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Enrichment of Human CCR6⁺ Regulatory T Cells with Superior Suppressive Activity in Oral Cancer

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Human oral squamous cell carcinoma (OSCC) constitutes an inflammatory microenvironment enriched with chemokines such as CCL20, which promote cancer cell invasion and tumor progression. We found that in OSCC there is a correlation between the expression of CCL20 and FOXP3 mRNA. Therefore, we hypothesized that OSCC may favor the recruitment and retention of regulatory T (Treg) cells that express the CCL20 receptor, CCR6. Interestingly, most (∼60%) peripheral blood Treg cells express CCR6, and CCR6⁺ Treg cells exhibit an activated effector/memory phenotype. In contrast, a significant portion (>30%) of CCR6⁻ Treg cells were found to be CD45RA⁺ naive Treg cells. Compared to CCR6⁻ naive or memory Treg cells, CCR6⁺ Treg cells exhibit stronger suppressive activity and display higher FOXP3 expression along with lower methylation at the Treg-specific demethylated region of the FOXP3 gene. This predominance of CCR6⁺ Treg cells was also found in the draining lymph nodes and tumor-infiltrating lymphocytes of OSCC patients with early or late clinical staging. Moreover, CCR6⁺ Treg cells isolated from tumor-infiltrating lymphocytes or draining lymph nodes maintained similar phenotypic and suppressive characteristics ex vivo as did their counterparts isolated from peripheral blood. These results suggest that CCR6 marks activated effector or memory Treg phenotypes with superior suppressive activity in humans.

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Abbreviations used in this article: LNC, lymph node cell; mets LNC, metastatic LNC; MFI, mean fluorescence intensity; OSCC, oral squamous cell carcinoma; Tconv, conventional T; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T.

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CCR6+ Treg cells compared with CCR6− Treg cells remain unclear. CCL20, or MIP-3α, is the only ligand known to bind to and activate chemokine receptor CCR6 in mice (32, 33). Although β-defensin-1 and -2, two small cationic antimicrobial peptides, also reportedly bind to CCR6 in humans, they are far less potent chemoattractants than CCL20 (34, 35). Interestingly, not only can OSCC cells express both CCR6 and CCL20, suppression of CCL20 expression can reduce cancer cell proliferation, migration, and invasion; however, the underlying mechanisms remain unclear (36).

In this study, we tested the hypothesis that through CCL20 expression, OSCC favors the recruitment and retention of CCR6+ Treg cells. Additionally, we characterized the phenotypic and functional differences between CCR6+ and CCR6− Treg cell subsets and confirmed that CCR6+ Treg cells largely feature an activated effector/memory phenotype, whereas CCR6− Treg cells contain both CD45RA− memory and CD45RA+ naive Treg cells. Activated CCR6+ Treg cells exhibit stronger suppressive activity than do CCR6− Treg cells due to enhanced IL-10 production and higher expression of CD25, CD39, and HLA-DR. Therefore, CCR6 expression delineates a functionally activated effector/memory subset of human Treg cells, which are recruited to OSCC by factors present within the inflammatory milieu.

Materials and Methods

Participants

Patients with OSCC were recruited from the Department of Oral Maxillary Facial Surgery or Otolaryngology Clinic at the National Taiwan University Hospital. Healthy donors were recruited among the laboratory personnel and other volunteers. The Institutional Review Board of National Taiwan University Hospital approved the protocol for collection of patient blood samples or tumor specimens, and written informed consent was obtained from each individual. None of the patients had received any cancer-related therapy before entering the study. PBMCs and cord blood mononuclear cells were isolated by Ficoll-Paque Plus (GE Healthcare) following the manufacturer’s protocol. CD4+ T cells were enriched by MagniSort bead-based negative selection (eBioscience). Purified cells were cultured in RPMI 1640 media (HyClone) supplemented with 10% certified FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Biological Industries).

For lymph node cell (LNC) isolation, lymph nodes were minced in a petri dish filled with RPMI 1640 medium (HyClone). Cell suspension was filtered through 40-µm filters (BD Biosciences). TILs were then enriched by discontinuous Percoll (GE Healthcare) gradient (25, 55, and 100%).

Flow cytometry

The following fluorochrome-conjugated mAbs were used for experiments: anti-CD45RA (clone MA-1; BioLegend), anti-CD25 (clone M-A251; BioLegend), anti-CD127 (clone eBioRDR5; eBioscience), anti-CCR6 (clone G043E3; BioLegend), anti-CD45RA (clone HI100; BD Biosciences), anti-CCR7 (clone G043H7; BioLegend), anti-ICOS (clone ISA-3; eBioscience), anti-HLA-DR (clone L243; eBioscience), anti-CD161 (clone HP-3G10; eBioscience), anti-CD38 (clone HB7; eBioscience), anti-CD39 (clone A1; eBioscience), anti-CD31 (clone WM-59; Bioscience), anti-CD–PD-1 (clone EH12.2H7; BioLegend), anti-FOXP3 (clone PCH101; eBioscience), anti-CTLA4 (clone 14D3; eBioscience), anti-Helios (clone 22F6; BioLegend), anti–HLA-DR (clone JES5-9D7; eBioscience), and anti–Ki-67 (clone 20RAj1; eBioscience). For surface staining, cells were stained with fluorochrome-labeled mAbs for 30 min at 4°C in 100 µl of staining buffer (PBS plus 4% FBS). Appropriate isotype Ab controls were used for each sample. For intracellular staining, cells were surface stained and subsequently fixed and permeabilized using a fixation/permeabilization kit (eBioscience) or Cytofix/Cytoperm kit (BD Biosciences) following the manufacturers’ instructions. For intracellular cytokine analysis, cells were stimulated with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) in the presence of monensin (2 µM; eBioscience) for 4 h prior to surface staining and fixation.

Flow cytometric analysis was performed on a FACSComp II or an LSRFortessa flow cytometer (BD Biosciences). Data were exported as FCS 3.0 for further analysis in FlowJo software (Tree Star).

Suppression assay

Freshly isolated PBMCs or lymphocytes from tissue were stained with mAbs specific to CD4, CD25, CD45RA, CD127, and CCR6, and cells were sorted on FACSaria (BD Biosciences) through the service provided by the Flow Cytometric Analyzing and Sorting Core (the First Core Laboratory, National Taiwan University College of Medicine). Specific Treg cell subsets (suppressors) were sorted depending on the experiments and cocultured with CD4+CD25+ T cells (responders) to test the suppressive ability of different Treg cell populations. Responder cells were labeled with 1 µM CFSE prior to suppression assays. CFSE-labeled responder cells were cultured at 2 × 104 per well and stimulated with anti-CD3/anti-CD28–coated Dynabeads (Invitrogen) alone or in combination with specific Treg cell subsets at specified ratios in 96-well round-bottom plates. Percent suppression of responder cell proliferation (measured by CFSE dilution) was calculated based on the following equation: [(proliferation of responder cells alone – proliferation of responder cells in the presence of Treg cell coculture/proliferation of responder cells alone] × 100.

To determine the role of IL-10 production by Treg cells in the suppression assay, IL-10 levels in the supernatant were determined by ELISA using the anti-IL-10 Ab (clone JES5-12G8). Anti-human IL-10 (clone JES5-9D7, 5 µg/ml) was added to the coculture to block the suppression.

Treg differentiation

Peripheral CD45RA−CD4+CD25− naive T cells were stimulated with 5 µg/ml plate-bound anti-CD3 Ab (clone OKT3), 1 µg/ml soluble anti-CD28 Ab (clone CD28.2), 100 IU/ml recombinant human IL-2 (Peprotech), and 5 ng/ml recombinant human TGF-β1 (PeproTech) in the presence or absence of human CCL20 (100 ng/ml; GenScript). After 6 d of incubation, the expression of FOXP3 was examined by FACS analysis.

Immunohistochemistry

Tumor tissues were embedded in Tissue-Tek OCT compound (Sakura) and were frozen at −80°C. Cryosections were cut into 5-µm-thick slices and were fixed in absolute acetone prior to staining with mAbs specific to CCL20 (Abcam) and FOXP3 (clone 236A/E7; eBioscience). Colors were developed with diaminobenzidine (Dako) or 3-amin-9-ethylcarbazole (BioGenex Laboratories) and sections were counterstained with instant hematoxylin. Sections were initially scanned and ~1000 inflammatory cells from at least three high-power (original magnification, ×200) fields of randomly selected cancer stromal tissue were counted for each case.

DNA methylation analysis

After extraction of genomic DNA, bisulfite conversion was performed using the EZ DNA Methylation-Direct kit (Zymo Research) according to the manufacturer’s instructions. Subsequently, the modified DNA was amplified by PCR and cloned into pGEM-T vector (Promega). PCR primers specific for FOXP3 promoter, Treg cell–specific demethylation region, and CCR6 differentially methylated region were previously described (37–39). Colonies (>10 colonies per region) were sequenced with a T7 or Sp6 primer. The sequenced data were analyzed using the QUMA Web tool (http://quma.cdb.riken.jp/).

Quantitative real-time PCR

Tumor tissues were disrupted using liquid nitrogen, and total RNA was extracted following a guanidinium thiocyanate–phenol–chloroform procedure (40). RNA was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega) following the manufacturer’s instruction. The quantitative real-time PCR was performed on an Applied Biosystems 7500 machine in the presence of FOXP3, CCL20, and ACTB primers (Genomics). Target gene transcription levels were measured and normalized to ACTB expression. The following primer sequences were used: FOXP3, 5'-CACCTCGTGCTGGGAAAATGG-3' and 5'-GGACCTTGCCTGGATGAT-3'; CCL20, 5'-GCAGATCGAAGCGAAGCT-3' and 5'-GACCTGATGTCACAGCCCTC-3'.

Statistical analysis

Data are presented as means ±SEM. A Student t test was used to compare the expression levels of indicated markers or the frequency of populations using GraphPad Prism 5 software (GraphPad Software). Differences with a
Results

FOXP3 tissue expression positively correlates with CCL20 in OSCC

Although CCR6+ Treg cells have previously been found in several human cancers (29–31), their functional characteristics and whether there is a correlation between CCR6 and CCL20 expression have remained unclear. To test the hypothesis that CCL20 is expressed in OSCC and is associated with the recruitment or retention of the CCR6+ Treg cells to the tumor microenvironment, both immunohistochemistry and RT-PCR analysis were performed. Consistent with previous findings in vitro (36), CCL20 was highly expressed in situ by OSCC tumor nests (Fig. 1A) with infiltrating FOXP3+ cells scattered around tumor cells (Fig. 1B). Double immunostaining confirmed that all FOXP3+ cells were infiltrating FOXP3+ cells scattered around tumor cells (Fig. 1C). In contrast, no FOXP3+ cells were detected in normal oral epithelium (data not shown). Treg cell CD4+ Treg cells (Fig. 1C). In contrast, no FOXP3+ cells were detected in normal oral epithelium (data not shown). Treg cell infiltration or recruitment into the TILs could be found during early stages of OSCC and were sustained at high levels in later stage tumors, based on the expression of FOXP3 in the whole tumor or in CD4+ T cells (Supplemental Fig. 1). Furthermore, the CCL20 mRNA expression positively correlated with the expression of FOXP3 and CCL20 mRNAs.

Phenotype of CCR6+ and CCR6− FOXP3+ Treg cells from healthy donor PBMCs

To determine the phenotypic and functional characteristics of the CCR6+ and CCR6− Treg cells, PBMCs were isolated from healthy donors or cord blood. Interestingly, among total CD4+ T cells, most FOXP3+ cells were CCR6+ Treg cells (~70%), although the frequency of CCR6+ cells among total Treg cells varied among the healthy donors tested (Fig. 2A, 2B). In contrast, <10% of total CCR6+Treg cells in cord blood expressed CCR6 (Fig. 2C, 2D), despite the fact that the frequency of Treg cells is similar between the two sources (~4.5% in both PBMCs and cord blood). Collectively, these results suggest that CCR6+ Treg cells in peripheral blood may exhibit a phenotype characteristic of effector and memory cells, whereas CCR6− Treg cells may exhibit naive characteristics, as is also found in mice (28). As a result, the expression of CCR6 may serve as a convenient marker to identify the effector/memory subset of Treg cells in humans.

The phenotypical and functional characteristics of peripheral CCR6+ and CCR6− Treg cell subsets were subsequently investigated. CCR6+ Treg cells from healthy donor PBMCs expressed significantly higher levels of FOXP3 (mean fluorescence intensity [MFI], 2476 ± 365.9) than did CCR6− Treg cells (MFI, 1660 ± 189.3) (Fig. 3A). Consistent with higher FOXP3 expression, staining for CD25, CTLA-4, HLA-DR, ICOS, and CD39 was significantly higher on CCR6+ Treg cells compared with CCR6− Treg cells (Fig. 3B). Moreover, CCR6+ Treg cells did not express CD45RA, whereas the CCR6− Treg cells contained both CD45RA+ and CD45RA− subsets. Additionally, the expression of CCR7, a marker commonly used in combination with CD45RA to identify naive T cells (43), was significantly lower in CCR6− than in CCR6+ Treg cells. Therefore, in the peripheral blood of healthy donors, the CCR6+ FOXP3+ Treg cell population predominantly contained the naive (CD45RA−CCR7+) Treg cell subset, whereas the CCR6−FOXP3+ cell population was mostly comprised of effector

\[ p \text{ value} < 0.05 \] were considered statistically significant. A Spearman correlation test was used to determine the strength of the association between FOXP3 and CCL20 mRNA expression levels.
memory Treg cells (CD45RA \(^2\) CCR7 \(^2\)) and, to a lesser extent, central memory cells (CD45RA \(^2\) CCR7 \(^+\)) (Supplemental Fig. 2A). Furthermore, the proportion of CD31\(^+\) cells was lower in CCR6\(^+\) Treg cells compared with that of CCR6\(^-\) Treg cells. CD31 (PECAM-1) marks recent thymic emigrants, and its expression is lost upon activation following TCR engagement (44). Additionally,

**FIGURE 3.** Phenotype of CCR6\(^+\) and CCR6\(^-\) FOXP3\(^+\) Treg cells from healthy donor PBMCs. (A) Histogram overlays indicate the expression of CCR6 among three different cell populations: FOXP3\(^+\)CCR6\(^+\) cells (blue), FOXP3\(^-\)CCR6 \(^-\) (purple) cells, and FOXP3\(^-\) (red) cells. CCR6\(^+\) Treg cells exhibit increased FOXP3 staining when compared with CCR6\(^-\) Treg cells. PBMCs were obtained from nine healthy donors, and the MFI values of FOXP3 were compared using a Student t test. (B) To further characterize CCR6\(^+\) Treg cells, a comprehensive panel assessment of PBMCs from healthy donors was used to characterize cell surface and intracellular molecule expression by Treg cells \((n = 6)\). Histogram overlays indicate the expression of each molecule within each cell populations as in (A): FOXP3\(^+\)CCR6\(^+\) cells (blue), FOXP3\(^+\)CCR6 \(^-\) (purple) cells, and FOXP3\(^-\) (red) cells and the percentage of positive cells positive or MFI value for each marker are indicated in each histogram. Statistical comparisons were performed using Student t test. * \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\).
CCR6+ Treg cells showed higher proportions of CCR4+, CXCR3+, Ki-67+, CD161+, TIGIT+, CD226+, PD-1+, or CD95+ cells. This suggests that CCR6+ Treg cells are indeed more activated than their CCR6− counterparts. Moreover, CCR6+ Treg cells showed fewer CD38+ and Helios+ cells (Fig. 3B). In contrast, in cord blood, naive Treg cells predominated, and a much smaller fraction of CCR6+ Treg cells was found (Supplemental Fig. 2B).

Besides surface and intracellular marker differences, DNA methylation status analyses showed that the FOXP3 promoter was highly demethylated in both CCR6+ and CCR6− Treg cell subsets, whereas the Treg-specific demethylated region was slightly more methylated in CCR6− Treg cells (Supplemental Fig. 2C). Collectively, these data suggest that CCR6 marks a phenotypically distinct Treg cell population that exhibits an activated effector/memory phenotype.

The suppressive activity of CCR6+ Treg cells is superior to that of CCR6− Treg cells

As shown in Fig. 3B, the phenotypic differences indicate that CCR6+ Treg cells may possess stronger suppressive activities. To test the hypothesis, peripheral CCR6+ and CCR6− Treg cell subsets were enriched from total CD4+ T cells based on high surface CD25 and low CD127 expression (15) (Fig. 4A). As shown in Fig. 4B, both CCR6+ and CCR6− Treg cells inhibited proliferation of conventional T (Tconv) cells in vitro. However, CCR6− Treg cells showed significantly higher suppressive activity than did CCR6+ Treg cells (percentage of suppression, 84.504 ± 4.29% for CCR6− versus 32.99 ± 11.299% for CCR6+ Treg; ratio of Tconv to Treg cells = 1:1) (Fig. 4C). Additionally, CCR6− Treg cells produced significantly higher amounts of IL-10 compared with CCR6+ Treg cells (Fig. 4D). Increased IL-10 protein levels were also detected in supernatants of Tconv and CCR6− Treg cell cocultures (Supplemental Fig. 3A). Additionally, IL-10 neutralization compromised the suppressive activity of CCR6−, but not CCR6+, Treg cells (Supplemental Fig. 3B). Taken together, these data suggest that CCR6+ Treg cells exhibit stronger suppressive activity partly through an IL-10-dependent manner.

We tested the suppressive ability of CCR6+ Treg cells in the presence of CCL20. As shown in Supplemental Fig. 3C, there was no influence of CCL20 on CCR6+ Treg suppressive activity. Furthermore, CCL20 also did not affect the induction of Treg cells from naive T cells (Supplemental Fig. 3D).

Phenotype and suppressive activities of naive and memory CCR6+ Treg cell subsets

A previous study found that based on the expression of CD45RA and FOXP3, human FOXP3+ cells can be divided into three subpopulations: CD45RA+FOXP3low (population I, resting Treg), CD45RA−FOXP3high (population II, effector Treg), and non-suppressive CD45RA−FOXP3low (population III) cells (18). However, CCR6 expression on these Treg cell populations was not assessed. To this end, we found that the frequency of CCR6+ cells in population I (17.93 ± 3.697%) was significantly lower than in populations II and III, in which >50% of each population expressed CCR6 (Fig. 5A). Based on the surface expression of CD45RA and CCR6, we found that Treg cells could be subdivided into three major populations: CD45RA+CCR6− (naive Treg), CD45RA−CCR6+ (CCR6+ memory Treg), and CD45RA−CCR6− (CCR6− activated effector/memory Treg) cells. CD45RA+CCR6− cells were not included in the subsequent analyses due to their low frequency (Fig. 5B). Phenotypic characterization revealed that naive Treg cells expressed the lowest levels of CD25, CD39, CTLA-4, ICOS, HLA-DR, PD-1, and Ki-67 compared with the other two memory Treg cell subsets (Fig. 5C). Although both CCR6+ and CCR6− memory Treg cells exhibited memory phenotypes based on higher HLA-DR and lower CCR7 expression, CCR6+ memory Treg cells expressed increased activation markers typically associated with activation of Treg cell function. Therefore, the increased expression of HLA-DR suggests CCR6+ effector/memory Treg cells indeed acquire a more activated status than do CCR6− Treg cells (20).

Several studies have indicated that the suppressive activity of naive Treg cells is relatively weak compared with that of memory Treg cells (18, 45, 46). Thus, the presence of naive Treg cells may account for the lower suppressive activity of total CCR6+ Treg cells demonstrated in Fig. 4. To compare the suppressive activity of naive, CCR6+, and CCR6− memory Treg cell subsets, individual subsets were sorted to analyze their suppressive abilities on Tconv cell proliferation in vitro. We found that among the three subsets, CCR6+ activated effector/memory Treg cells exhibited the strongest suppressive activity, followed by CCR6− memory Treg cells, with naive Treg cells being the weakest suppressor cells (Fig. 6). Taken together, these results indicate that CCR6+ activated effector/memory Treg cells exhibit superior suppressive activity in vitro compared with CCR6− memory or naive Treg cells.

FIGURE 4. CCR6+ Treg cells exhibit superior suppressive activity compared with CCR6− Treg cells. CD4+CD25+ (Tconv) cells and CD4+CD25−CD127low (Treg) cells from peripheral blood of healthy donors were isolated to test their suppressive activities in vitro. (A) Desired cellular subsets were sorted based on the representative plots. Purity of Treg cells was validated by FOXP3 staining. (B) Tconv cells were labeled with CFSE before culture alone or before coculture with unlabeled Treg cell subsets at specified ratios in the presence of anti-CD3/CD28 beads for 4 d. Histogram plots were gated on CFSE+ cells and thus represent Tconv cell proliferation. Numbers above the histogram plots indicated Tconv/Treg ratio. The data were further summarized as percentage suppression of division of Tconv cells as described in Materials and Methods (C) (n = 3). **p < 0.01, ***p < 0.001. (D) Following the experiment in (B), during the last 4 h of coculture (Tconv/Treg ratio = 1:0.5), cells were stimulated with PMA and ionomycin to induce IL-10 production. Flow cytometry plots show CFSE dilution of Tconv cells and IL-10 production by Tconv and Treg cells. Inset shows the percentage of events in each quadrant.
**FIGURE 5.** Phenotype of naive and memory Treg subsets. (A) Treg cell subpopulations were first separated based on the expression of FOXP3 and CD45RA, as detailed by Miyara et al. (18), and CCR6 expression was subsequently determined for each subpopulation. (B) Because CD45RA+ cells are mostly CCR6-, we further separated the FOXP3+ cells into three populations based on CCR6 and CD45RA expression. (C) Each Treg cell population from healthy donor PBMCs (n = 6) was analyzed by a comprehensive panel to characterize Treg cells. The percentage of cells positive or the MFI for each molecule was compared across different cellular populations. Statistical comparisons were performed using a Student t test. CCR6+CD45RA- Treg cells exhibit the highest levels of activation markers. *p < 0.05, **p < 0.01, ***p < 0.001.

Enhanced suppressive function of CCR6+ Treg cells in TILs and draining lymph nodes

OSCC and its precancerous lesions, oral lichen planus or oral submucosal fibrosis, are associated with a chronic inflammatory microenvironment (4). CD4 and CD8 lymphocytes constitute the major populations of TILs in OSCC (5). As a result, we hypothesized that if CCL20 is a functional ligand of CCR6 and is capable of recruiting CCR6+ Treg cells from peripheral blood, CCR6+ Treg cells found in OSCC should exhibit similar phenotypical and functional characteristics as those present in PBMCs. Therefore, we analyzed the distribution frequency and the characteristics of CCR6+ Treg cells in PBMCs, draining LNCs, metastatic LNCs (mets LNCs), and TILs from OSCC patients. Similar to PBMCs isolated from healthy donors (Fig. 2A), the frequency of CCR6+ cells was significantly higher in FOXP3+ Treg cells than FOXP3- Tconv cells in PBMCs (59.62 ± 6.2.394% versus 28.65 ± 2.175%), LNCs (51.92 ± 3.453% versus 27.64 ± 3.174%), mets LNCs (71.63 ± 1.398% versus 31.37 ± 2.388%), and TILs (69.91 ± 3.569%...
versus 44.13 ± 2.437%) (Fig. 7A). The FOXP3 expression level in CCR6+ Treg cells was also significantly higher than in CCR6− Treg cells from PBMCs (MFI, 2482 ± 197.5 versus 1883 ± 142.6), LNCs (MFI, 2404 ± 301.2 versus 1612 ± 195.6), and TILs (MFI, 3868 ± 519.4 versus 2745 ± 369.3). In line with previous reports (6, 47–49), the frequency of Treg cells and FOXP3 expression were both significantly higher in TILs than PBMCs (Fig. 7B and data not shown). Most importantly, we found that the frequency of CCR6+ Treg cells was significantly higher in TILs than PBMCs or LNCs (Fig. 7C), and CCR6+ Treg cells from different sources consistently expressed higher FOXP3 levels compared with their CCR6− counterparts (Fig. 7D).

In contrast to PBMCs, the frequency of naive Treg cells in TILs was extremely low (Fig. 7E), indicating that TIL/Treg cells consist only of memory Treg cells. In addition to the reduction in naive Treg cells, an increased activated effector memory subset and decreased central memory subset were found in TIL/Treg cells (Supplemental Fig. 4). Moreover, the frequency of CCR6+ activated effector/memory Treg cells was significantly higher in TILs than in PBMCs.

Because naive Treg cells were nearly absent in TILs, we sorted only CCR6+ activated effector/memory Treg cells and CCR6− memory Treg cells to test their suppressive activities (Fig. 8A). CCR6+ activated effector/memory Treg cells in TIL or draining lymph nodes from three patients exhibited consistently stronger suppressive activity than did CCR6− memory Treg cells (Fig. 8B). Corresponding with this stronger suppressive capacity, TIL/CCR6+ activated effector/memory cells expressed higher CTLA-4, CD25, HLA-DR, and ICOS compared with TIL/CCR6− memory Treg cells (Fig. 8C). Taken together, these results demonstrate that CCR6+ activated effector/memory Treg cells are enriched in situ in the OSCC microenvironment and exhibit similar phenotypic and functional characteristics as those cells found in the peripheral blood.

Discussion

Treg cells are a functionally unique subset of CD4+ T lymphocytes that play a critical role in regulating immune homeostasis. Our results indicate that CCR6 could serve as a convenient marker to identify activated effector/memory Treg cells, which exhibit superior in vitro suppressive activity regardless of being isolated from peripheral blood, lymph nodes, or tumors. Moreover, the unique microenvironment of OSCC led to the discovery that CCL20 is important for the recruitment or retention of the CCR6+ Treg cells into the tumor. In the present study, Treg cells were identified by the absence of CD127 together with the high expression of CD25. These markers have been widely used to define functional human Treg cells in either healthy donors or in patients with inflammatory diseases (15, 50). Additionally, we found that functional distinct subsets exist among CD4+FOXP3+ effector/memory Treg cells. The CD45RA−CCR6− memory Treg cells possess moderate suppressive activity, CD45RA−CCR6+ Treg cells possess strong suppressive functions, and CCR6− naive Treg cells exhibit little suppressive activity, consistent with other reports (18, 45, 46). Interestingly, we previously showed that among the CCR6+FOXP3+ cells isolated from TILs of OSCC but not from PBMCs, there are cells that are capable of producing inflammatory cytokines such as IL-17 (6). It has been reported that human peripheral Treg cells contain heterogeneous subsets that phenotypically mirror effector Th cells (51). Taken together, our and other reports suggest that the FOXP3+ Treg cells in either the peripheral blood or the tissue sites such as tumors of OSCC are a heterogeneous population, yet CCR6 expression still marks a unique Treg cell characteristics of superior suppressive activity.

CCR6 expression alone defines effector/memory-like Treg cells in mice (28). Kleinewietfeld and colleagues (28) revealed that CD25+CCR6+ Treg cells, similar to CD25−CCR6+ effector T cells, exhibit markers of activation, memory, and expansion as well as rapid IL-10 production upon in vitro activation. However, no difference in suppressive capacity between CCR6+ and CCR6− Treg cells was found in mice (28). In the present study, we found that in humans, CCR6 may also serve as a marker of activated effector/memory Treg cells. However, in contrast to mice, human CCR6+ Treg cells from both PBMCs or TILs exhibit superior suppressive capacity. Although anti–IL-10 Ab neutralization partially reduced the suppressive function of CCR6+ Treg cells, they still exhibited stronger suppressive activity than did CCR6− Treg cells. This is possibly due to increased expression of other suppressive molecules, such as HLA-DR, CTLA-4, and CD39, associated with the superior suppressive activity of CCR6+ Treg cells.

It has been considered that CCL20 could also play a critical role in mucosal homeostasis, whereas local inflammation can lead to upregulation of CCL20 mRNA in various tissues (52, 53). This suggests that, in addition to the maintenance of mucosal homeostasis, CCL20 also contributes to the recruitment of CCR6+expressing cells to sites of local inflammation. It is possible that, upon local inflammation, effector/memory-like CCR6+ Treg cells might be rapidly attracted to local tissue to prevent uncontrolled inflammation. Indeed, it has been reported that CCR6 is required for Treg cells to control the CNS inflammatory disease experimental allergic encephalomyelitis in mice (54). In line with these findings, we found that CCR6+ Treg cells are enriched in TILs and metastatic lymph nodes compared with PBMCs. Therefore, the CCR6+ Treg cells in the peripheral circulation seem to facilitate a first-aid patrolling mechanism to counteract or resolve unwarranted immune activation in inflamed tissues, as well as the inflammatory microenvironment in OSCC.

It is now widely recognized that inflammation is required for cancer progression in many tissues, including the colon and liver, in humans and in mice (55). It is generally thought that Treg cells...
help tumors escape antitumor immunity, yet the role of these cells in the prognosis and clinical outcomes of OSCC are controversial. It has been suggested that the infiltration of Treg cells may play a role in inhibition of chronic inflammation that promotes tumor cell invasion and metastasis in OSCC (9–12). However, direct experimental evidence regarding the role of Treg cells in the development or progression of OSCC is limited. In a 4-nitroquinoline-1-oxide–induced tongue SCC model, an increase of Treg cells in the peripheral blood and regional lymph nodes was found at ∼24 wk when compared with controls (56). However, the presence of Treg cells in the TIL or their correlation with either the timing or severity of the induced carcinogenesis was not reported. Nevertheless, the anti-inflammatory role of Treg cells in inhibiting carcinogenesis has been found in the mouse model of colon cancer promoted by innate immune inflammation caused by the carcinogenic bacteria Helicobacter hepaticus (57). Similar to colon cancer, the OSCC microenvironment during cancer progression is complicated with the infiltrating microflora (58). Both our results and analysis of the Cancer Genome Atlas dataset (59) showed that the Treg cell infiltration or recruitment in the TILs started during the early stage of OSCC (Supplemental Fig. 1). It is possible that Treg cells infiltrating during the early or late stage of OSCC may

FIGURE 7. CCR6+ Treg cells are enriched in OSCC. (A) The percentage of CCR6+ Treg cells was determined in PBMCs (n = 33), LNCs (n = 10), mets LNCs (n = 3), and TILs (n = 15). In all of the anatomical compartments analyzed, CCR6+ cells predominated among FOXP3+ cells, and CCR6+ cells exhibited higher FOXP3 MFI values than did the other subpopulations. (B) Frequency of FOXP3+ cells among total CD4+ cells. (C) Frequency of CCR6+ cells among FOXP3+ cells. (D) MFI of FOXP3 staining in T cells from different anatomical compartments, including PBMCs, LNCs, mets LNCs, and TILs. (E) Representative flow cytometry results showing that CD45RA+ CCR6+ Treg cells are absent in OSCC TILs. Plots were gated on CD4+FOXP3+ cells. In contrast, CD45RA+ CCR6+ activated/memory Treg cells predominated in TILs. Comparisons between groups were performed by a Student t test. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not statistically significant.
TILs were isolated from OSCC immediately after surgery and sorted TILs and draining lymph node. (between CCR6+ and CCR6 Treg cells. the recruitment or retention of these CCR6+ Treg cells into OSCC subsets, and the CCL20/CCR6 axis may play an important role in are phenotypically and functionally distinct human Treg cell 2

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