The Frequency and Suppressor Function of CD4⁺CD25⁺⁺Foxp3⁺ T Cells in the Circulation of Patients with Squamous Cell Carcinoma of the Head and Neck
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Abstract

Objective: Immune escape is a characteristic feature of head and neck squamous cell carcinoma (HNSCC). Regulatory T cells (Treg) might contribute to HNSCC progression by suppressing antitumor immunity, and their attributes in patients are of special interest.

Methods: Multicolor flow cytometry was used to study the frequency and phenotype of Treg in peripheral blood lymphocytes of 35 patients with HNSCC and 15 normal controls (NC). CD4⁺CD25⁺⁺ T cells were purified by fluorescence-activated cell sorting and tested for regulatory function by coculture with carboxyfluorescein diacetate succinimidylester–labeled autologous CD4⁺CD25⁺ responder cells.

Results: The percentages of circulating CD4⁺CD25⁺ T cells were increased in HNSCC patients (5 ± 3%) versus NC (2 ± 1.5%). In patients, this cell subset largely contained CD4⁺CD25⁺⁺ Foxp3⁺ T cells and only few CD25⁺⁻Foxp3⁻ cells. In addition, the frequency of Treg positive for CD62L, CTLA-4, Fas, FasL, and Foxp3 was greater in the circulation of patients than in NC (P < 0.0001). In HNSCC patients, Treg mediated significantly higher suppression (78 ± 7%) compared with Treg in NC (12 ± 4%) with P < 0.0001. Surprisingly, higher Treg frequency (P < 0.0059) and levels of suppression (P < 0.0001) were observed in patients with no evident disease (NED) than in untreated patients with active disease (AD).

Conclusions: The frequency of T cells with suppressor phenotype and function (Treg) was significantly greater in HNSCC patients who were NED after oncologic therapy relative to those with AD. This finding suggests that oncologic therapy favors expansion of Treg.

Patients with head and neck squamous cell carcinoma (HNSCC) have benefited from recent advances in radiation therapy, chemotherapy, and surgical techniques. However, despite new treatment modalities and their success in terms of organ preservation and overall quality of life, survival rates for this disease have not improved in many years (1). More recent studies have examined the role of host immune responses in HNSCC progression, suggesting that T lymphocytes may play a role in control of tumor growth. Several naturally processed and presented HNSCC-associated antigens have been identified and are known to be recognized by specific CD4⁺ and CD8⁺ T lymphocytes (2). Most immunologic studies have focused on the analysis of CD8⁺ T cells, which have been shown to mediate antitumor immunity (3). The role of CD4⁺ T cells is more complex, as subsets of CD4⁺ T cells are involved in initiating and maintaining antitumor responses (4, 5), as well as down-regulating these responses. In HNSCC subjects, antitumor functions of CD8⁺ T lymphocytes are often compromised (6).

In recent years, the concept has emerged that peripheral tolerance to tumors is maintained and enhanced by T cells with immunoregulatory function (Treg). In cancer, Treg frequency is increased in the peripheral circulation, and their accumulations in the tumor may be predictive of significantly reduced patient survival (7). We have previously described an enrichment of CD4⁺CD25⁺ T cells among tumor-infiltrating or circulating lymphocytes in HNSCC patients (8). Such increases in Treg could potentially present a significant problem, as these cells could be interfering with antitumor immune responses and inhibit responses to immunotherapies. However, phenotypic characteristics of Treg in HNSCC patients are incompletely understood, and their functional characteristics have not been evaluated.

To date, three types of CD4⁺ Treg cells have been partly characterized in humans: (a) CD4⁺CD25⁻Foxp3⁻ regulatory (Tr1) cells, which arise in the periphery upon encountering antigen in a tolerogenic environment via a process that is interleukin-10 (IL-10) dependent (9–11); (b) naturally occurring CD4⁺CD25⁺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 (12–14); and (c) Th3 cells, which are dependent on IL-4 for functional differentiation (15).

Currently, isolation and expansion of human Treg cells subsets into functionally active, disease-specific T cells is, however, difficult due to (a) the paucity of Treg cells in the peripheral blood and (b) the lack of specific identity markers for Treg cells. In humans, CD4+CD25+ T cells are mixed populations, including suppressor CD4+CD25high T cells as well as CD4+CD25low T cells, which are nonsuppressive, activated CD4+ T cells. Furthermore, expression of Treg markers such as CTLA-4 or GITR can vary depending on cell activation, and these markers have not been useful for discriminating nTreg from effector T-cell populations. Similarly, Foxp3 expression, although more specific for Treg, may also be up-regulated on effector cells following activation (16). Also, due to its intracellular localization, Foxp3 cannot be used for isolation of living Treg cells. Recently, two groups have independently shown that expression of CD127, the alpha chain of the IL-7 receptor, discriminates CD127low Treg cells from CD127high conventional T cells within the CD25+CD45RO+/RA effector/memory and the CD45RA*RO naïve compartments in the human peripheral blood and lymph nodes (17). Nevertheless, T effector cells, which fail to differentiate into CD127high memory T cells after activation, down-regulate CD127 (18). These CD127low pseudoeffector cells persist in the peripheral circulation, displaying the hallmarks of activated effector cells but are unable to mediate effector functions (19).

In our previous studies, we have developed flow cytometry-based methods for the characterization of phenotypic and functional attributes of human circulating CD4+ Treg (20). Here, we use these methods to evaluate Treg in the peripheral blood of HNSCC patients with active disease (AD) as well as no evident disease (NED) after oncologic therapy. We show that the Treg subset within CD3+CD4+ is expanded and strongly depressive in the peripheral circulation of HNSCC patients. Furthermore, in HNSCC patients who are NED after oncologic therapy, the frequency and suppressor function of Treg are higher than those in patients with AD. This finding suggests that oncologic therapy might contribute to the survival and expansion of highly active Treg in patients with cancer.

### Materials and Methods

**HNSCC patients and healthy volunteers.** Blood samples were obtained from 35 HNSCC patients and 15 age-matched healthy volunteers as controls (NC). All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh. All patients were seen at the Outpatient Otolaryngology Clinic at the University of Pittsburgh Cancer Institute (UPCI) between January 2006 and April 2007. The patient cohort included males and females with a mean age of 60 years (range, 23–82 years). The NC group included 10 males and 5 females with a mean age of 60 years. A total of 15 patients had AD. All NED patients (n = 20) underwent surgical resection of their tumor with a curative intent, and 14 of these received radiotherapy and/or chemotherapy. Of the 15 AD patients, 11 had untreated primary tumors, and 4 had a recurrent disease. The therapy, if given, was terminated from 3 weeks to 12 months before the time of phlebotomy for this study. The age, sex, and clinicopathologic characteristics of the patients are listed in Table 1.

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**Collection of peripheral blood mononuclear cells.** 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. Peripheral blood mononuclear cells (PBMC) were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

**Antibodies.** The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD3-EC, anti-CD4-PC5, anti-CD8-PC5, anti-GITR-FTIC, anti-CD25-FTIC, anti-CD62L-FTIC, anti-HLA-DR-FTIC, anti-Foxp3-FTIC, anti-CD122-FTIC (IL-2Rβ), anti-CD45RA-FTIC, anti-CD45RO-FTIC, anti-Fas-FTIC, anti-CD127-FTIC, anti-CCR7-FTIC, anti-TGFβ1-FTIC, anti-FasL-PE (NOK-1,42 kDa), anti-CD132-PE (IL-2Rγδ), anti-CD45PE-FTIC, anti-CD152-PE (CTLA-4), anti-CR4-PE, 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-FTIC, anti-FasL-PE (BD Pharmingen), anti-CD127-FTIC, anti-CCR7-FTIC [R&D Systems, Inc.], anti-TGFβ1-FTIC (Antigenix America Inc.), anti-CD127-FTIC, and anti-Foxp3-FTIC (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⁺⁺ and CD8⁺CD25⁺⁺ T cells and the expression of nTreg markers, including intracellular and surface expression of transforming growth factor β1 (TGFβ1) and IL-10, PBMCs (at least 2 × 10⁵ cells per tube) were stained with mAbs included in the above-described panel for 15 min at 4°C. Appropriate isotype Ab controls were used in all experiments. Cells were washed and examined by four-color flow cytometry, as previously described (21).

Intracellular staining for Foxp3, CD152 (CTLA-4), and IL-10 was done as previously described (21). Intracytoplasmic expression of TGFβ1 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 μg/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 RT, washed once with PBS containing 0.5% (v/v) bovine serum albumin (BSA; w/v) and 2 mmol/L EDTA, permeabilized with PBS containing 0.5% BSA and 0.1% (v/v) saponin and stained with pre-titrated anti–CTLA-4-PE, anti–Foxp3-FITC, anti–TGFβ1-FITC, or anti–IL-10-PE mAb for 30 min at RT. Cells were further washed twice with PBS containing 0.5% BSA.
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 their characteristic forward (FSC) and side-scatter (SSC) properties. FSC an SSC were set in a linear scale. For analysis, $1 \times 10^5$ lymphocytes were acquired. Furthermore, analysis gates were restricted to the CD3+CD4+, CD4+CD25+, CD8+CD3+, CD8+CD25high, and CD4+CD25high T-cell subsets, 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–sorted, fresh CD4+CD25+ T-cell populations were tested for regulatory function by coculture analysis with at least $0.5 \times 10^5$ carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled autologous CD4+CD25- responder cells per well at the suppressor/responder (S/R) ratios of 1:1, 1:5, and 1:10. Soluble OKT3 (1 µg/mL; American Type Culture Collection) and soluble anti-CD28 mAb (1 µg/mL) were used for stimulation in the presence of 150 IU IL-2/mL for 5 days. CFSE-labeling of R cells was done as previously described (21, 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 the CD4+CD25+ R cells in the coculture assay and if decreasing the number of CD4+CD25+ T cells relative to the number of CD4+CD25+ R 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, so that they could be tested regardless of levels of expansion.

All CFSE data were analyzed using the ModFit software provided by Verity Software House (Topsham). The percentages of suppression were calculated based on the proliferation index (PI) of responder cells alone compared with the PI of cultures containing responders and Treg. The program determines the percent of cells within each peak, and the sum of all peaks in the control culture 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 $5 \times 10^4$ 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 IL-2/mL in the lower chambers of the plates. Autologous Treg or control cells (CD4+CD25-) were added to the upper chambers at the S/R ratio of 1:1 or 1:5.

**Statistical analysis.** Differences between groups were assessed using Kruskal-Wallis test. Two-group differences reported in this manuscript were tested with the Wilcoxon test, with $P$ values adjusted by resampling (2,000 permutations; ref. 23). Suppressor function at three different ratios of S/R cells was analyzed by a two-way factorial ANOVA for square root–transformed suppression.

**Results**

Frequency of CD4+CD25+ T cells in the peripheral blood of HNSCC patients. PBMC obtained from HNSCC patients with
NED (n = 20), AD (n = 15), and NC (n = 15) were first examined for the frequency of CD4+CD25+ T cells. The percentages of CD4+CD25+ and CD4+CD25high T cells in the total CD3+CD4+ T-cell population were determined by flow cytometry. Figure 1A to C illustrates the gating strategy used to identify these cell subsets. We also quantified surface expression of CD25 based on mean fluorescence intensity (MFI). As previously described (22), T cells with CD25 expression ≥120 were considered as CD25high (average MFI, 160 ± 25), those with a MFI ≥ 50 were considered as CD25intermediate (average MFI, 75 ± 35), and those with a MFI ≤ 50 were considered as CD25low (average MFI, 25 ± 9). The percentages of circulating CD4+CD25+ T cells were significantly increased (P ≤ 0.0001) in patients with HNSCC (4.8 ± 2.9) relative to those in NC (1.2 ± 0.5; see Fig. 1D). In patients, the expanded subset of CD4+CD25high T cells contained few CD25low/interm T cells compared with NC, as shown in Fig. 1E. The patients were grouped into those who were disease-free (NED) at the time of phlebotomy and those studied before any therapy (AD). The frequency of CD4+CD25+ in the CD3+CD4+ gate was significantly greater (P < 0.001) in NED than AD patients (6 ± 0.8% versus 2.9 ± 0.7%; Fig. 2). Unexpectedly, the percentage of CD4+CD25high T cells was also greater in NED than AD patients (2.6 ± 1.32 versus 1 ± 0.3; P = 0.0059; see Fig. 2). The expansion of CD4+CD25high T-cell subset in NED patients was accompanied by that of CD25low/interm cells. NED patients had 3.8 ± 2.4%, and AD patients had 1.8 ± 0.3% of CD25low/interm T cells in the CD3+CD4+ compartment. Not only percentages but also absolute numbers of CD4+CD25+ T cells were increased in the circulation of NED patients compared with NC or patients with AD (preliminary results). These findings suggest that the hematologic recovery in NED patients involves the expansion of activated T cells as well as Treg.

Because the subset of CD4+CD25+ T cells may include activated helper T cells as well as Treg, we next analyzed the ratios of CD25high to CD25low/interm T cells in patients with NED and AD and in NC. For this analysis, the NED patients were divided in two groups: (a) patients who were only treated with surgery (NED Surg) and (b) patients who were treated with surgery plus radio- and/or chemotherapy (NED RTCT). Similarly, patients with AD were divided into two groups: (a) untreated patients with primary tumors (primary disease: PD); and (b) patients previously treated by surgery and/or radiotherapy/chemotherapy with recurrent disease (RD). As shown in Fig. 3, the CD25high/CD25low/interm ratio was not significantly different in AD and NED patients, remaining at close to 1:1 in all cases. Thus, oncologic therapy seemed to induce significant expansion of both the CD25high Treg subset and the CD25low/interm activated memory/effector T-cell subset. Indeed, the CD25low/interm fraction was somewhat (NSD) greater in NEDRTCT patients relative to NED Surg (Fig. 3). Importantly, patients with AD and recurrent disease (RD) had a slight increase of the total CD25+ T-cell subset relative to those with active primary disease (PD; Fig. 3). In contrast to HNSCC patients, the CD4+CD25+ T-cell population in NC comprised a larger subset of CD25low/interm T cells, with few CD25high T cells (Fig. 1E) and the CD25high /CD25low/interm ratio of 1:2 (Fig. 3). These data indicate that in NC, the CD25+ subset is mainly comprised of CD25low/interm activated effector and memory T cells, but contains relatively few Treg.

Some of the patients with NED were followed for up to 12 months after the termination of oncologic therapy. The frequency and suppressor function of Treg remained significantly elevated (P ≤ 0.0001) relative to NC as well as HNSCC patients with AD (data not shown). Our preliminary follow-up studies at later time points support the data reported for the earlier time points after termination of oncologic therapies.

**Frequency of CD8+CD25+ T cells in the peripheral blood of HNSCC patients.** Gating on CD3+CD8+ T cells, we observed that the subset of CD8+CD25+ T cells, was decreased (P ≤ 0.0034) in HNSCC patients relative to NC (data not shown). CD8+CD25+ T cells represented 1.5 ± 0.5% of the total circulating CD8+ T cells in the NC, but only 0.3 ± 0.7% in HNSCC patients (data not shown). This observation is consistent with our previously described data and suggests that circulating CD8+CD25+ T cells, presumably representing activated effectors, are dying in HNSCC patients (23). No differences were seen in the frequency of CD8+CD25+ T cells among patients with AD versus NED.

**Phenotypic analysis of the peripheral CD4+CD25high T-cell fraction.** To phenotypically characterize Treg, we next gated on the CD4+CD25high T-cell subpopulation and, using multiparameter flow cytometry, determined the percentages of CD4+CD25high T cells positive for CD62L, GITR, HLA-DR, CD45RO, CD45RA, Fas, Fasl, CD122, CD132, CD127, CTLA-4, CCR4, CCR7, Foxp3, TGFβ1, and IL-10. As shown in Fig. 4A, the CD4+CD25high T-cell population contained close to 60% of memory (RO+/RA) CD4+ T cells. This result indicates that Treg in humans largely belong to the memory T-cell compartment as previously suggested (24). The CD4+CD62L+ T cells also represented 60% of the CD4+CD25high subset. A majority of the CD4+CD25high T cells in HNSCC patients up-regulated Fas, CTLA-4, and Foxp3 (P ≤ 0.0001) relative to Treg in NC (Fig. 4A). More CD4+CD25high T cells were positive for the migration and lymph node homing receptors, CCR7 and
CD62L, in PBMC of HNSCC patients than in NC, but only CCR7 expression was significantly different with \( P \leq 0.0001 \). Another chemokine receptor, CCR4, was found on nearly all CD4\(^+\)CD25\(^{high}\) T cells in PBMC of HNSCC patients, but only on \( \sim 40\% \) of CD4\(^+\)CD25\(^{high}\) T cells in NC (\( P \leq 0.0001 \)). In contrast to HNSCC patients, cells in the CD4\(^+\)CD25\(^{high}\) compartment of NC significantly up-regulated GITR, CD122, CD132, and HLA-DR, with \( P \leq 0.0001 \) for all markers (Fig. 4A). Reciprocally, CD4\(^+\)CD25\(^{high}\) T cells down-regulated these markers in patients (\( P \leq 0.0001 \)).

Among HNSCC patients, the frequency of Foxp3 T cells was increased (\( P \leq 0.034 \)) in the CD4\(^+\)CD25\(^{high}\) T-cell subset in NED compared with AD patients (Fig. 4B). Noteworthy, FasL was found to be exclusively expressed on CD4\(^+\)CD25\(^{high}\) T cells in NED patients (Fig. 4C). In NC and AD patients, no cells positive for FasL within the CD3\(^+\)CD4\(^+\) and CD4\(^+\)CD25\(^{high}\) T-cell subsets were present (Fig. 4C). No membrane-bound or intracytoplasmic IL-10 and TGF\(\beta\) were detected within the CD3\(^+\)CD4\(^+\) and CD4\(^+\)CD25\(^{high}\) T-cell populations of NC or HNSCC patients. All analyzed T cells were also negative for CD127 (data not shown).

The low frequency of circulating CD8\(^+\)CD25\(^{high}\) T cells in the peripheral blood in HNSCC patients limited our study of Treg markers expressed on these T cells. However, we have detected IL-10\(^+\) CD8\(^+\)CD25\(^{high}\) T cells in 3 HNSCC patients (data not shown).
The frequency of circulating CD4*CD25\textsuperscript{high} T cells and clinical variables. To obtain some insights into the role of Treg in disease progression, we measured the frequency of regulatory and activated CD4* T cells in HNSCC patients with different tumor stage (T1/T2 versus T3/T4) or nodal status (N0 versus N1/2) as defined at diagnosis. No significant difference (P \leq 0.65) in the frequency of CD4*CD25* or CD4*CD25\textsuperscript{high} T cells was observed in HNSCC patients presenting with the T3/T4 stage relative to the T1/T2 stage disease or those presenting with the N0 stage relative to the N1/2 stage (data not shown). Also, no significant differences were observed in the frequency of CD4*CD25\textsuperscript{high}Foxp3* T cells between these patient cohorts (P \leq 0.143). However, a significantly greater frequency of CD4*CD25\textsuperscript{high} T cells, which were also positive for Foxp3, was observed in patients who received oncologic therapy (NED) relative to patients who had AD at the time of blood draw (P \leq 0.0049). These data suggest that oncologic therapy resulted in an expansion of the CD3*CD4*CD25\textsuperscript{high}Foxp3* subset of T cells (Treg). Also, the expanded Treg acquired Fasl expression in NED patients (Fig. 4C), indicating the capability to induce apoptosis in Fas\textsuperscript{+} effector cells as well as increased sensitivity of Treg to Fas-mediated lysis.

Suppressor function of circulating CD4*CD25\textsuperscript{high} T cells in patients and NC. To perform functional analysis of fresh CD4*CD25\textsuperscript{high} T cells, CD4*CD25\textsuperscript{high} and CD4*CD25* cells were purified from PBMC in HNSCC (n = 20) and NC (n = 10) by single-cell sorting. The purity of sorted CD4* CD25* ranged from 92% to 96%. To measure the inhibition of proliferation of autologous CD4*CD25* T cells (R) by CD4*CD25\textsuperscript{high} T cell (S), flow cytometry was used with gates set to include all CD3*CFSE* cells, as illustrated in Fig. 5A. At the S/R ratio of 1:1, the mean percent inhibition was 12 \pm 6% for autologous CD4*CD25* R in NC (Fig. 5B). When CD4*CD25\textsuperscript{high} T cells isolated from HNSCC were cocultured with autologous CD4*CD25* R cells (Fig. 5B), they almost completely inhibited R cell proliferation (P \leq 0.0001). No significant differences in suppressor function of CD4*CD25\textsuperscript{high} cells between NED and AD patients were observed (P \leq 0.4714) at the S/R ratio of 1:1 (Fig. 5C). When, however, S cells were diluted to give the S/R ratios of 1:5 and 1:10 in the cocultures, it became apparent that Treg isolated from NED patients mediated significantly stronger suppression (P \leq 0.0011 at the 1:5 ratio and P \leq 0.0038 at the 1:10 ratio) relative to Treg obtained from AD patients (Fig. 5C). The greater suppression levels seen with cells of NED patients were consistent with an increased proportion of CD4*CD25\textsuperscript{high}Foxp3* Treg in the circulation of these patients (Fig. 5C). This observation underscores the need for measuring suppression at more than one S/R ratio when making comparisons between individuals. Because suppressor activity in HNSCC patients was associated with the frequency of circulating CD25\textsuperscript{high} T cells positive for Foxp3 (P \leq 0.0001), it follows that the CD4*CD25\textsuperscript{high}Foxp3* T-cell subset was functionally less active in AD than NED patients, suggesting an alteration in the frequency as well as quality of suppression after oncologic therapy.

Suppressor function versus disease activity, stage, and nodal involvement in HNSCC patients. It was important to determine whether Treg-mediated suppression was associated with prognostically useful parameters in HNSCC such as T stage as well as N stage at the time of surgery or disease activity defined at the time of blood draw for this study. We related suppression levels measured at all S/R ratios to clinicopathologic findings (Fig. 6). The “no-interaction” analysis showed that only the disease status was significantly associated with suppression levels mediated by Treg, demonstrating a much stronger suppression by Treg obtained from patients with NED as also indicated above (Fig. 5C). Suppression was not associated with the T stage. The weak interaction statistic between the N stage and Treg suppression levels could perhaps be explained by relatively small, although significant, differences in suppression levels measured at the S/R ratios of 1:2 and 1:5, with Treg obtained from N0 versus N1/2 patients (P = 0.0441 versus 0.0337).

Mechanisms responsible for suppression. We show that CD4*CD25\textsuperscript{high}Foxp3* T cells in PBMC do not express TGFr1 or IL-10 in contrast to Treg in tumor-infiltrating lymphocytes isolated from HNSCC patients (22). To test the hypothesis that circulating Treg function is mediated via direct cell-to-cell contact, CD4*CD25* Treg isolated from PBMC were co-cultivated at the S/R ratio of 1:2 or 1:5, with CFSE-labeled autologous CD4*CD25* R cells responding to OKT3 and anti-CD28 Ab in the presence or absence of Transwell inserts. The inserts permit diffusion of soluble factors, but prevent cell-to-cell contact. Coincubation in the presence of Transwell inserts resulted in almost complete abrogation of suppression, as shown in Fig. 7. These results suggest that cell-to-cell contact between S and R is necessary for suppression mediated by circulating Treg patients with HNSCC.

Discussion

We have previously reported the expansion of Treg in the peripheral circulation and at the tumor site in HNSCC patients (8, 25), which is consistent with the data for other human cancers (7, 26). In this study, we show that circulating CD4*CD25\textsuperscript{high} Treg subset in HNSCC patients comprises cells with a characteristic phenotype, which mediate powerful suppression of T responder cells in a cytokine-independent manner. Similar to Treg in NC, these Treg express a memory phenotype (CD45RO*/CD45RA-) and up-regulate Foxp3, CTLA-4, and Fas (21). However, CD4*CD25\textsuperscript{high} Treg in HNSCC patients significantly up-regulate all of these markers and are also positive for the T-lymphocyte homing markers, CD62L and CCR7, suggesting that they have the capability to migrate to lymph nodes and possibly to the tumor site. Treg isolated from PBMC of patients and NC also up-regulate the chemokine receptor CCR4. It has been recently shown that the chemokine CCL22 (CCR4L) is abundantly expressed in ovarian cancer cells in tissues as well as ascites and is responsible for attracting peripheral CD4*CD25*Foxp3* T cells to the tumor (7). We also reported additional evidence for chemokine-mediated localization of human Treg to HNSCC in situ (27).

Circulating CD4*CD25\textsuperscript{high} T cells in HNSCC patients have a higher suppressor function relative to those in NC. Only a small proportion of CD4*CD25* T cells in the peripheral circulation of NC represents CD4*CD25\textsuperscript{high} cells (Treg), whereas the majority are CD4*CD25\textsuperscript{low/interm} non-Treg T cells. Although CD4*CD25\textsuperscript{high} T cells in NC up-regulate T-cell activation markers, HLA-DR, GITR, and CD132 (IL2-Ry), they mediate...
Fig. 5. Suppression of proliferation by CD4⁺CD25⁺⁺⁺T cells isolated from PBMC of NC or HNSCC patients. Autologous CD4⁺CD25⁺ T cells were labeled with CFSE and stimulated with soluble OKT3 and CD28 Ab 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 (left). Analysis gates on the ModFit program were restricted to the CD3⁺CD4⁺ and CD4⁺CFSE⁺ T-cell subsets. Right, CD4⁺CD25⁺ responder T cell(s) isolated from PBMC of one representative NC labeled with CFSE and stimulated with OKT3 and anti-CD28 Ab in the absence of Treg (positive control). B, left, suppressor CD4⁺CD25⁺⁺⁺T cell(s) from PBMC isolated from NC (white column) or HNSCC patients (black column) was added to autologous CD4⁺CD25⁺⁺⁺ responders (R) at the start of the culture (S/R ratios were 1:1). Right, CFSE plots for responder cell (CD4⁺CD25⁺⁺⁺) proliferation in the presence of autologous Treg (1:1) in one representative NC and one representative HNSCC patient. Flow cytometry was done on day 5. Cell division represented by the dilution of CFSE was analyzed using the ModFit software. The data are the mean percentages of suppression ± SD. C, left, flow dot plots for Foxp3 expression on CD4⁺CD25⁺⁺⁺T cells in one representative AD patient and one representative NED patient. Right, suppression mediated by Treg of patients with AD versus NED. CD4⁺CD25⁺⁺⁺T cells isolated from PBMC of HNSCC patients (AD or NED) were added to autologous CD4⁺CD25⁺⁺⁺ responders at the start of the culture (at the S/R ratios of 1:1, 1:5, and 1:10). Note that no difference was observed in Treg functions between NED and AD at the 1:1 ratio. *p ≤ 0.0038, significant differences.
only weak suppression largely because in NC, this T-cell subset is dominated by activated CD4+CD25+ T effector and memory T cells (20, 21). Suppressor function correlated with the frequency of Foxp3+ Treg (P ≤ 0.0001) in HNSC patients and NC, confirming our previously reported results that Foxp3 expression on CD4+CD25high Treg clones was required for suppression (21). However, Foxp3 expression is not confined to Treg, as it has been reported that subsets of CD8+ T cells as well as tumor cells might also be Foxp3+ (28, 29). The role of this transcription factor in the process of suppression is yet to be determined.

It has been reported in the literature that the frequency of regulatory CD4+ T cells increases during tumor progression, and that their in vivo depletion is associated with up-regulated antitumor immune responses in humans (7, 30). Surprisingly, we show that oncologic therapy is associated with the expansion of CD4+CD25high Treg positive for Foxp3 and Fasl in patients with NED treated with surgery alone or surgery and radiotherapy/chemotherapy. We have previously reported that HNSCC patients with AD have low absolute numbers of CD4+ and CD8+ T cells (31). Here, we show that the hematologic recovery in response to oncologic therapy results in the expansion of the CD4+CD25+ compartment, including CD4+CD25high/Foxp3+ Treg. When the ratio of CD25high/CD25low/internm cells in NED and AD cohorts was compared, it remained constant at 1:1, suggesting that the oncologic therapy indeed increases both Treg and activated T effector cell subsets in HNSCC patients. Not only was the proportion of CD4+CD25+ T and CD4+CD25high/Foxp3+ cells increased in the patients, but suppressor function of these cells was significantly elevated. The observed expansion of Treg expressing Foxp3 correlated with increased suppression mediated by these cells in NED patients (P < 0.001). Furthermore, this expansion and elevated suppressor function persisted for months after the termination of oncologic therapies.

It is well documented that many of the Foxp3 target genes are key modulators of T-cell activation and function (32). Therefore, the increase of Foxp3 in NED relative to AD patients could be explained by strong T-cell activation induced by oncologic therapy. Altogether, our data indicate that the peripheral CD4+CD25+ T-cell pool in HNSCC patients is enriched in CD4+CD25high/Foxp3+ Treg with potent suppressor function, which is significantly related to disease activity of the time of blood draw. The observed higher frequency of Treg with powerful suppressor functions in NED patients versus AD patients or NC is counterintuitive, but it may reflect effects of oncologic therapy on T cell subsets in patients with HNSCC. The effect of oncologic therapy on Treg might be related to three phenomena: (a) homeostatic regulation after radiotherapy/chemotherapy-induced lymphopenia resulting in the expansion of a total lymphocyte pool; (b) radiation-induced increased expression of chemokine ligands on tissue cells resulting in recruitment of chemokine receptor-positive T cells in the microenvironment; and (c) activation and expansion of de novo induced Treg and T responder cells by inflammatory cytokines derived from the strong inflammatory response that usually induced accompanied radiotherapy/chemotherapy. The immune system controls the levels and the activation state of each cellular compartment through homeostatic regulation, a process that is triggered during development and also after the induction of a lymphopenic state by external stimuli (33). Radiotherapy/chemotherapy may have profound effects on the peripheral blood cell count, due to increased availability of homeostatic cytokines and increased interactions of T cells with APCs. It has been proposed that lymphodepletion removes endogenous cellular elements that act as sinks for cytokines that are responsible for augmenting the activity of tumor-reactive T cells (34). Thus, T cells recruited and surviving after radiotherapy/chemotherapy receive strong stimuli (cytokines and enhanced APC–T cell interactions) that trigger T-cell activation and expansion. It has been reported that adoptive transfer of tumor-reactive T cells into lymphodepleted hosts results in significantly increased antitumor responses (34, 35). A similar mechanism(s) might be involved in the activation and expansion of the activated T responder and memory T-cell compartments in HNSCC patients who receive oncologic therapy. Our findings suggest that monitoring of Treg frequency and function as well as depletion of Treg after oncologic therapy might be crucial to allow the development of effective antitumor T-cell responses.

Recent studies show that proportions of circulating Treg increase after acute inflammation (36) and after radiotherapy (37). We hypothesize that proinflammatory cytokines might induce, recruit, and/or activate and expand Treg. In support of this hypothesis, Dhodapkar et al. have shown recently that DC treated ex vivo with inflammatory cytokines are the most
effective DC subsets in inducing the expansion of Foxp3\textsuperscript{high} regulatory T cells with suppressive function (38). Surprisingly, however, we observed that the frequency of Treg in the circulation of HNSCC patients remained elevated even after surgical removal of the tumor in the absence of radiotherapy/chemotherapy. This observation suggests that inflammatory cytokines or other immune suppressors released by the tumor remain active in the absence of the tumor. It is also possible, however, that occult metastatic disease present in HNSCC patients after oncologic therapy is responsible for tumor-derived immune suppressive factors, which mediate peripheral immune tolerance. If so, then the frequency and levels of suppressor function of Treg might be a predictor of recurrence and should be monitored and followed.

Our data also indicate that radiotherapy/chemotherapy selectively increases the expression of FasL on CD4\textsuperscript{+}CD25\textsuperscript{high} T cells in NED patients, but not AD patients. This finding suggests that oncologic therapy can induce FasL expression on Treg. Indeed, we have recently shown that cisplatin selectively induces FasL expression on CD4\textsuperscript{+}CD25\textsuperscript{high} Treg but not CD4\textsuperscript{+}CD25\textsuperscript{−} T cells obtained from NC and HNSCC patients.\textsuperscript{1} FasL\textsuperscript{+} Treg might be capable of inducing cell death in activated (Fas\textsuperscript{+}) effector T cells, thus contributing to tumor-associated immune suppression. Additional studies are in progress in our laboratory to explore the use of the Fas/FasL pathway as one mechanism of suppression mediated by Treg. In this context, we have preliminary evidence that FasL\textsuperscript{+} Treg isolated from HNSCC patients with NED induced apoptosis in activated autologous CD8\textsuperscript{+}CD25\textsuperscript{−} T cells and, thus, partially suppressed their proliferation (39). Venet et al. (36) have confirmed the engagement of the Fas/FasL pathway in Treg suppressive functions by showing that percentages of FasL\textsuperscript{+} Treg increased in the circulation of patients with septic shock, and that these FasL\textsuperscript{+} Treg induced apoptosis in monocytes. Killing by Treg of monocytes and/or macrophages could decrease their protumoral effects during cancer development. This implies that nTreg could have a beneficial effect in some cancers by transiently down-regulating tumor-associated inflammation (40, 41). However, we believe that proinflammatory cytokines are continuously produced in the tumor microenvironment, re-attract immune cells into the tumor and, simultaneously, trigger the induction of Treg that suppress and kill tumor-specific T effector cells. Radiotherapy/chemotherapy or even surgery alone could alter the cytokine balance in the microenvironment, inducing Treg activation and expansion, as well as their suppressor function, which is, in part, mediated via the Fas/FasL pathway. Therefore, it is reasonable to expect that targeting FasL on Treg might be a novel strategy to block Treg-mediated suppression in subjects who have hyperfunctional Treg, such as, e.g., NED patients after oncologic therapy.

The importance of our study is that it provides a detailed analysis of the phenotypic profile and functional attributes of circulating CD4\textsuperscript{+}CD25\textsuperscript{high} T cells in HNSCC patients with AD as well as those who are NED. Furthermore, it shows that the phenotype and function of Treg in NED patients are profoundly influenced by oncologic therapy. Further studies of molecular mechanisms that are involved in the origin, function, and interactions of Treg with different immune cell subsets are urgently required to help develop immunotherapeutic strategies that might overcome tumor immune escape.

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\textsuperscript{1} Unpublished data.


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The Frequency and Suppressor Function of CD4+CD25\textsuperscript{high} Foxp3\textsuperscript{+} T Cells in the Circulation of Patients with Squamous Cell Carcinoma of the Head and Neck

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