

Functionally distinct subsets of CD4⁺ regulatory T cells in patients with laryngeal squamous cell carcinoma are indicative of immune deregulation and disease progression

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Received June 26, 2014; Accepted October 7, 2014

DOI: 10.3892/or.2014.3553

Abstract. CD4⁺ regulatory T cells (Tregs) mediate immune tolerance in laryngeal squamous cell carcinoma (LSCC). However, Tregs are functionally heterogeneous. Recently, we reported that three distinct Treg subsets (resting Tregs, activated Tregs and cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells) vary in the peripheral circulation of patients with head and neck squamous cell carcinoma (HNSCC); however, the potential implication of these Treg subsets in LSCC immunity is unclear. Here, we report that activated Tregs and cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells were increased in LSCC patients compared with healthy donors (HD) ($p < 0.001$, $p < 0.001$), whereas resting Tregs were decreased ($p < 0.001$). Activated Tregs inhibited the proliferation of CD4⁺CD25⁺ T cells ($p < 0.001$) and secreted lower levels of interleukin-2 ($p < 0.001$), interferon- γ ($p < 0.001$) and tumor necrosis factor- α ($p < 0.001$) compared with the cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells. Importantly, activated Treg prevalence was correlated with tumor stage ($p = 0.001$) and nodal status ($p = 0.007$). The prevalence of naïve CD4⁺ ($p < 0.001$), naïve CD8⁺ ($p = 0.002$), and Th1 T-cell

subsets ($p < 0.001$, $p < 0.001$) was decreased in the LSCC patients. In conclusion, our findings showed that activated Tregs with suppressive activity are a distinct subset of Tregs in LSCC, and correlate with disease progression. Several immune system abnormalities in LSCC patients are represented by expansion of functionally activated Tregs, both in the circulation and tumor microenvironment along with decreased frequencies of naïve T-cell populations and Th1-cell populations.

Introduction

Laryngeal squamous cell carcinoma (LSCC), a common type of head and neck squamous cell carcinoma (HNSCC), accounts for approximately 2.4% of new malignancies worldwide every year (1,2). LSCC patients often display considerable variability in survival, despite new advances in treatment (2).

In the past few years, it has become clear that the complex interaction between LSCC and immune system members plays an important role in determining tumor progression (3). Among these members, CD4⁺ regulatory T cells (Tregs), from either the thymus or the periphery, play an important role in antitumor immune responses, in which they have been associated with suppressive activities against tumor-specific T-cell responses (3,4). Several studies have shown that Treg prevalence increases in peripheral blood and many types of human malignancies, such as ovarian, gastric and esophageal cancer (5-9).

Despite efforts to better understand the role of Tregs in human malignancies, isolating Treg subsets is difficult due to the lack of Treg-specific markers. Human Tregs with suppressive activities were initially described in 1995 based on elevated CD25 expression (10). However, subsequent studies showed that these CD4⁺CD25⁺ T cells are mixed populations, composed of suppressor CD4⁺CD25^{high} as well as CD4⁺CD25^{low} T cells, which are nonsuppressive, and activated CD4⁺ T cells (11). Until 2003, Foxp3 was thought to be a key transcription factor for the development and function of Tregs (12,13). Although

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Key words: Foxp3, regulatory T cells, immune suppression, Th1, laryngeal squamous cell carcinoma

Foxp3 expression is specific for Tregs, it cannot be used to isolate living cells to study Treg heterogeneity since Foxp3 is located intracellularly. Hence, several surface markers, such as CD127 and CD45RA, have been studied during the functional evaluation of Tregs (14-16). For example, Liu *et al* (14) demonstrated that CD127 expression is inversely correlated with FoxP3 and the suppressive function of human Tregs. In particular, Miyara *et al* (16) recently found that Tregs in healthy populations and patients with autoimmune disease can be defined by three functionally distinct subsets on the basis of CD45RA expression: CD45RA⁺Foxp3^{low} resting Tregs, CD45RA⁺Foxp3^{high} activated Tregs, both of which are suppressive *in vitro*, and cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ nonsuppressive T cells. The frequency and function of these Treg subsets vary in different diseases (16-18).

When assessing the role of Tregs it is important not only to examine them directly, but also to investigate their relationship to other important antitumor members, as Tregs have been associated with suppressive activities against tumor-specific T cells. Studies have shown that Th1 cells and CD8⁺ T cells play an important role in the control of tumor growth. For example, CD8⁺ T cells were shown to mediate antitumor immunity (19). Th1 cells, a subset of CD4⁺ T cells, constitutively express IFN- γ and TNF- α , and play a role in priming tumor-specific cytotoxic T lymphocytes (CTLs) through the release of soluble IL-2 in the proximity of CTLs (20). Moreover, induction of MHC class I-restricted tumor-specific immunity requires epitope linkage between Th1 and CTL epitopes, important for CTL induction (21). Recently, we reported that functionally distinct Treg subsets (CD45RA⁺Foxp3^{low} resting Tregs, CD45RA⁺Foxp3^{high} activated Tregs, and cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells) vary in the peripheral circulation of HNSCC patient subgroups (22); however, the details of distinct Treg subsets and the correlation between these Treg subsets and tumor-specific T cells in the peripheral circulation of LSCC have not been demonstrated.

To investigate the potential implication of functionally distinct Treg subsets in LSCC immunity, we used the CD45RA, Foxp3, and CD25 markers to evaluate both the frequency and various functions of distinct Treg subsets in the peripheral blood of LSCC patients in relation to CD4⁺ and CD8⁺ T cells, tumor stage and nodal status.

Materials and methods

Patients and healthy donors. From March to November 2013, 42 LSCC patients were enrolled. Patients were diagnosed at the Department of Otorhinolaryngology, The First Affiliated Hospital of Sun Yat-Sen University without any previous oncological treatment. Healthy age-matched donors (20 males and 1 female with a mean age of 43 years) were enrolled as controls. The main clinical and pathological characteristics of the patients are presented in Table I. Clinical staging and the anatomic site of the tumors were assessed according to the 6th edition of the Union Internationale Contre le Cancer (UICC, 2008) tumor-node-metastasis classification of malignant tumors.

Ethics statements. The study protocol (No. 2012-349) was approved by The Ethics Committee of The First Affiliated

Hospital of Sun Yat-Sen University, and was used for research purposes only. Patient and healthy donor (HD) informed consent was obtained before enrollment.

Collection of peripheral blood. Peripheral blood lymphocytes (PBLs) were isolated from peripheral venous blood as we previously described (22). Isolated cells were immediately resuspended in 100 μ l flow cytometry staining buffer (eBioscience, San Diego, CA, USA) for surface and intracellular staining.

Antibodies and reagents. Freshly obtained human PBLs were stained with the following anti-human monoclonal antibodies: anti-CD3-eFluor 605NC (0.25 μ g/100 μ l), anti-CD4-FITC (1.0 μ g/100 μ l), anti-CD8-PE-Cy7 (0.06 μ g/100 μ l), anti-CD25-APC (0.125 μ g/100 μ l) and anti-CD45RA-eFluor 450 (0.5 μ g/100 μ l) for surface staining; and anti-Foxp3-PE (0.25 μ g/100 μ l), antitumor necrosis factor- α (TNF- α)-Alexa Fluor 700 (0.25 μ g/100 μ l), anti-interleukin-2 (IL-2)-PE-Cy7 (0.125 μ g/100 μ l), anti-interferon- γ (IFN- γ)-APC-eFluor 780 (0.25 μ g/100 μ l) and anti-interleukin-17 (IL-17)-PerCP-Cy5.5 (0.125 μ g/100 μ l) for intracellular staining. Soluble anti-CD3 (OKT3, 0.5 μ g/ml) and anti-CD28 (CD28.2, 2 μ g/ml) mAb were used for *in vitro* activation of T cells. All antibodies and isotype controls were purchased from eBioscience (San Diego).

Multicolor flow cytometry. Multicolor flow cytometry was conducted using a Ten-Color (3 laser: 488 nm blue, 638 nm red and 405 nm violet) Gallios Flow Cytometer (Beckman Coulter, Hercules, CA, USA) equipped with Gallios software v1.0. The acquisition and analysis gates for PBLs (5×10^4) were determined by characteristic forward and side-scatter properties of lymphocytes. Furthermore, analysis gates were restricted to the CD3⁺CD4⁺, CD3⁺CD8⁺, and CD4⁺CD25⁺CD45RA⁺ T-cell subsets, as appropriate. Cells expressing surface and intracellular markers were acquired and analyzed on a logarithmic scale from FL1 to FL9. Following doublet discrimination, a CD25 vs. Foxp3 dot plot gated on CD3⁺CD4⁺ T cells was created to determine Tregs (Fig. 1Aa). In particular, following doublet discrimination, a CD45RA vs. Foxp3 dot plot gated on CD3⁺CD4⁺ T cells was created to determine the different levels of Foxp3 expression (Foxp3^{low} and Foxp3^{high}); CD45RA⁺ T cells with Foxp3 expression were defined as CD45RA⁺Foxp3^{low} T cells (I), CD45RA⁺ T cells exceeding a certain level of Foxp3 expression on CD45RA⁺ T cells were defined as CD45RA⁺Foxp3^{high} T cells (II), CD45RA⁺ T cells with the same level of Foxp3 expression by CD45RA⁺Foxp3^{low} T cells were defined as CD45RA⁺Foxp3^{low} T cells (III) (Fig. 1Ab).

Surface and intracellular staining. To determine the frequency of CD4⁺ and CD8⁺ T cells, and their naïve phenotypes, mAbs against surface markers CD3, CD4, CD8 and CD45RA were added to the cell suspension (1×10^7 cells/100 μ l) and incubated on ice for 30 min in the dark. Appropriate isotype Ab controls were used. Cells were washed twice and examined by multicolor flow cytometry.

To determine the frequency of Treg subsets, Foxp3⁺CD8⁺ T cells, and Th1 cells, both cell surface and intracellular staining was performed. To examine the secretory function, intracytoplasmic expression of IL-2, IL-17, TNF- α and IFN- γ

Table I. Clinicopathological features of the LSCC patients.

Characteristics	Number
Age (years)	
Mean (range)	46 (38-81)
Gender	
Male	40
Female	2
Total	42
Tumor site	
Glottic region	37
Supraglottic region	3
Subglottic region	2
Tumor stage	
T ₁	12
T ₂	10
T ₃	14
T ₄	6
Nodal stage	
N ₀	35
N ₁	5
N ₂	2
N ₃	0
M stage	
M ₀	42
M ₁	0
Alcohol history	
Yes	31
No	5
Prior	3
Unknown	3
Smoking history	
Yes	36
No	2
Prior	3
Unknown	1

LSCC, laryngeal squamous cell carcinoma.

was assessed after stimulation of freshly isolated PBLs for 5 h with a cocktail of phorbol 12-myristate 13-acetate (PMA), ionomycin, and Golgi stop (brefeldin A and monensin) (eBioscience). Briefly, following surface staining, cells were fixed and permeabilized on ice with fixation/permeabilization buffer (eBioscience) for 1 h in the dark. Cells were then washed twice and incubated with intracellular mAbs against Foxp3, IL-2, IL-17, TNF- α , and IFN- γ for 1 h at room temperature in the dark. After intracellular staining, cells were washed twice and examined by multicolor flow cytometry. Appropriate isotype Ab controls were included for each sample.

In vitro suppression of Treg subsets and isolation by flow cytometry. Six separate experiments were performed. Stained

cells (mAbs against CD3, CD4, CD25, and CD45RA) at a concentration of 5×10^7 cells/100 μ l were sorted using a FACS cell sorter (BD Influx, BD Biosciences). Three Treg subsets were prepared as live cells as we previously described (22): Foxp3^{low}CD45RA⁺ cells, which were CD25⁺⁺ (I), Foxp3^{high}CD45RA⁺ cells, which were CD25⁺⁺⁺ (II), and Foxp3^{low}CD45RA⁺ cells, which were CD25⁺⁺ (III) were prepared by sorting as CD25⁺⁺CD45RA⁺, CD25⁺⁺⁺CD45RA⁺, and CD25⁺⁺CD45RA⁺ cells, respectively.

After sorting, 1×10^4 responder cells (CD25⁺CD45RA⁺CD4⁺ T cells) were labeled with 1 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE) (eBioscience) and co-cultured with unlabeled CD25⁺⁺CD45RA⁺, CD25⁺⁺⁺CD45RA⁺, or CD25⁺⁺CD45RA⁺CD4⁺ T cells and assessed for their suppressive activity. Soluble anti-CD28 (2 μ g/ml) and plate-bound anti-CD3 (0.5 μ g/ml) were used to activate T cells in 96-well round-bottom plates, and cells were harvested and analyzed by flow cytometry after 86 h of co-culture. All CFSE data were analyzed using the ModFit software provided by Verity Software House (Topsham, USA). The percentages of suppression were determined based on the proliferation index (PI) of responder cells alone (100% proliferation, 0% suppression) compared with the PI of responders co-cultured (1:1 ratio) with Treg subset.

Statistical analysis. Statistical analysis was performed with SPSS software (SPSS standard version 13.0, IBM, Chicago, IL, USA). Differences between groups were assessed using the Mann-Whitney U test, Student's t-test, or Kruskal-Wallis test. The correlation between Treg subsets and clinical factors (tumor stage and nodal status) was determined by one-way ANOVA.

Results

Distinct Treg subsets in LSCC patients. We first examined the frequency of Tregs. Our results showed that the Treg percentage was increased in PBLs of 42 LSCC patients compared with 21 HD ($8.42 \pm 1.30\%$, median: 8.57% vs. $6.37 \pm 1.30\%$, median: 6.43% , $p < 0.001$) (Fig. 1Ba), consistent with previous findings (21). The percentage of the three CD4⁺ Treg subsets was then evaluated based on CD45RA and Foxp3 expression. The novelty of this study was that the percentage of CD45RA⁺Foxp3^{high} activated Tregs ($2.47 \pm 0.75\%$, median: 2.42% vs. $0.79 \pm 0.26\%$, median: 0.74% , $p < 0.001$) and of cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells ($5.36 \pm 0.93\%$, median: 5.46% vs. $3.85 \pm 0.77\%$, median: 3.93% , $p < 0.001$) was increased in the LSCC patient PBLs compared with HD PBLs. In contrast, the percentage of CD45RA⁺Foxp3^{low} resting Tregs ($0.59 \pm 0.23\%$, median: 0.57% vs. $1.73 \pm 1.12\%$, median: 1.31% , $p < 0.001$) was decreased in the LSCC patient PBLs compared with HD PBLs (Fig. 1Bb-d).

Suppressive and secretory functions of distinct Treg subsets. The suppressive activity of each Treg subset from the LSCC patients ($n=6$) was assessed by their ability to suppress the proliferation of an autologous T-cell population (CD25⁺CD45RA⁺CD4⁺). When each Treg subset isolated from LSCC patients was co-cultured (1:1 ratio) with autologous CD25⁺CD45RA⁺CD4⁺ responder cells, both activated and resting

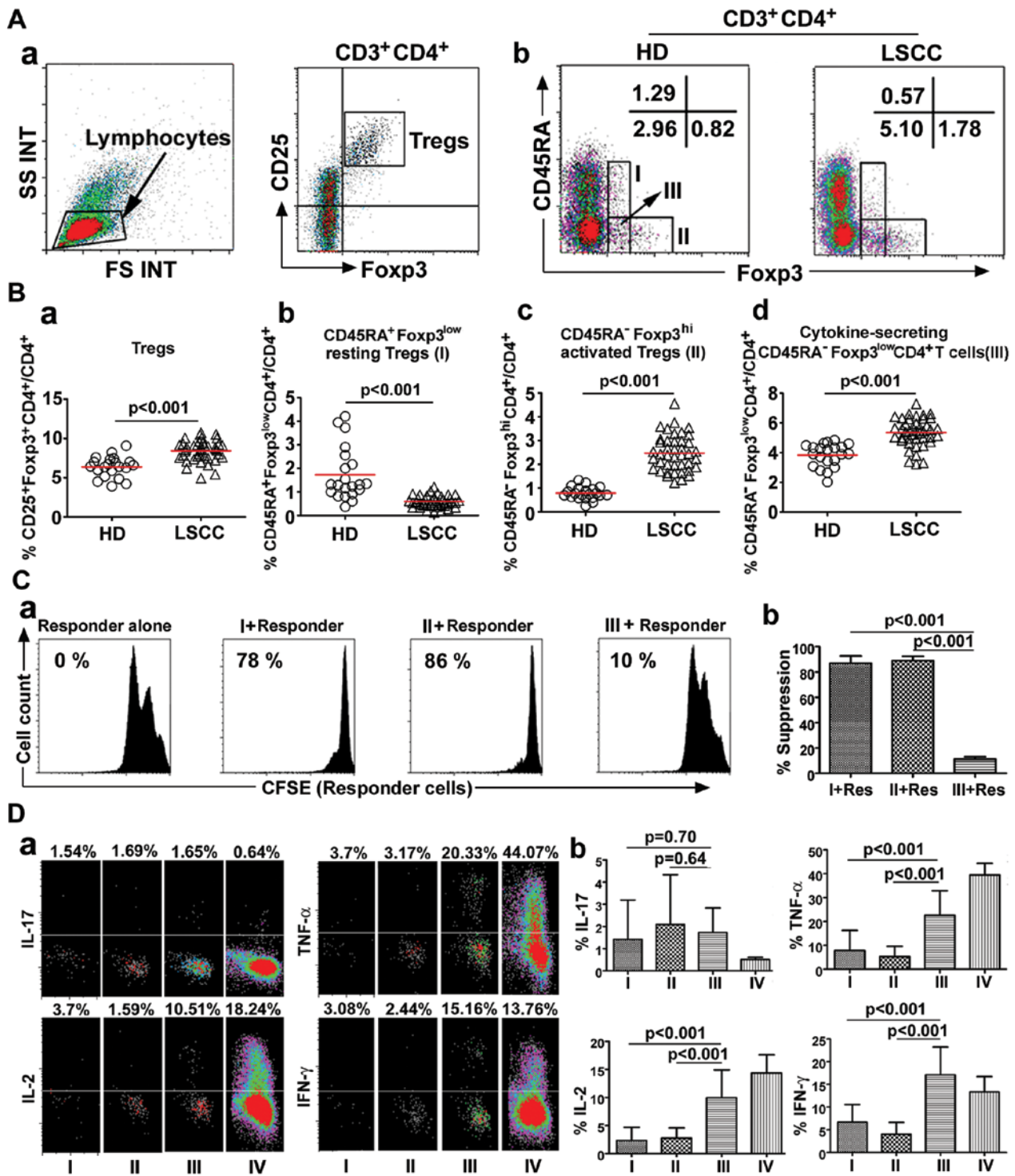


Figure 1. Functionally distinct subsets of CD4⁺ Tregs in 42 LSCC patients and 21 HD. (A) CD4⁺ Tregs defined by Foxp3 and CD25 expression (a). Flow cytometry of each Treg subset (I, CD45RA⁺Foxp3^{low} resting Tregs; II, CD45RA⁺Foxp3^{hi} activated Tregs; III, cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells) isolated from HD and LSCC patients. Flow dot plots for one representative HD and LSCC patient (b). (B) The percentage of Tregs (a) and each Treg subset (b-d) isolated from HD and LSCC patients (HD vs. LSCC, mean \pm SD, $p < 0.001$). Each dot represents an individual sample. Red horizontal bars represent mean percentage. Statistical comparisons were performed using the Kruskal-Wallis test. (C) CFSE dilution by labeled CD4⁺CD25⁺CD45RA⁺ responder T cells assessed after TCR-stimulated co-culture with the indicated Treg subset. Percentage of suppression is indicated. Flow plots for one representative LSCC patient (a). The histogram represents the mean percentages of suppression \pm SD ($n = 6$). Statistical comparisons were performed using the Student's *t*-test (b). (D) Production of IL-17, IL-2, IFN- γ , and TNF- α by each fraction after stimulation with PMA + ionomycin. Flow dot plots for one representative LSCC patient (a). The histogram represents the cytokine expression profiles of each Treg subset ($n = 5$). Res, responder cells; I, CD45RA⁺Foxp3^{low} resting Tregs; II, CD45RA⁺Foxp3^{hi} activated Tregs; III, cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells; IV, CD4⁺CD25⁺ T cells. Statistical comparisons were performed using the Student's *t*-test (b).

Tregs consistently induced a greater percentage of suppression compared with cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺

T cells ($89.12 \pm 3.25\%$ vs. $11.29 \pm 1.87\%$, $p < 0.001$; $86.98 \pm 5.71\%$ vs. $11.29 \pm 1.87\%$, $p < 0.001$, respectively) (Fig. 1C).

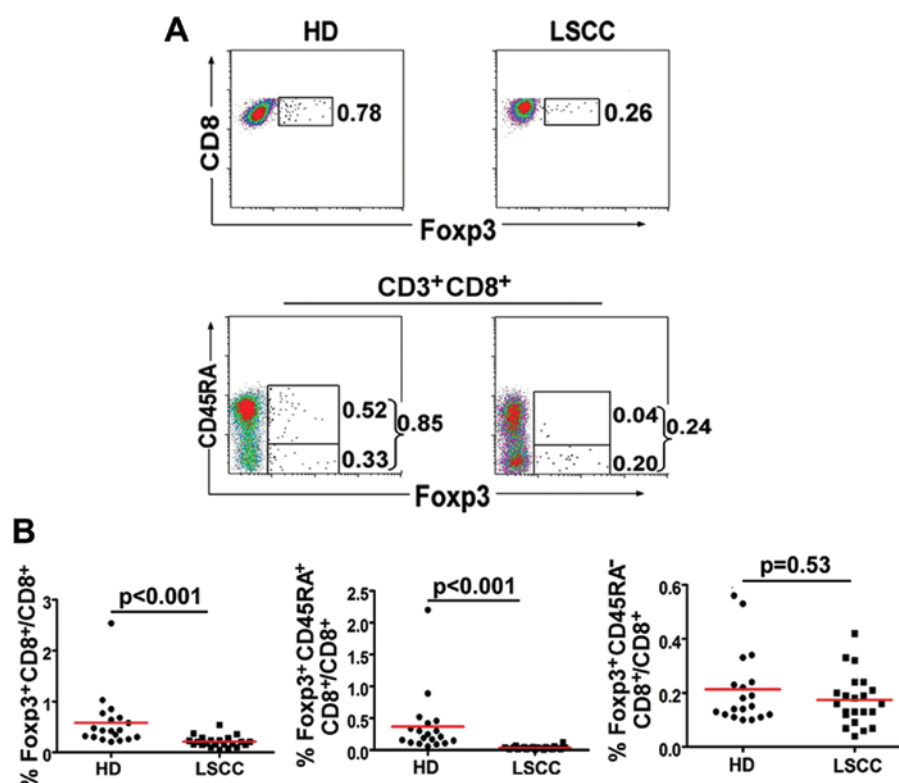


Figure 2. Percentage of CD8⁺Foxp3⁺ T cells in 21 LSCC patients and 19 HD. (A) Flow dot plots for one representative HD and LSCC patients. (B) The percentages of CD8⁺Foxp3⁺ T cells and CD8⁺Foxp3⁺CD45RA⁺ T cells were significantly lower in LSCC patients. Statistical comparisons were performed using the Mann-Whitney U test.

Moreover, the functional cytokine patterns in sorted Treg subsets from 5 LSCC patients were also studied after *ex vivo* stimulation. Those results suggested that cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells secreted significantly higher amounts of IL-2, IFN- γ and TNF- α than did the activated or resting Tregs ($p<0.001$), whereas IL-17 production remained the same ($p>0.05$) (Fig. 1D).

Frequency of Foxp3⁺CD8⁺ T cells in LSCC patients. It has been reported that CD8⁺ T cells might also express Foxp3, indicating that Foxp3 expression is not confined to Tregs (23). Thus, we evaluated Foxp3 expression in CD8⁺ T cells. The results revealed that PBL CD8⁺Foxp3⁺ T cells in 21 LSCC patients were decreased compared with these cells in 19 HD ($0.23\pm0.11\%$, median: 0.20% vs. $0.59\pm0.53\%$, median: 0.43%, $p<0.001$). Moreover, the percentage of naïve CD8⁺Foxp3⁺CD45RA⁺ T cells in the LSCC patients was decreased compared with HD ($0.04\pm0.02\%$, median: 0.03% vs. $0.37\pm0.49\%$, median: 0.21%, $p<0.001$) (Fig. 2).

CD4⁺ and CD8⁺ T cells in LSCC patients. Cancer patient PBL CD4⁺ or CD8⁺ T cells may decrease because of Treg suppressive activities (24,25). However, our results showed that there was no significant difference in the percentage of CD4⁺ ($36.01\pm9.75\%$, median: 34.12% vs. $34.62\pm9.22\%$, median 35.46%, $p=0.65$) and CD8⁺ ($29.77\pm8.42\%$, median: 28.44% vs. $31.98\pm7.88\%$, median: 30.48%, $p=0.39$) T cells (Fig. 3A). Notably, the naïve CD4⁺ ($23.94\pm11.92\%$, median: 23.59% vs. $45.90\pm10.69\%$, median: 50.48%, $p<0.001$) and naïve CD8⁺ ($51.81\pm11.29\%$, median: 51.65% vs. $65.42\pm13.89\%$,

median: 71.75%, $p=0.002$) T cells were significantly lower in the LSCC patients than in HD (Fig. 3B and C).

Th1 cells in LSCC patients. Although studies of non-cancerous diseases have shown that a decrease in Th1 cells can be attributed to Treg suppressive activities (26,27), the change in LSCC Th1 cells is unknown. In the present study, IFN- γ and TNF- α were used to identify Th1 cell levels in a small cohort of 8 LSCC patients and 5 HD. Our preliminary results showed that the percentage of Th1 cells in CD25⁺CD45RA⁺CD4⁺ T cells was decreased in LSCC patients compared with HD ($4.20\pm2.07\%$ vs. $10.68\pm0.93\%$, $p<0.001$). The percentages of IFN- γ -TNF- α ⁺ effector CD25⁺CD45RA⁺CD4⁺ T cells did not differ between LSCC patients and HD ($36.10\pm5.76\%$ vs. $38.24\pm1.84\%$, $p=0.36$) (Fig. 4A and B).

To understand which Th1 subsets varied in LSCC patients, Th1 cells were separated into two subsets (IFN- γ ⁺IL-2⁺ and IFN- γ ⁺IL-2⁻) using our previously described method (28). The results showed that Th1 subsets were decreased in the LSCC patients compared with HD (IFN- γ ⁺IL-2⁺ Th1 cells: $1.38\pm0.53\%$ vs. $3.89\pm0.28\%$, $p<0.001$; IFN- γ ⁺IL-2⁻ Th1 cells: $2.82\pm1.59\%$ vs. $6.79\pm0.74\%$, $p<0.001$). The percentages of IFN- γ IL-2⁺ effector T cells did not differ between LSCC patients and HD ($11.45\pm4.01\%$ vs. $11.68\pm1.28\%$, $p=0.88$) (Fig. 4C and D).

Relationship between circulating Treg subsets and clinical variables. Glottic squamous cell carcinoma is a common type of LSCC, and lymph node metastasis is uncommon in patients with glottic squamous cell carcinoma (especially in patients

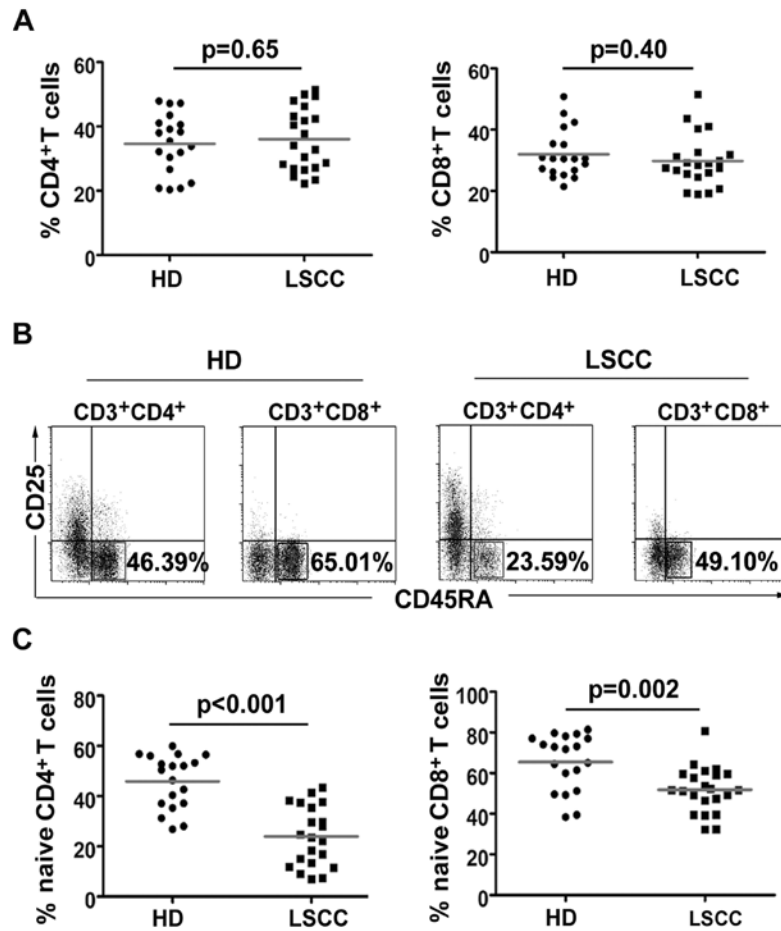


Figure 3. Percentage of CD4⁺ and CD8⁺ T cells in 21 LSCC patients and 19 HD. (A) The histograms represent the percentage of CD4⁺ and CD8⁺ T cells in HD and LSCC patients. (B) Flow dot plots of naïve CD4⁺ T cells and naïve CD8⁺ T cells for one representative HD and LSCC patient. (C) The histograms represent the percentage of each subset of naïve T cells in healthy donors and LSCC patients. Statistical comparisons were performed using the Mann-Whitney U test.

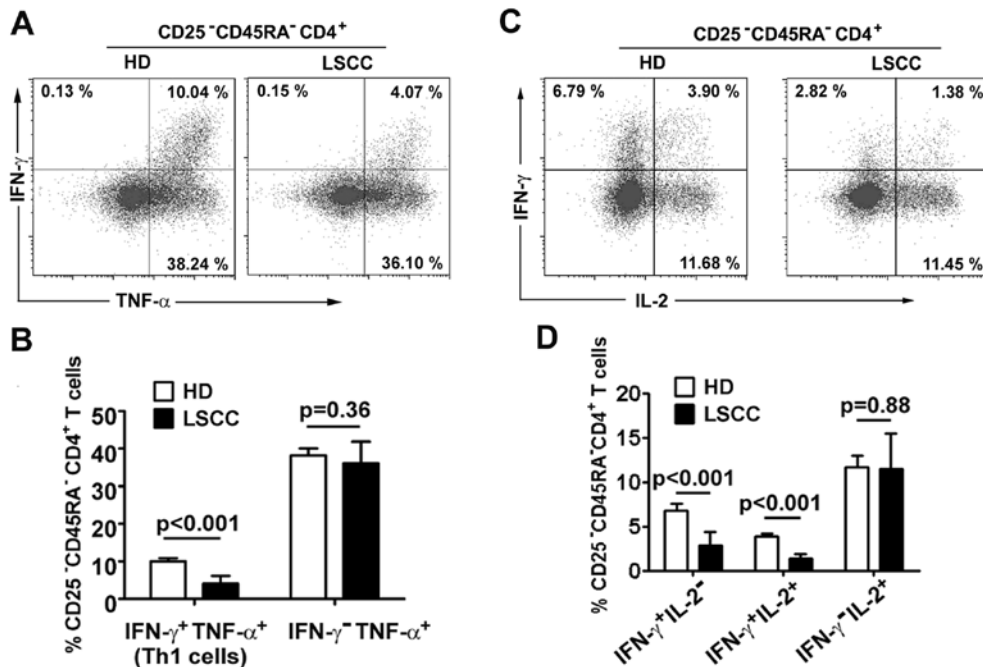


Figure 4. Percentage of Th1 cells in 8 LSCC patients and 5 HD. (A) Flow dot plots of Th1 cells (IFN- γ ⁺TNF- α ⁺CD25⁻CD45RA⁻CD4⁺ T cells) for one representative HD and LSCC patient. (B) The percentage of Th1 cells was significantly lower in LSCC patients than in HD. The percentage of IFN- γ ⁺TNF- α ⁺ effector T cells did not differ between LSCC patients and HD. (C) Flow dot plots of two Th1 subsets (IFN- γ ⁺IL-2⁺ and IFN- γ ⁻IL-2⁺) for one representative HD and LSCC patient. (D) The percentages of IFN- γ ⁺IL-2⁺ and IFN- γ ⁻IL-2⁺ Th1 subsets were significantly lower in LSCC patients than in HD. The percentage of IFN- γ ⁻IL-2⁺ effector T cells did not differ between LSCC patients and HD. Statistical comparisons were performed using the Student's t-test.

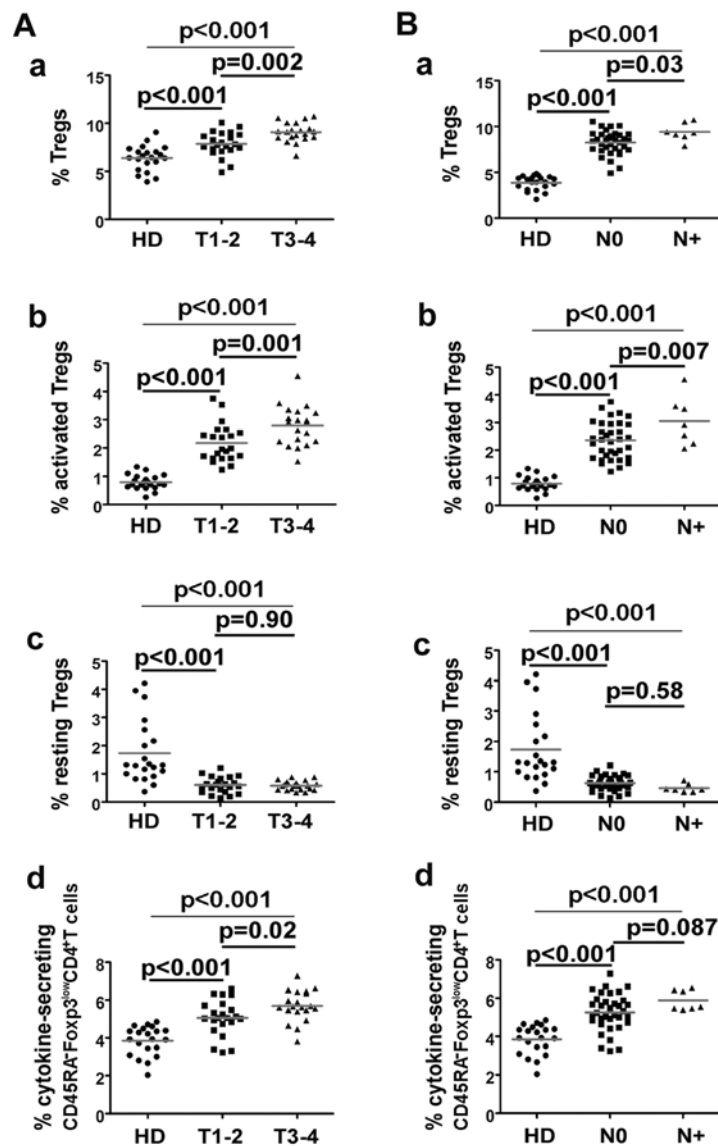


Figure 5. Relationship between Treg subsets and clinical variables. (A) Forty-two LSCC patients were subgrouped according to tumor stage. The percentage of Tregs (a) and each Treg subset (b-d) in PBLs were compared for 21 HD and LSCC patients with T₁₋₂ (n=22) and T₃₋₄ (n=20). (B) Forty-two LSCC patients were subgrouped according to nodal status. The percentage of Tregs (a) and each Treg subset (b-d) in PBLs were compared for 21 HD and LSCC patients with N⁺ (n=7) and N₀ (n=35). Statistical comparisons were performed using one-way ANOVA.

with T₁ to early T₃) due to the lack of lymphatic drainage in the glottic region. In the present study, 37 of the 42 LSCC patients had glottic squamous cell carcinoma; only 7 patients had nodal involvement. Thus, any conclusions regarding the difference between the two populations (N₀ and N⁺) must not be overstated since the number of patients in each category was unbalanced (i.e. 35 vs. 7).

The clinical impact of circulating Treg subsets on tumor stage and nodal status was examined. First, the percentage of Tregs was higher in 20 T₃₋₄ patients (9.07±1.01%) than in 22 T₁₋₂ patients (7.84±1.28%, $p=0.002$) and 21 HD (6.37±1.30%, $p<0.001$) (Fig. 5Aa). Furthermore, Tregs were increased in 7 N⁺ patients (9.39±0.98%) when compared with Tregs in 35 N₀ patients (8.23±1.28%, $p=0.03$) and 21 HD (6.37±1.30%, $p<0.001$) (Fig. 5Ba).

We also aimed to ascertain whether activated Tregs correlated with tumor progression. Interestingly, activated Tregs were elevated in T₃₋₄ patients (2.80±0.70%) relative to T₁₋₂

patients (2.18±0.67%, $p=0.001$) and HD (0.79±0.26%, $p<0.001$) (Fig. 5Ab). In addition, activated Tregs were elevated in N⁺ patients (3.05±0.88%) relative to N₀ patients (2.36±0.67%, $p=0.007$) and HD (0.79±0.26%, $p<0.001$) (Fig. 5Bb). The percentage of resting Tregs did not differ between patients with T₃₋₄ and T₁₋₂ (0.58±0.17% vs. 0.61±0.29%, $p=0.90$) (Fig. 5Ac) or with N⁺ and N₀ (0.46±0.14% vs. 0.62±0.24%, $p=0.58$) (Fig. 5Bc). Cytokine-secreting CD45RA^{low}Foxp3^{low}CD4⁺ T cells were elevated in T₃₋₄ patients compared with T₁₋₂ patients (5.69±0.82% vs. 5.06±0.95%, $p=0.02$) (Fig. 5Ad), but did not differ between N⁺ and N₀ patients (5.88±0.52% vs. 5.25±0.97%, $p=0.087$) (Fig. 5Bd).

Discussion

There is mounting evidence that Tregs are involved in the control of immune regulation in many types of human malignancies, with a particular focus on T-cell suppression (5-9). Tregs have

been reported to be negative prognostic factors for ovarian (5), hepatocellular (6), gastric and esophageal cancer (7). However, in contrast to these observations, Pretscher *et al* showed that higher Treg levels did not show any significant influence on the outcome of oro- and hypopharyngeal carcinoma patients (8). In addition, HNSCC studies indicate that elevated Treg levels are prognostic factors and predict better locoregional control and overall survival (9). This apparent contradiction regarding the role of Tregs in cancer prognosis might be explained by the functional heterogeneity of Tregs. For example, Zhou *et al* showed that CD4⁺Foxp3⁻ T cells transiently express lower levels of Foxp3, leading to the generation of T cells with a pathogenic memory (29). Allan *et al* postulated that activated CD4⁺ T cells express Foxp3, but lack regulatory activity (30). Hence, identification of distinct Treg subsets and their functional abilities might be intriguing for the antitumor immunity field.

Despite a number of studies performed on the role of Tregs in LSCC (15,23,31,32), the characteristics of functionally distinct Treg subsets in LSCC are poorly understood. Recently, one functional study reported by Drennan *et al* (15), showed that suppressive activities of CD127^{low/-} Tregs (including CD4⁺CD25^{inter}CD127^{low/-} and CD4⁺CD25^{high}CD127^{low/-} Tregs) increased in the peripheral circulation of laryngeal and oropharyngeal patients. The decrease was associated with advanced stage and nodal involvement, supporting the need to delineate the prevalence and function of different Treg subsets in LSCC, requiring further assessment of immunotherapeutic strategies. Hence, we sought to analyze the percentage and function of three distinct Treg subsets in LSCC patients at the time of diagnosis. Tregs were significantly higher in LSCC patients than in healthy age-matched donors, in agreement with previous studies (23,31,32). Nonetheless, a new finding was that the percentage of activated Tregs with highly suppressive activities increased in LSCC patients, and that this correlated with tumor stage and nodal status. Although resting Tregs also showed highly suppressive activities, the percentage of this Treg subset decreased, suggesting that resting Tregs may be swiftly converted into activated Tregs immediately after migrating from the thymus or having been peripherally generated (16).

Another interesting finding of the present study is that cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells increased in parallel with activated Tregs. We found that this Treg subset secreted elevated levels of effector cytokines, but did not have suppressive function *in vitro*. It may be that cytokine-secreting CD45RA⁺Foxp3^{low}CD4⁺ T cells are an heterogeneous Treg subset specific to LSCC. They might also be non-Tregs that differentiate into effector T cells, as others have proposed (16). The increased percentage of this subset might be the result of antigen exposure in the tumor microenvironment, similar to what is observed for CD8⁺ T cells in renal cancer (33). In our opinion, investigators should carefully identify distinct Treg subsets rather than whole Treg populations when studying LSCC. Taken together, these data suggest that activated Tregs might be potential targets in LSCC immunotherapy. In addition, the Foxp3 expression in CD8⁺ T cells was evaluated, as this T cell subset might also be Foxp3⁺ (23). Our data showed that the small percentage of CD8⁺Foxp3⁺ T cells in LSCC patients was decreased compared with HD, and that CD8⁺CD45RA⁺Foxp3⁺

T cells were rare in LSCC patients. Nevertheless, the ontogeny and function of Foxp3 expression in CD8⁺ T cells are still far from certain, owing to their scarcity in number and thus the difficulty in evaluation.

The complex functions of human Tregs and their interaction with other antitumor immune system members make the study of antitumor immunity challenging (3,4). Previous HNSCC studies have shown that a decreased frequency of peripheral CD4⁺ or CD8⁺ T cells may be attributed to Treg suppressive activities (24,25). However, one striking finding of our study is that the percentages of naïve CD4⁺ and naïve CD8⁺ T cells were decreased in the LSCC patients compared with these percentages in HD, whereas the percentages of CD4⁺ and CD8⁺ T cells were not. We hypothesized that Tregs may suppress proliferation of naïve CD4⁺CD45RA⁺ and naïve CD8⁺CD45RA⁺ T cells, but not all CD4⁺ and CD8⁺ T cells. The functional study of Treg subsets partially supported our hypothesis that among Tregs, both activated and resting Tregs had highly suppressive activities on autologous naïve CD4⁺CD45RA⁺ T cells.

It is now generally agreed that antitumor cellular immune responses are induced and maintained by Th1 cells (20,21). Although Th1 cells can be suppressed by Tregs in other diseases, such as autoimmune disease and allergies (17,26,27), the interplay between Tregs and Th1 cells is poorly understood in human malignancies. Our preliminary results, perhaps most excitingly, showed that the percentage of Th1 cells (including IFN- γ ⁺IL-2⁺ and IFN- γ ⁺IL-2⁻ Th1 subsets) was decreased in LSCC patients compared with HD, which suggests that Th1 cells may be suppressed by activated Tregs. Although we did not test this hypothesis, the present data support this speculation and suggest a complex interaction between Th1 cells and activated Tregs in LSCC. Further studies that focus on the interaction between Tregs and Th1 subsets may shed more light on the immunosuppressive activities of Tregs with respect to antitumor immunity.

In conclusion, this study provides evidence to support the notion that activated Tregs suppress CD4⁺CD25⁻ T-cell proliferation, and that functionally activated Tregs are correlated with disease progression in LSCC patients. An increase in activated Tregs might reduce T-cell-mediated antitumor immunity, as represented by the decrease in CD4⁺ T-cell subpopulations in LSCC patients. Thus, the present findings provide important information relevant to the future design of immunotherapeutic strategies for LSCC.

Acknowledgements

This study was supported by The National Natural Science Foundation of China (grant no. 81271055/H1301).

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