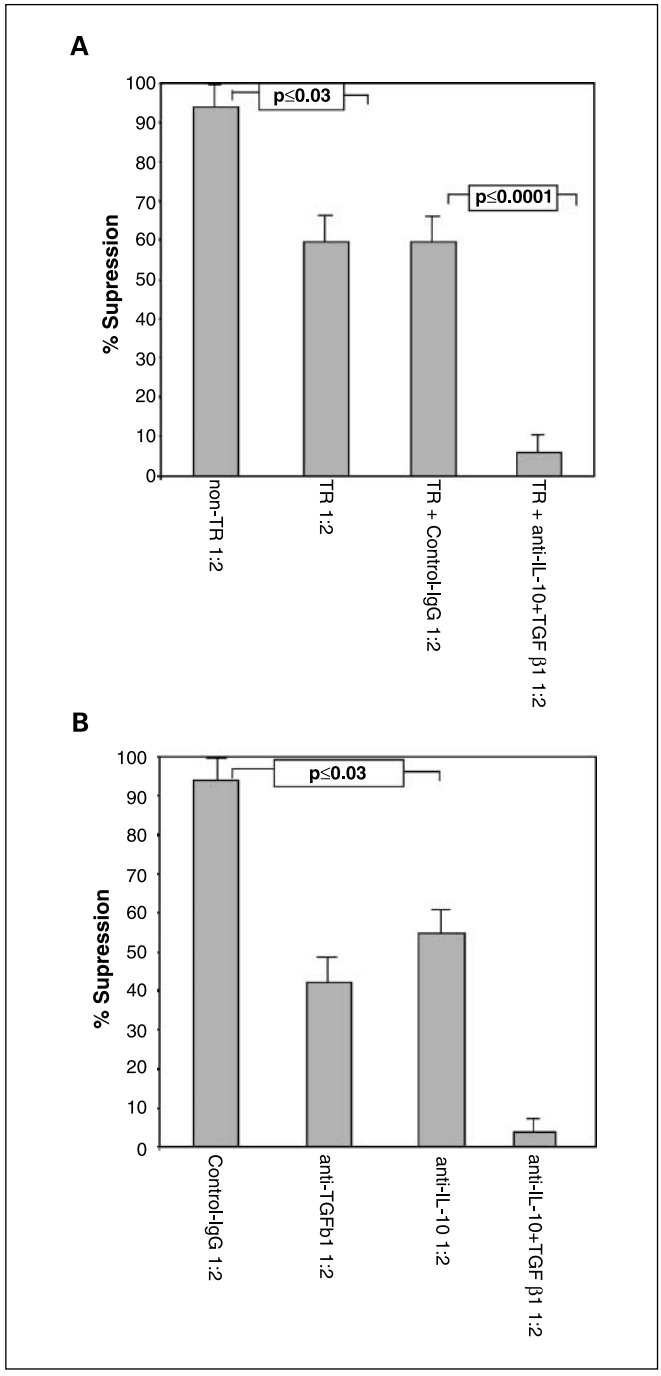

Fig. 8. Potential mechanisms used to suppress proliferation of responder cells by $CD4^+CD25^{high}$ T cells in TIL of HNSCC patients. Autologous $CD4^+CD25^-$ T cells were labeled with CFSE and stimulated with OKT3 and anti-CD28 antibody in the presence of IL-2 as described in Materials and Methods. $CD4^+CD25^{high}$ T cells from TIL were added at the start of the culture (suppressor to responder ratio of 1:2). In some experiments, Transwell inserts were used to separate responder from Treg. Neutralizing anti-IL-10 and/or anti-TGF-β1 antibody or nonblocking IgG anti-mouse antibody were added to the Transwell and non-Transwell assays. Flow cytometry was done on day 5. *A,* left, suppression of responder proliferation separated from Treg by Transwell inserts (*TR*) compared with assays that are not separated by inserts at the suppressor to responder ratio of 1:2; right, suppression of responder proliferation separated from Treg by Transwell inserts in the presence of nonblocking IgG anti-mouse antibody or neutralizing anti-IL-10 + anti-TGF-β1 antibody at the suppressor to responder ratio of 1:2. *B,* suppression of responder proliferation in the absence of cell-to-cell interactions and the presence of nonblocking IgG anti-mouse antibody, neutralizing anti-IL-10 or anti-TGF-β1 antibody, or neutralizing anti-IL-10 + anti-TGF-β1 antibody at the suppressor to responder ratio of 1:2. Results of three representative experiments done with TIL of different HNSCC patients.

neutralizing anti-IL-10 or TGF-β1 antibodies to Treg in the absence of Transwell inserts did not reduce suppression beyond the level seen with the insert alone (i.e., ~50%; Fig. 8B). These results suggest that not only cell-to-cell contact but also soluble factors (cytokines) are involved in suppression. However, in the absence as well as presence of cell-to-cell interaction, blocking of both IL-10 and TGF-β1 was necessary to completely abrogate suppression (Fig. 8A and B). In aggregate, these data suggest that suppression is mediated by soluble factors produced by Treg and able to diffuse through the membrane when cell-to-cell contact is abrogated. However, cell-to-cell contact contributes to suppression mediated by Treg-derived cytokines.

The role of $CD4^+CD25^{high}$ T-cell subsets in tumor progression. Most patients (n = 12) whose tumors were used for TIL isolation had advanced disease (stage T_3/T_4). The tumors were infiltrated by $CD3^+CD4^+CD25^+Foxp3^+$ T cells (Figs. 3 and 5) as determined by immunofluorescence of frozen tumor sections. These Treg were strongly suppressive and produced IL-10 and TGF-β1. Thirteen of 15 of the HNSCC patients died of their disease within 3 to 4 years of surgery. Our observations suggest that the presence at the tumor site of $CD4^+CD25^{high}Foxp3^+$ T cells with strong suppressor functions seems to be a characteristic feature of T_3/T_4 stage tumors and might be associated with a poor prognosis.

Discussion

Several studies have reported that accumulation of TIL at the tumor site is associated with a better prognosis (5–8). However, other studies show that infiltrating $CD4^+$ T cells can favor the tumor to the detriment of the host (10). Specifically, the subpopulation of $CD4^+CD25^{high}Foxp3^+$ T cells referred to as Treg may impair the ability of the host immune system to defend against tumor progression (10, 24). An improved understanding of the phenotype and function of Treg within TIL could help establish a more reliable prognostic role of TIL in patients with solid tumors. Further, such an understanding is necessary to foster the development of targeted approaches that would eliminate Treg and perhaps specifically augment anti-tumor immune responses. Indeed, our study suggests that the type and functional attributes of tumor-infiltrating $CD4^+$ T cells, but not their quantity or localization, may prove to be the most critical prognostic determinants.

In contrast to our results, Badoual et al. (4) recently showed that the percentage of $CD4^+Foxp3^+$ T cells positively correlated with a better prognosis in patients with HNSCC. In addition, recent studies in follicular lymphoma (25, 26) and Hodgkin's lymphoma (27) showed that the presence of tumor-infiltrating $CD4^+Foxp3^+$ Treg cells was associated with a better patient survival. Results of these studies are counterintuitive, and it is important to note that, in these studies, only the *in situ* frequency of $CD4^+Foxp3^+$ T cells was correlated with clinical outcome (4, 25, 27). None examined the phenotypic characteristics and suppressor functions of $CD4^+CD25^{high}Foxp3^+$ T cells in TIL.

We show for the first time that Treg localizing to tumor tissue in HNSCC comprise a unique subset of $CD4^+CD25^{high}$ T cells, which secrete IL-10 and TGF-β1 and mediate strong suppressor function. Intratumoral $CD4^+CD25^{high}Foxp3^+$ T cells have phenotypic and functional characteristics that differ from those of circulating $CD25^{high}$ Treg. Phenotypically, Treg found in TIL combine characteristic features of both nTreg and Tr1

cells; on the one hand, they express the phenotype consistent with activated memory T cells ($CD25^{high}CD45RO^{+}CD45RA^{-}$) and up-regulate Foxp3, CTLA-4, and CD95 as previously described by us for circulating nTreg in normal donors (22) and HNSCC patients (19). On the other hand, Treg in TIL up-regulate IL-10 and TGF-β1 and express CD132 (IL-2Rγ), which are features is characteristics of tumor-induced Tr1 cells (28). These novel findings introduce several questions about the origin of Treg at the tumor site and interactions between the host and human Treg subsets. Whether the regulatory cells are recruited from the periphery and accumulate at the tumor or whether the tumor microenvironment converts $CD4^{+}CD25^{-}$ T cells present *in situ* to $CD4^{+}CD25^{high}$ Treg is currently unclear. Treg in TIL might also comprise a subset of "transition" cells (i.e., nTreg activated by tumor antigens in the tumor microenvironment and converted into Tr1 T cells). Recent observations suggest that nTreg cells are involved in the differentiation of IL-10–secreting Tr1 T cells *in vitro* (29) and *in vivo* (30). Most circulating Treg are positive for the lymphocyte homing markers CD62L and CCR7 as also previously reported (22). This finding indicates that they can migrate to lymph nodes as well as to the tumor. Notably, close to ≤90% of Treg in TIL are $GITR^{+}$, although only few circulating Treg express GITR, and this suggests that Treg at the tumor site acquire GITR expression perhaps as consequence of local activation. Thus, considerable reshaping of infiltrating lymphocytes seems to be ongoing in the tumor microenvironment.

We also report for the first time that virtually all tumor-infiltrating Treg are positive for the chemokine receptor CCR4. We and others have previously shown that Treg in PBMC from patients with cancer and from normal controls up-regulate CCR4 (31). The functional significance of CCR4 expression on tumor-infiltrating Treg remains uncertain. Curiel et al. (10) recently showed that the chemokine CCL22 (CCR4L), which is abundantly expressed in ovarian cancer tissues and tumor cells in ovarian ascites, preferentially attracts the peripheral $CD4^{+}CD25^{+}Foxp3^{+}$ T cells to the tumor. Several recent reports associated this expression of CCR4 with protection of T cells from apoptosis (32). Because TIL are especially sensitive to apoptosis in the tumor microenvironment (33), this possibility is intriguing and should be further investigated.

Our phenotypic analysis of the $CD4^{+}CD25^{+}$ T-cell subset in TIL indicates for the first time in humans that close to half of the $CD25^{high}$ Treg in TIL and PBMC down-regulate CD45RO expression but do not concomitantly gain CD45RA. This loss of CD45RO expression might be related to apoptosis sensitivity of $CD25^{high}$ Treg. It is well documented that $CD45RO^{high}$ memory T effector cells ($CD25^{low/interm}$) are highly sensitive to activation-induced cell death, whereas $CD45RA^{+}$ naive T cells are protected (34). Induction of apoptosis in human T cells by ligation of CD45 has been described (35). The loss of CD45RO on Treg may increase their resistance to apoptosis relative to tumor-specific $CD45RO^{+}$ T effector cells that are sensitive to apoptosis, contributing to an increased Treg compartment in patients with cancer.

Cytokines, and in particular IL-10 and TGF-β1, play major roles in the generation of nTreg (36–38) and function of Tr1 cells (11, 12), although their individual contributions to differentiation of Treg subsets and suppressor function are still debated. In the initial studies, using *in vitro* assay systems, it was shown that strong suppressive activity exerted by $CD4^{+}CD25^{high}Foxp3^{+}$ Treg requires cell-to-cell contact (13, 14), and it was thus considered likely that the suppression was dependent on ligand-receptor interactions at the cell surface. However, more recent reports suggest that some subsets of human $CD4^{+}CD25^{high}$ cells produce IL-10 and/or TGF-β1 (39–41). We show that $CD4^{+}CD25^{high}$ T cells in TIL isolated from HNSCC patients or *in situ* produce IL-10 and TGF-β1 and that neutralizing antibodies to these cytokines completely restored T responder cell proliferation in an autologous system. This suggests that soluble factors play a central role in immune suppression mediated by Treg in TIL. Interestingly, however, our data also show that Transwell inserts that prevent cell-to-cell contact between suppressor and responder also significantly block suppression (50%). The fact that the combination of neutralizing antibodies to IL-10 and TGF-β1 completely abrogated suppression in the presence or absence of the Transwell insert, whereas Transwell inserts in the absence of the blocking antibodies reduced suppression by 50%, suggests that these cytokines might be required for activation of surface molecules on Treg that are involved in suppression mediated by cell-to-cell contact. Thus, it is possible that, although cell-to-cell contact contributes to suppression, it is dependent on Treg-derived cytokines, which regulate/induce mechanisms directly responsible for blocking of responder cell functions. Our data suggest that mechanisms involving cytokines and also requiring cell-to-cell contact contribute to suppression mediated by Treg.

Our data also suggest that suppressor functions of Treg present in TIL may be related to poor survival in HNSCC patients with advanced disease. Frequently, newly diagnosed HNSCCs are locoregionally advanced (stages III and IV) at the time of diagnosis (42). Unfortunately, current oncologic therapy does not provide complete tumor control for advanced disease, and prognosis for advanced local HNSCC remains disappointing (43). It has been previously reported that advanced primary tumors of ovary are infiltrated by suppressor $CD4^{+}CD25^{+}Foxp3^{+}$ T cells (10), whereas early tumors are characterized by a significantly lower Treg infiltration (44). These results indicate that Treg infiltrates into the tumor might reflect disease progression and that the nature of the tumor microenvironment, including accumulation of Treg, may influence the outcome of patients with cancer. Our data with TIL obtained from tumors of patients with advanced disease stages fit well with this explanation.

This study contributes to a better characterization of $CD4^{+}$ T cells present in the tumor tissues and peripheral circulation of patients with HNSCC by analysis of the surface markers as well as cytokine profile and function, thus providing a broader basis for the development of more reliable prognostic factors. It also emphasizes the fact that in patients with cancer the tumor induces tolerance via activation and/or induction of Treg both locally and systemically. Additionally, the observation that Treg in TIL and PBMC have a different phenotype and use different mechanisms to suppress T-cell proliferation might have strong implications for the design and implementation of future immune therapies.

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References

1. Allan CP, Turtle CJ, Mainwaring PN, Pyke C, Hart DN. The immune response to breast cancer, and the case for DC immunotherapy. Cytotherapy 2004;6:154–63.
2. Whiteside TL. Down-regulation of ζ-chain expression in T cells: a biomarker of prognosis in cancer? Cancer Immunol Immunother 2004;53:865–78.
3. Ogino T, Bandoh N, Hayashi T, Miyokawa N, Harabuchi Y, Ferrone S. Association of tapasin and HLA class I antigen down-regulation in primary maxillary sinus squamous cell carcinoma lesions with reduced survival of patients. Clin Cancer Res 2003;9:4043–51.
4. Badoual C, Hans S, Rodriguez J, et al. Prognostic value of tumor-infiltrating $CD4^+$ T-cell subpopulations in head and neck cancers. Clin Cancer Res 2006;12:465–72.
5. Labarriere N, Pandolfino MC, Gervois N, et al. Therapeutic efficacy of melanoma-reactive TIL injected in stage III melanoma patients. Cancer Immunol Immunother 2002;51:532–8.
6. Kuss I, Hathaway B, Ferris RL, Gooding W, Whiteside TL. Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck. Clin Cancer Res 2004;10:3755–62.
7. Fukunaga A, Miyamoto M, Cho Y, et al. $CD8^+$ tumor-infiltrating lymphocytes together with $CD4^+$ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. Pancreas 2004;28:e26–31.
8. Cho Y, Miyamoto M, Kato K, et al. $CD4^+$ and $CD8^+$ T cells cooperate to improve prognosis of patients with esophageal squamous cell carcinoma. Cancer Res 2003;63:1555–9.
9. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of $FOXP3^+$ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res 2006;12:5423–34.
10. 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;10:942–9.
11. Groux H, O'Garra A, Bigler M, et al. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737–42.
12. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. Immunol Rev 2001;182:68–79.
13. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. *Ex vivo* isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. J Exp Med 2001;193:1303–10.
14. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded *in vitro* without loss of function. J Exp Med 2001;193:1295–302.
15. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med 2001;193:1285–94.
16. Carrier Y, Yuan J, Kuchroo VK, Weiner HL. Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-β T cell-transgenic mice. J Immunol 2007;178:179–85.
17. Albers AE, Ferris RL, Kim GG, Chikamatsu K, DeLeo AB, Whiteside TL. Immune responses to p53 in patients with cancer: enrichment in $tetramer^+$ p53 peptide-specific T cells and regulatory T cells at tumor sites. Cancer Immunol Immunother 2005;54:1072–81.
18. Schaefer C, Kim GG, Albers A, Hoermann K, Myers EN, Whiteside TL. Characteristics of $CD4^+CD25^+$ regulatory T cells in the peripheral circulation of patients with head and neck cancer. Br J Cancer 2005;92:913–20.
19. Strauss L, Bergmann C, Johnson J, Whiteside TL. Functional $CD4^+CD25^{high}Foxp3^+$ T cells in the circulation of patients with head and neck squamous cell carcinoma (HNSCC) and correlation with advanced disease. Arch Otolaryngol Head Neck Surg 2006;132:883–4.
20. Loddenkemper C, Schernus M, Noutsias M, Stein H, Thiel E, Nagorsen D. *In situ* analysis of $FOXP3^+$ regulatory T cells in human colorectal cancer. J Transl Med 2006;4:52.
21. Whiteside TL, Miescher S, MacDonald RH, von Fliedner V. Separation of tumor infiltrating lymphocytes from tumor cells in human solid tumors. A comparison between velocity sedimentation and discontinuous density gradients. J Immunol Methods 1986;90:221–33.
22. Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human $CD4^+CD25^+Foxp3^+$ regulatory T cells cultured with rapamycin. J Immunol 2007;178:320–9.
23. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745–63.
24. Beyer M, Schultze JL. Regulatory T cells in cancer. Blood 2006;108:804–11.
25. Carreras J, Lopez-Guillermo A, Fox BC, et al. High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. Blood 2006;108:2957–64.
26. Lee AM, Clear AJ, Calaminici M, et al. Number of $CD4^+$ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. J Clin Oncol 2006;24:5052–9.
27. Alvaro T, Lejeune M, Salvado MT, et al. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res 2005;11:1467–73.
28. Bergmann C, Strauss L, Zeidler R, Lang S, Whiteside TL. Expansion and characteristics of human T regulatory type 1 cells in co-cultures simulating tumor microenvironment. Cancer Immunol Immunother. 2007 Jan 31, Epub ahead of print.
29. Seo N, Hayakawa S, Takigawa M, Tokura Y. Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4(+) T-regulatory cells and systemic collapse of antitumour immunity. Immunology 2001;103:449–57.
30. Dieckmann D, Plottner H, Dotterweich S, Schuler G. Activated $CD4^+$ $CD25^+$ T cells suppress antigen-specific $CD4^+$ and $CD8^+$ T cells but induce a suppressive phenotype only in $CD4^+$ T cells. Immunology 2005;115:305–14.
31. Hirahara K, Liu L, Clark RA, Yamanaka K, Fuhlbrigge RC, Kupper TS. The majority of human peripheral blood $CD4^+CD25^{high}Foxp3^+$ regulatory T cells bear functional skin-homing receptors. J Immunol 2006;177:4488–94.
32. Kim JW, Ferris RL, Whiteside TL. Chemokine C receptor 7 expression and protection of circulating $CD8^+$ T lymphocytes from apoptosis. Clin Cancer Res 2005;11:7901–10.
33. Reichert TE, Strauss L, Wagner EM, Gooding W, Whiteside TL. Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma. Clin Cancer Res 2002;8:3137–45.
34. Kohlmeier JE, Chan MA, Benedict SH. Costimulation of naive human CD4 T cells through intercellular adhesion molecule-1 promotes differentiation to a memory phenotype that is not strictly the result of multiple rounds of cell division. Immunology 2006;118:549–58.
35. Gregori S, Mangia P, Bacchetta R, et al. An anti-CD45RO/RB monoclonal antibody modulates T cell responses via induction of apoptosis and generation of regulatory T cells. J Exp Med 2005;201:1293–305.
36. Liu H, Hu B, Xu D, Liew FY. $CD4^+CD25^+$ regulatory T cells cure murine colitis: the role of IL-10, TGF-β, and CTLA4. J Immunol 2003;171:5012–7.
37. Shevach EM. $CD4^+$ $CD25^+$ suppressor T cells: more questions than answers. Nat Rev Immunol 2002;2:389–400.
38. Nakamura K, Kitani A, Fuss I, et al. TGF-β1 plays an important role in the mechanism of $CD4^+CD25^+$ regulatory T cell activity in both humans and mice. J Immunol 2004;172:834–42.
39. Freeman CM, Chiu BC, Stolberg VR, et al. CCR8 is expressed by antigen-elicited, IL-10-producing $CD4^+CD25^+$ T cells, which regulate Th2-mediated granuloma formation in mice. J Immunol 2005;174:1962–70.
40. Tsuji NM, Mizumachi K, Kurisaki J. Antigen-specific, $CD4^+CD25^+$ regulatory T cell clones induced in Peyer's patches. Int Immunol 2003;15:525–34.
41. Larmonier N, Marron M, Zeng Y, et al. Tumor-derived CD4(+)CD25(+) regulatory T cell suppression of dendritic cell function involves TGF-β and IL-10. Cancer Immunol Immunother 2007;56:48–59.
42. Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. N Engl J Med 1993;328:184–94.
43. Hamberger AD, Fletcher GH, Guillamondegui OM, Byers RM. Advanced squamous cell carcinoma of the oral cavity and oropharynx treated with irradiation and surgery. Radiology 1976;119:433–8.
44. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 2003;9:4404–8.