

Serum from patients with oral squamous cell carcinoma remodels the tumor immune escape ecological niche by promoting regulatory T-cell differentiation and T-cell exhaustion

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Abstract. Oral squamous cell carcinoma (OSCC) ranks as the sixth most prevalent malignancy worldwide, and is characterized by high morbidity and mortality rates. Elucidating the molecular and cellular mechanisms of tumor-directed immune escape through ecological niche remodeling is crucial for advancing tumor biotherapy. The serum of patients with cancer contains not only tumor biomarkers but also immune regulators secreted by immune cells and/or cancer cells. Notably, the interstitial fluid within the cancer ecological niche is derived from serum. The cross-talk between serum and cancer cells determines the future of cancer cells, either cell survival or death. The present study revealed that serum from patients with OSCC could remodel the cancer immune escape ecological niche by promoting antigen-induced regulatory T-cell (Treg) differentiation and T-cell exhaustion. When serum from patients with OSCC was added to a

phytohemagglutinin-stimulated peripheral blood mononuclear cell (PBMC) culture system, the Treg subset was significantly increased compared with that in the culture system treated with fetal bovine serum. Moreover, when the serum of patients with OSCC was added to a PBMC culture system stimulated with tumor antigens, the activation of the CD3⁺ subset was significantly inhibited, and high levels of IL-4, IL-10 and TGF- β were detected in the supernatant; moreover, CD3⁺ T cells expressed high levels of T-cell immunoglobulin and mucin-domain containing-3 and programmed death ligand 1, which is known to induce T-cell apoptosis and exhaustion. Finally, the antitumor effect of T cells were significantly decreased. These results indicated that the serum of patients with cancer can promote the inhibition and exhaustion of anti-tumor T cells, thereby remodeling the tumor immune escape ecological niche.

Introduction

Oral squamous cell carcinoma (OSCC) is a challenging disease with an increasing incidence rate, particularly among younger patients (1,2). In 2022, ~389,485 new cases of lip and oral cavity cancer were diagnosed worldwide, resulting in ~188,230 deaths; most of those cases were identified in Asia (3). Despite advancements in diagnostic and management strategies in recent decades, the 5-year survival rate of patients with OSCC is still ~50% (4,5). OSCC is the predominant subtype of head and neck squamous cell carcinoma (HNSCC), which presents distinct pathological characteristics that differ from those of cancers at other sites; a hallmark feature of OSCC is its prominent keratinization (6,7). Because of the unique risk factors specific to the oral cavity, OSCC has been studied separately from other types of HNSCC. OSCC is characterized by high morbidity and mortality rates (8,9).

The development of OSCC involves multiple molecular events influenced by an the genetic predisposition of the individual and exposure to environmental carcinogens. Notably, smoking, alcohol, tobacco and betel quid use have been reported as potential carcinogen risk factors contributing to the occurrence of OSCC (10,11).

The molecular and cellular mechanisms underlying the pathogenesis of OSCC are relatively poorly understood. The

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Abbreviations: CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; FBS, fetal bovine serum; FOXP3, Forkhead box P3; HNSCC, head and neck squamous cell carcinoma; HS, human healthy serum; HTS, human tumor serum; OSCC, oral squamous cell carcinoma; PBMC, peripheral blood mononuclear cell; PD-1, programmed death 1 receptor; PD-L1, programmed death ligand 1; PHA, phytohemagglutinin; TIM3, T-cell immunoglobulin and mucin-domain containing-3; Treg, regulatory T cell

Key words: serum, patients with cancer, ecological niche, regulatory T cells, T-cell exhaustion

ecological theory of cancer has led researchers to investigate cancer from ecological and evolutionary principles and perspectives (12,13). According to cancer ecology, the formation and development of cancer involves invasion, variation, selection and evolutionary progression. During this process, cancer cells remodel the microenvironment and form a cancer immune escape mechanism, which is necessary for the survival and proliferation of cancer cells. Identification of the molecular and cellular mechanisms of cancer ecological niche remodeling is important for the exploration of new targets and strategies for cancer biotherapy (14).

The mechanism by which cancer cells evade immune responses via ecological niche remodeling is not completely understood. Previous studies have shown that cancer immune escape is caused by diverse complex processes, including i) cancer cells expressing and secreting immune negative modulatory molecules, such as programmed death ligand 1 (PD-L1), TGF- β and IL-10; ii) abnormal activation, proliferation and differentiation of immune cell subpopulations in the tumor microenvironment, such as regulatory T cells (Tregs) and M2 macrophages; and iii) impaired homing of immune cells to the tumor site (15-18). Targeting immune negative modulatory molecules and rescuing the function of immune cells are the major efforts of current tumor biotherapies (19,20).

Immunodeficiency is a universal phenomenon in patients with cancer, particularly at the later stages. This status leads to poor defenses against bacterial and viral infections, the lack of cancer cell elimination by the immune system and the absence of a response to biotherapy (21). T-cell exhaustion causes T cells to lose their ability to kill tumor cells, reducing the immune surveillance function of the body, which is also detrimental to treatment efficacy and the survival time of patients (22). In terms of immunotherapies, the presence of Tregs and exhausted T cells weakens the antitumor effects of immunotherapeutic drugs, reducing the responsiveness of patients to immunotherapies and affecting treatment outcomes. Therefore, an improved understanding of the mechanisms underlying serum-induced Treg differentiation and T-cell exhaustion is crucial for optimizing tumor immunotherapeutic strategies and improving treatment efficacy in patients (23).

As an essential component of the human circulatory system, serum comprehensively reflects the physiological and pathological status of the body and contains a multitude of bioactive molecules derived from tumor tissues, and various organs and systems throughout the body (24-26). Studying serum can indicate the impact of tumors on the immune system from a holistic perspective, rather than being limited to the local tumor microenvironment. Moreover, previous studies have shown that certain components in serum can remotely regulate the functions of immune cells and participate in the processes of tumorigenesis, development and metastasis (24,25). Therefore, research on patient serum has unique advantages and importance for revealing the systemic mechanisms of tumor immune escape.

However, a literature search using PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) revealed limited research discussing tumor immune escape mechanisms involving serum-derived factors. Only one article that specifically addresses the relationship between serum and immune escape in patients with breast cancer was identified (27).

The present study aimed to investigate the immunomodulatory effects of serum from patients with OSCC on peripheral blood T-lymphocyte differentiation, with the ultimate goal of elucidating the mechanisms underlying serum-induced immunosuppression.

Materials and methods

Ethics statement. Patients and healthy controls were recruited to the present study between February 2023 and August 2024, and provided written informed consent. The present study was approved by the Committee for Ethics Approval of the West China Hospital of Stomatology, Sichuan University (approval no. WCHSIRB-D-2022-491; Chengdu, China).

Serum collection from patients with OSCC and healthy volunteers. We referred to the sample size ($n=10$) selection in previous similar studies and conducted preliminary experiments by stimulating T lymphocytes with either serum from patients with cancer or from healthy donor, followed by comparative analysis of CD3⁺ T-cell subsets using flow cytometry (28,29). The results of the preliminary experiments revealed that when the sample size was 10, the expected effect size could be detected, and the experimental results were highly reproducible. Furthermore, a statistical power analysis was performed using GraphPad Prism 6.0 (Dotmatics). On the basis of the data from the preliminary experiments, the calculated sample size of $n=10$ in the current study was considered sufficient to meet the statistical requirements and had enough statistical power to support the conclusions.

A total of 10 patients with OSCC (five women and five men) were recruited from the Department of Head and Neck Oncology of West China Hospital of Stomatology, Sichuan University. The inclusion criteria for patients with OSCC were: Aged 30-80 years; histopathologically confirmed primary OSCC; and newly diagnosed, treatment-naïve patients with Tumor-Node-Metastasis stages I-IV (American Joint Committee on Cancer 8th edition) (30). The serum used in the present study was collected before surgery. The exclusion criteria for patients with OSCC were: Systemic or current use of nonsteroidal anti-inflammatory drugs, antibiotics, chemotherapy drugs, similar hormonal compounds or blood transfusions; or a history of other malignancies within the past 5 years. The clinical characteristics of the 10 patients are shown in Table SI. In addition, 10 healthy volunteers (five women and five men; age range, 22-59 years; mean age, 37 years) without prior dental treatments were recruited from the State Key Laboratory of Oral Diseases, Sichuan University. All of the patients and healthy volunteers were informed of the aim and procedures of the study, and the medical information were used for the current study only. Fresh patient (human tumor serum, HTS) and healthy donor (human healthy serum, HS) serum samples were collected and stored at -80°C .

Isolation of peripheral blood mononuclear cells (PBMCs). PBMCs from healthy donors have relatively uniform and stable functional states, which can reduce the interference of factors such as immune system disorders and underlying diseases on the experimental results (31), enabling a clear observation of the direct effects of serum from patients with OSCC on

immune cells. When PBMCs from patients with cancer are stimulated with autologous serum for a long period of time, the status of T cells, such as surface markers and T-cell subsets changes (32). Restimulating these PBMCs with serum from patients with cancer may therefore yield limited scientific value. Therefore, in the present study, PBMCs from healthy donors were used to explore the effects of cancer serum.

Fresh PBMCs from healthy volunteers were isolated via density gradient centrifugation using Ficoll-Paque Plus. Briefly, 10–20 ml fresh peripheral blood was collected, diluted with an equal volume of PBS, then gently layered onto Ficoll-Paque Plus (cat. no. 17144002; 1:2; Cytiva) using a pipette at a 45° angle, maintaining a clear interface between phases. Then, centrifugation was performed at 400 x g for 30 min at 19±1°C using a swing-bucket rotor with brake function disabled. After centrifugation, the PBMCs were collected and washed twice with RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.). PBMCs (1x10⁷ cells/ml) were suspended in RPMI 1640 medium supplemented with 20% indicated serum [heat-inactivated fetal bovine serum (FBS; HyClone; Cytiva), HTS or HS] and 100 U/ml penicillin/streptomycin.

Tumor antigen preparation. The Cal-27 OSCC cell line was purchased from American Type Culture Collection. Cal-27 cells were seeded in 10-cm Petri dishes and incubated in a humidified atmosphere of 5% CO₂ at 37°C. DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin was used for cell culture. When the cells reached 80–90% confluence, they were digested with 0.25% trypsin-EDTA (HyClone; Cytiva) and washed twice with PBS. Total protein was extracted from cells lysed in RIPA buffer (MedChemExpress) supplemented with a protease inhibitor cocktail (APeXBIO Technology LLC) via ultrasonication (Q800R3; Qsonica LLC) at 20 kHz for 10 min at 4°C. The supernatant was collected by centrifugation at 13,000 x g for 10 min at 4°C. The protein concentration was assessed using a BCA kit.

Effects of serum on antigen-induced T-cell differentiation, proliferation and activation. As aforementioned, fresh PBMCs from healthy volunteers were isolated via density gradient centrifugation using Ficoll-Paque Plus. Subsequently, the PBMCs were labeled with 10 µM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; APeXBIO Technology LLC) for subsequent experiments. Briefly, the PBMCs were washed once with serum-free RPMI 1640 and were then incubated in serum-free RPMI 1640 supplemented with 10 µM CFSE at 37°C in the dark for 15 min. After incubation, the PBMCs were washed twice with 5% FBS in RPMI 1640 and once with serum-free RPMI 1640. A total of 1x10⁶/well CFSE-labeled PBMCs were seeded in a 12-well plate and cultured in RPMI 1640 medium supplemented with the following serums: 20% FBS, 20% HTS, 20% HS, 10% FBS + 10% HTS, 10% FBS + 10% HS or 10% HTS + 10% HS, and 30 µg/ml phytohemagglutinin (PHA; nonspecific activator of T cells; MilliporeSigma) or 20 µg/ml total protein antigen from Cal-27 cells was added to each group. After incubation at 37°C in an atmosphere containing 5% CO₂ for 3–14 days, all of the cells were harvested and analyzed. Briefly, serum and PHA-induced PBMCs were harvested and stained with anti-human CD3-APC (cat. no. FC0003; 1:100; Signalway

Antibody LLC), anti-human CD4-PE (cat. no. 300508; 1:100; BioLegend, Inc.) or anti-CD8-PE (cat. no. 344705; 1:100; BioLegend, Inc.) antibodies at 4°C for 30 min, and the cells were collected and analyzed with a CytoFLEX S (Beckman Coulter, Inc.). The differentiation and proliferation of T cells were analyzed with Kaluza 3.2 software (Beckman Coulter, Inc.).

In addition, PBMCs induced by serum and PHA as aforementioned were harvested and stained with the eBioscience™ Human Regulatory T-Cell Staining Kit #3 (cat. no. 88-8995; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, PBMCs were stained with anti-human CD4-FITC (1:100) and CD25-PE (1:100) at 4°C for 30 min and washed twice with PBS. Subsequently, 1 ml fixation/permeabilization working solution was added, and the mixture was incubated at room temperature for 1 h. The samples were subsequently washed three times with 2 ml 1X permeabilization buffer and resuspended in 100 µl 1X permeabilization buffer with 5 µl anti-human Forkhead box P3 (FOXP3) PE-Cyanine5 (1:20) at room temperature for 30 min. The samples were then washed twice with 2 ml 1X permeabilization buffer and detected with a CytoFLEX S. CD4⁺CD25⁺FOXP3⁺ Treg subsets were analyzed with Kaluza 3.2 software.

PBMCs were cultured in medium supplemented with FBS or HTS and stimulated with 20 µg/ml total protein antigen from Cal-27 cells for 14 days. The PBMCs were then harvested and stained with anti-human CD3-FITC (cat. no. 317306; 1:100; BioLegend, Inc.), anti-human programmed death 1 receptor (PD-1)-PE (cat. no. 329906; 1:50; BioLegend, Inc.), anti-human T-cell immunoglobulin and mucin-domain containing-3 (TIM3)-PE (cat. no. 345005; 1:50; BioLegend, Inc.) or anti-human CD69-APC (cat. no. 310909; 1:50; BioLegend) antibodies at 4°C for 30 min. Subsequently, the cells were collected and detected with a CytoFLEX S, and exhausted and activated markers on the T cells were analyzed with Kaluza 3.2 software.

cell stimulation and cytotoxicity assays use 16–24 h as the standard duration, since this period is the peak time for T-cell activation, T cells release cytokines, perforin and granzymes in response to antigen stimulation. On the basis of established methodologies in the field (33), an 18 h coculture duration was selected for the present study. PBMCs were cultured in medium supplemented with FBS or HTS and stimulated with 20 µg/ml total protein antigen from Cal-27 cells for 14 days. PBMCs were then harvested and labeled with CFSE. Subsequently, 7x10⁶ cells/ml PBMCs were cocultured with 7x10⁵ Cal-27 cells/ml in a 12-well plate for 18 h at 37°C and 5% CO₂. Images of the plates were captured every 30 min via the IncuCyte S3 ZOOM Live-Cell analysis system (Essen Bioscience) for a total of 10 h. Six images per well at x10 magnification were collected at each time point. After 10 h, the cells were collected and stained at 4°C for 30 min using the Annexin V-APC/PI kits (cat. no. KGA 1107-100; Nanjing KeyGen Biotech Co., Ltd.). Annexin V+PI+CFSE- apoptotic Cal-27 cells were detected with a CytoFLEX S and were analyzed using Kaluza 3.2 software. The representative gating strategies for flow cytometry data are shown in Appendix S1.

ELISA. PBMCs were cultured in medium supplemented with FBS or HTS and 20 µg/ml total protein antigen from

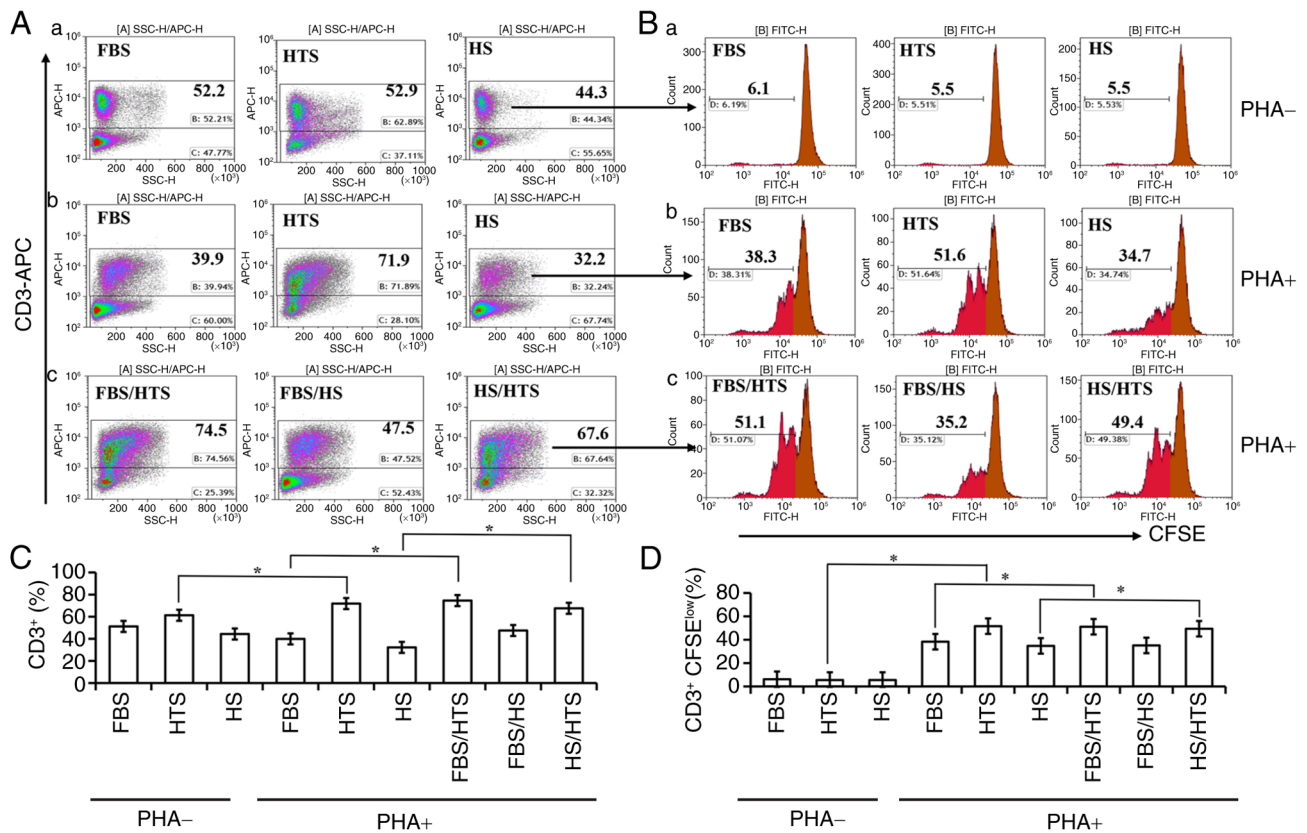


Figure 1. Effects of HTS on T-lymphocyte differentiation and proliferation. Peripheral blood mononuclear cells were pre-labeled with CFSE and cultured in medium containing 20% FBS, HTS or HS, with or without 30 μ g/ml PHA for 72 h. Cells were then collected, stained with CD3-APC and analyzed by flow cytometry using Kaluza 2.1 software. (A) Differentiation of CD3⁺ T cell subsets after stimulation with: (Aa) FBS, HTS or HS without PHA; (Ab) FBS, HTS or HS with PHA; (Ac) FBS + HTS, FBS + HS or HTS + HS with PHA. (B) Proliferation of CD3⁺ T cells after stimulation with: (Ba) FBS, HTS or HS without PHA; (Bb) FBS, HTS or HS with PHA; (Bc) FBS + HTS, FBS + HS or HTS + HS severally with PHA. (C) Percentage of CD3⁺ T-cell subsets in each group. (D) Percentage of CFSE^{low} CD3⁺ T-cell subsets in each group. The figures are representative of three independent experiments. Data are presented as the mean $\pm$ SD of each group. * P <0.05. CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; FBS, fetal bovine serum; HS, human healthy serum; HTS, human tumor serum; PHA, phytohemagglutinin.

Cal-27 cells for 14 days. The supernatants were collected, and the concentrations of IL-4 (cat. no. CSB-E04633h), IL-10 (cat. no. CSB-E04593h) and TGF- β (cat. no. CSB-E04725h-IS) were determined using ELISA kits (Cusabio Technology, LLC) according to the manufacturer's instructions. All of the experiments were performed in triplicate and the absorbance was measured at 450 nm; the standard curve and sample concentrations were calculated automatically using a microtiter plate reader (Varioskan Flash; Thermo Fisher Scientific, Inc.).

Data analysis. Statistical analysis was performed using GraphPad Prism 6.0 (Dotmatics). Comparisons of continuous variables between groups were conducted via unpaired Student's t-test (two-tailed). The association between different serums and T-cell differentiation and proliferation were analyzed using two-way ANOVA followed by the post hoc Tukey's multiple comparisons test. Before performing ANOVA and t-test, normality tests and tests for homogeneity of variance were conducted. The normality of the data was tested using the Shapiro-Wilk test, and the results revealed that all of the data conformed to a normal distribution (P >0.05). The homogeneity of variance was tested using Levene's test, and the results indicated that the variances of all the groups were homogeneous (P >0.05). Therefore, the data in this study

meet the assumptions of ANOVA and t-test, and the statistical methods used are reasonable and valid. P <0.05 was considered to indicate a statistically significant difference. Data are presented as the mean $\pm$ SD unless otherwise indicated.

Results

HTS promotes PHA-induced T-cell differentiation and proliferation. PBMCs were cultured in medium supplemented with 20% serum and 30 μ g/ml PHA for 72 h. The proliferation and differentiation of T cells were analyzed by flow cytometry. PHA-induced T-cell activation and proliferation are usually used to evaluate the function of T cells *in vitro*. HTS was added to the culture system of the PHA-induced PBMCs for 72 h. The percentage of the CD3⁺ T-cell subset was not significantly different between FBS, HTS and HS alone (without PHA) (Fig. 1Aa and C). After 30 μ g/ml PHA was added to the culture system, the percentage of CD3⁺ T cells in groups stimulated with HTS, FBS + HTS or HTS + HS were significantly increased compared with after the addition of FBS or HS alone or in combination (Fig. 1Ab, Ac and C). To explore the mechanism by which the number of CD3⁺ T-cell subsets increases, the proliferation of CD3⁺ T cells was analyzed under PHA stimulation with different serum samples and

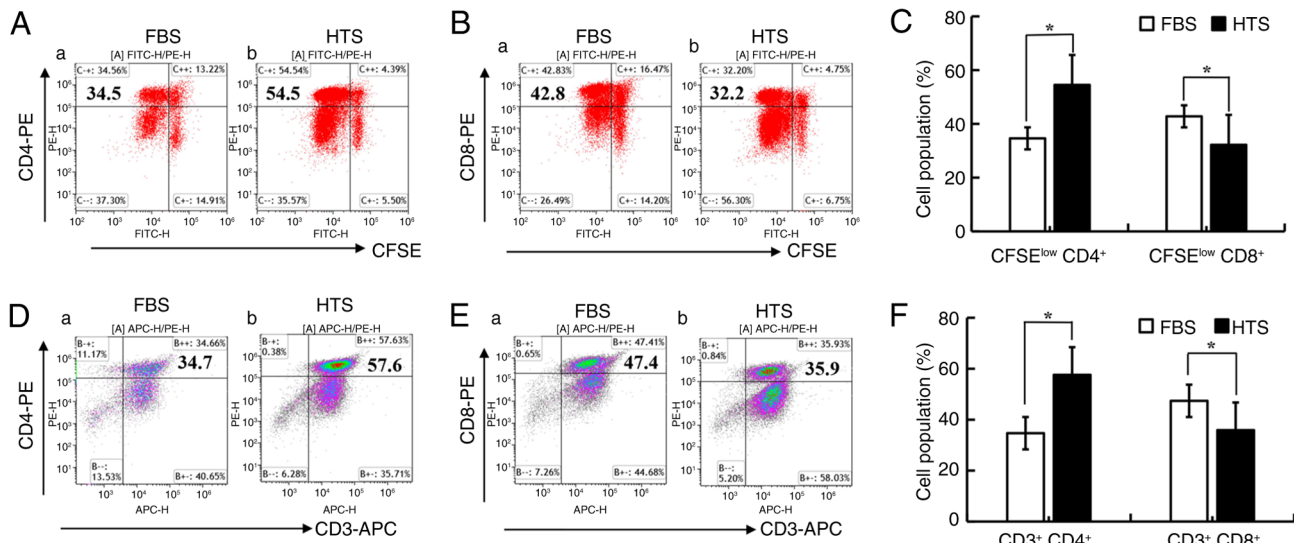


Figure 2. Effect of HTS on the proliferation and differentiation of CD4⁺ and CD8⁺ T-cell subsets. Peripheral blood mononuclear cells were pre-labeled with CFSE and cultured in medium containing 20% FBS or HTS, and 30 μ g/ml phytohemagglutinin for 72 h. Proliferation and differentiation of the T-cell subsets were analyzed by flow cytometry using Kaluza 2.1 software. (A) Proliferation of CD4⁺ T-cell subsets after stimulation with: (Aa) FBS and (Ab) HTS. (B) Proliferation of CD8⁺ T-cell subsets after stimulation with: (Ba) FBS and (Bb) HTS. (C) Proliferation of CD4⁺ and CD8⁺ T-cell subsets. (D) Differentiation of CD3⁺CD4⁺ T-cell subsets after stimulation with: (Da) FBS and (Db) HTS. (E) Differentiation of CD3⁺CD8⁺ T-cell subsets after stimulation with: (Ea) FBS and (Eb) HTS. (F) Differentiation of CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cell subsets. The figures are representative of three independent experiments. Data are presented as the mean $\pm$ SD for each group. * P <0.05; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; FBS, fetal bovine serum; HTS, human tumor serum.

their combination. Notably, there was no difference between cells treated with FBS, HTS and HS without PHA stimulation (Fig. 1Ba and D). When HTS was added to the PHA-induced PBMC culture system, there was a larger decrease in CFSE than in that without HTS (Fig. 1Bb, Bc and D), indicating that the proliferation of CD3⁺ T cells increased with HTS. These results indicated that HTS could promote PHA-induced CD3⁺ T-cell differentiation and proliferation. As shown in Fig. 1, there was no difference between FBS and HS groups; therefore, in the subsequent experiments, FBS was used as the only control.

HTS has different effects on the differentiation and proliferation of CD4⁺ T and CD8⁺ T-cell subsets. The previous results demonstrated that HTS could promote the proliferation and differentiation of PHA-induced CD3⁺ T cells. Subsequently, PBMCs were prelabeled with CFSE, stimulated with 30 μ g/ml PHA and cultured in medium supplemented with FBS or HTS for 72 h. The cells were collected, and the proliferation and differentiation of CD4⁺ and CD8⁺ T-cell subsets were analyzed with Kaluza software 2.1. Compared with that of FBS-treated cells (Fig. 2Aa), the proliferation of CD4⁺ T cells was significantly increased after PBMCs were stimulated with PHA and cultured in medium supplemented with HTS (Fig. 2Ab and C). By contrast, the CD8⁺ T-cell subset was significantly decreased (Fig. 2Ba, Bb and C). In addition to the proliferation of the T-cell subsets, the differentiation of CD4⁺ T cells increased, and the number of CD8⁺ T cells decreased after PBMCs were cultured in medium supplemented with HTS and 30 μ g/ml PHA compared with in the FBS group (Fig. 2D and C). These data indicated that the serum of patients with OSCC had different effects on PHA-induced CD4 and CD8 T cells, and can promote the proliferation and differentiation of CD4⁺ T cells and inhibit CD8⁺ T cells.

HTS promotes Treg differentiation. Tregs are one of the CD4⁺ T-cell subsets that have strong immune inhibitory functions in the body. Most studies have shown that in the peripheral blood of patients with cancer and in the tumor microenvironment, the percentage of Treg subsets is significantly increased (34-36). An increase in the Treg subset in the tumor microenvironment is an important mechanism of tumor immune escape. The present study demonstrated that HTS can promote the proliferation and differentiation of CD4⁺ T cells induced by PHA; therefore, Treg changes in CD4⁺ subsets were further analyzed. Compared with FBS (Fig. 3A and B), HTS significantly promoted the differentiation of T cells into Tregs (Fig. 3C and D).

HTS promotes tumor antigen-induced T-cell inhibition and exhaustion. During the progression of tumor formation, long-term and chronic tumor antigen stimulation, and the induction of T-cell exhaustion and inhibition are the main mechanisms of tumor immune escape. PBMCs were stimulated with Cal-27 total protein antigen for 14 days, and the cells and the supernatant were collected. The T-cell exhaustion markers TIM3 and PD-1, and the active T-cell marker CD69 were analyzed by flow cytometry. The concentrations of the immune-negative molecules, IL-4, IL-10 and TGF- β in the supernatant were assessed via ELISA. As shown in Fig. 4A and B, compared with FBS, HTS significantly promoted the levels of PD-1 and TIM3 in tumor-induced CD3⁺ T cells. On the other hand, the number of CD69⁺ active T cells was significantly decreased after HTS was added to the tumor antigen-stimulated PBMC system. In addition to the concentrations of exhausted T-cell markers, the concentrations of IL-4, IL-10 and TGF- β in the culture supernatant were significantly increased in the HTS group compared with those

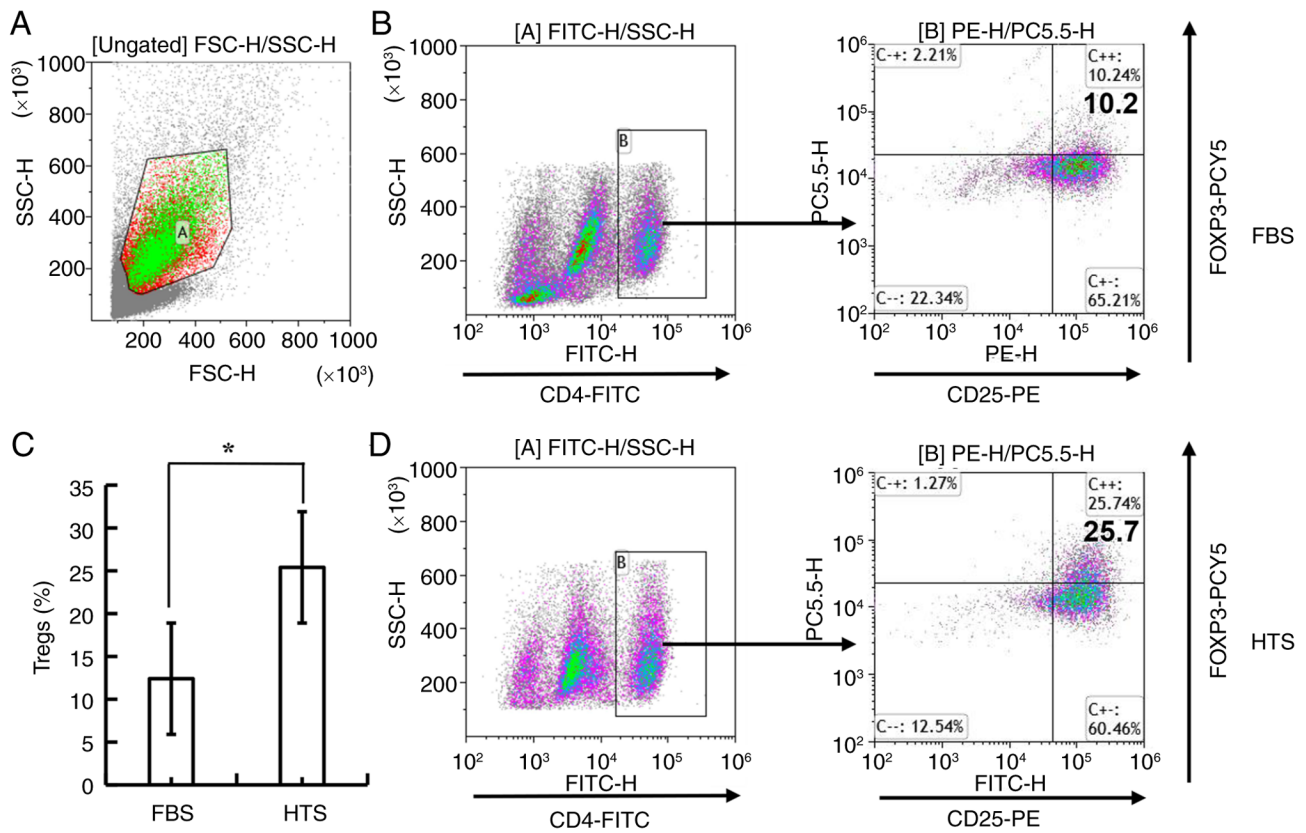


Figure 3. Effect of HTS on Treg differentiation. Peripheral blood mononuclear cells were stimulated with 30 μ g/ml PHA and cultured in medium supplemented with FBS or HTS for 72 h. The CD4⁺CD25⁺FOXP3⁺ Treg subset was analyzed by flow cytometry using Kaluza 2.1 software. (A) FSC and SSC gating to exclude debris, cell aggregates and dead cells, focusing on single, viable cells. (B) Treg cell subset in PBMCs stimulated with PHA and FBS. (D) Treg cell subset in PBMCs stimulated with PHA and HTS. (C) Percentage of Tregs in PBMCs after stimulation with PHA and different serums. The figures are representative of three independent experiments. Data are presented as the mean $\pm$ SD for each group. * $P < 0.05$ vs. FBS. Cy5, Cyanine5; FBS, fetal bovine serum; FOXP3, Forkhead box P3; FSC, forward scatter; HTS, human tumor serum; PHA, phytohemagglutinin; SSC, side scatter; Treg, regulatory T cell.

in the FBS group (Fig. 4D). These results indicated that the serum of patients with OSCC can promote T-cell exhaustion and the secretion of negative immune regulators induced by tumor antigens. As a result, the activation of antitumor T cells may be prohibited.

HTS inhibits the antitumor effect of T cells. The present results revealed that HTS not only promotes the differentiation of Treg cells but also inhibits the activation of T cells, promoting the expression of negative immune regulators. Tumor antigens and HTS or FBS pre-activated T cells were used as the effector cells and were cocultured with Cal-27 cells for 18 h. The antitumor effect was then analyzed by flow cytometry. Compared with that of FBS-treated T cells, the percentage of apoptotic Cal-27 cells (PI⁺Annexin V⁺CFSE⁻) was significantly lower after coculture with HTS-treated T cells (Fig. 5A and B). These data indicated that the antitumor effect of antigen-specific T cells was significantly inhibited by HTS. In the FBS group, when Cal-27 cells were cocultured with the tumor antigen-preactivated T cells for 1 h (Fig. 5C), the T cells began to move and adhere to the cancer cells, which is the first step in which T cells kill cancer cells, and after 6 h, most of the T cells had adhered to the cancer cells. Conversely, in the HTS group, there were no notable changes between the 1 and 6 h groups (Fig. 5D). These results indicated that HTS could inhibit the antitumor effect of tumor antigen-specific T cells.

Discussion

OSCC is an aggressive tumor (37,38); despite substantial advances in diagnosis, surgery and chemoradiotherapy, there has been little improvement in its prognosis in recent decades (37,39,40). The comprehensive elucidation of the molecular and cellular mechanisms of OSCC is imperative for early detection and treatment, and for improving patient survival.

The immune system of the body can usually eliminate cancer cells through the cancer immune cycle (41). First, cancer antigens are captured by antigen-presenting cells and the antigens are presented to naïve CD8⁺ T cells (42,43). T cells are activated and differentiated into effector CD8⁺ T cells (44). Next, the effector T cells enter the blood circulation and infiltrate the tumor site. Finally, effector T cells specifically recognize and eliminate tumor cells by releasing granzymes and perforins (45,46). However, during the progression of cancer, the remodeling of the cancer-directed immune escape microenvironment interrupts the immune cycle, and cancer cells escape the recognition and elimination by T cells (43,47).

One mechanism by which tumor cells evade the immune system involves cancer cells secreting and expressing a number of immunosuppressive molecules, such as PD-L1 and TGF- β , which induce severe immune cell hypoplasia, abnormality

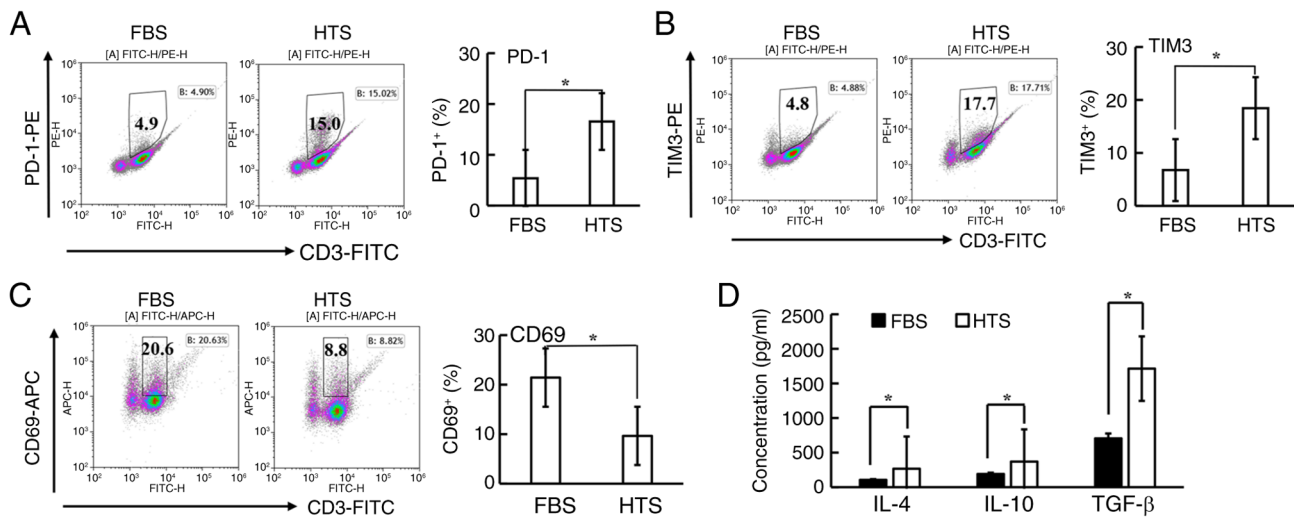


Figure 4. Effects of HTS on T-cell expression and secretion of immune molecules. Peripheral blood mononuclear cells were stimulated with tumor cell total protein antigen and cultured in medium containing FBS or HTS for 14 days. Supernatants were collected at day 14 to measure the concentrations of IL-4, IL-10 and TGF- β using ELISA kits. Cells were harvested at day 14, stained with anti-human CD3-FITC, PD-1-PE, TIM3-PE and CD69-APC, and the surface markers were analyzed by flow cytometry using Kaluza 2.1 software. Expression of (A) PD-1, (B) TIM3 and (C) CD69 on T cells. (D) Secretion of immune inhibitory molecules by HTS-induced T cells. Figures are representative of three independent experiments. Data are presented as the mean $\pm$ SD of each group. * $P < 0.05$ vs. FBS. FBS, fetal bovine serum; HTS, human tumor serum; PD1, programmed death ligand 1; TIM3, T-cell immunoglobulin and mucin-domain containing-3.

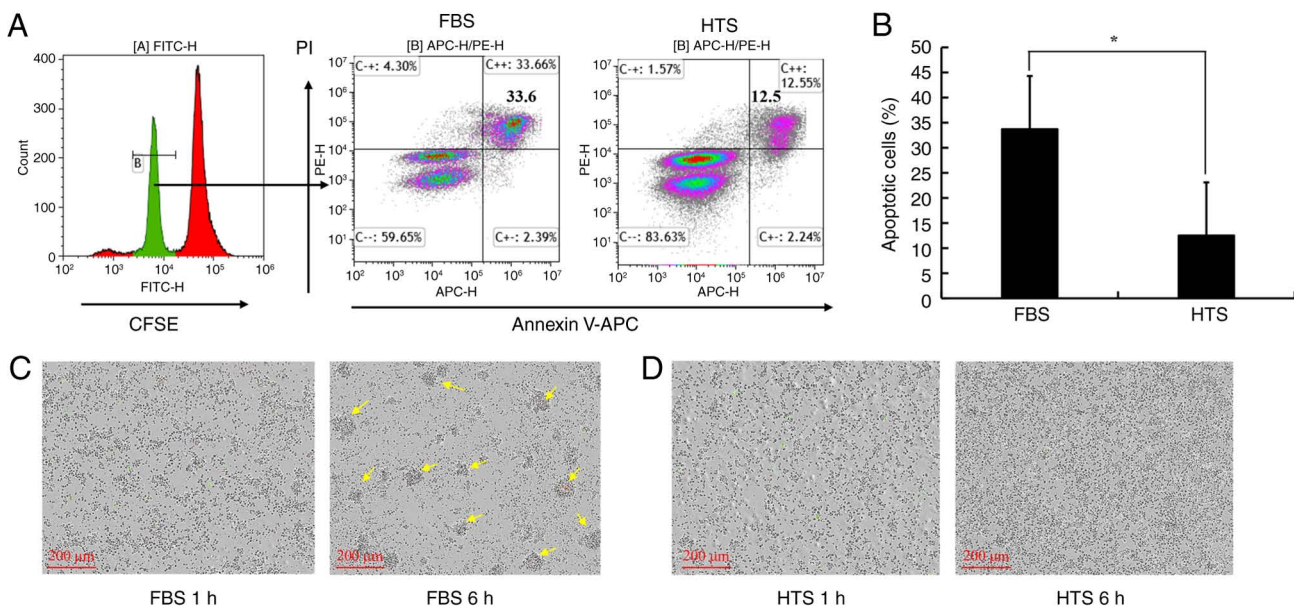


Figure 5. Effects of tumor antigen and HTS on T cell-mediated cancer cell death. PBMCs were stimulated with Cal-27 total protein antigen and cultured in medium containing 20% FBS or HTS for 14 days. PBMCs were collected and labeled with CFSE and co-cultured with Cal-27 cells at a ratio of 2:1 in the IncuCyte Rs3 system for 18 h. Cells were then collected and labeled with Annexin V-APC/PI kits. The Annexin V⁺PI⁺CFSE⁺ cell subset was analyzed by flow cytometry using Kaluza 2.1 software. (A) Apoptosis of cancer cells after co-culture with PBMCs. (B) Percentage of apoptotic cancer cells. (C) T cells were co-cultured with Cal-27 cells in medium containing 20% FBS for 1 and 6 h (yellow arrows indicate the adherence of T cells to cancer cells). (D) T cells were co-cultured with Cal-27 cells in medium containing 20% HTS for 1 and 6 h. Figures are representative of three independent experiments. Data are presented as the mean $\pm$ SD of each group. * $P < 0.05$ vs. FBS. CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; FBS, fetal bovine serum; HTS, human tumor serum; PBMC, peripheral blood mononuclear cell.

and dysfunction (48). Targeting immune inhibition and T-cell exhaustion in the tumor microenvironment, and rescuing the function of antitumor immune cells are the effective tumor biotherapy methods. Illustrating the mechanism by which the tumor immune system evades microenvironment remodeling and exploring specific targets for rescuing the function of T cells constitute an important area of cancer research.

Human serum contains a variety of proteins, peptides, fats, carbohydrates, growth factors, hormones and inorganic substances, and these substances can act as biomarkers of specific diseases (49,50). The serum of patients with cancer contains not only normal molecules, but also several biomarkers of cancer, such as TGF, IL, chemokines and tumor antigens, which are secreted by cancer cells, immune cells

and other somatic cells (24,26), most of which act as negative immune factors that inhibit immune cell differentiation and activation. For example, TGF- β is a critical molecule that promotes Treg differentiation, and tumor antigens (such as TP53 protein in OSCC) can persistently stimulate immune cells in a chronic manner and induce exhaustion (51-53). However, the underlying molecular mechanism is a potential future direction that remains to be studied. The current study investigated the ability of the serum of patients with OSCC to remodel the tumor immune escape microenvironment.

In the cancer immune cycle, the critical step is the activation of naïve CD8⁺ T cells and active CD8⁺ T cells, which are different from effector cells (54). In the tumor microenvironment, the CD4⁺ Treg subset is increased, and CD8⁺ T-cell exhaustion is a common characteristic of patients with cancer (55). This characteristic is also a mechanism of cancer immune escape. The present study collected HTS before clinical treatment, and PBMCs from healthy donors were stimulated with PHA and HTS for 72 h; FBS and HS were used as controls. PHA was selected as the stimulant because it is a classic polyclonal activator of T cells, which can nonspecifically activate T cells in PBMCs, inducing their proliferation and differentiation (56). PHA has been widely used in immunological research, and the experimental results obtained with this method have high reliability and comparability (57,58). Compared with FBS and HS, both HTS alone and combined with HS or FBS significantly promoted PHA-induced CD3⁺ T-cell proliferation and differentiation. However, there was no difference between HTS alone and the combination of FBS and HS. There was no difference between the FBS and HS groups. Notably, HTS had no effect on CD3⁺ T cells without PHA stimulation. PHA is a nonspecific polyclonal T-cell stimulator (58), which can promote human T-cell activation and proliferation nonspecifically via the PHA receptor on T cells (59). These results indicated that HTS may affect the process of CD3⁺ T-cell activation.

T cells are divided into CD4⁺ and CD8⁺ subpopulations according to their surface markers and functions (60). In the cancer immune cycle, CD8⁺ T cells are the major antitumor effector cells at the final step (61,62). They eliminate cancer cells via specific contact with them. CD4⁺ T cells regulate the activation, proliferation and effects of CD8⁺ T cells via the secretion of a series of cytokines, such as IL-2, IL-4 and IL-10 (63-65). The present study demonstrated that HTS promoted antigen-induced CD3⁺ T-cell proliferation and differentiation into CD3⁺CD4⁺ T cells but not CD3⁺CD8⁺ T cells. As a result, after PBMCs were stimulated with PHA and cultured in medium supplemented with 20% HTS for 72 h, the percentage of CD4⁺ T cells increased significantly compared with that of FBS-treated cells. On the other hand, the CFSE-labeled proliferation assay revealed that the CD4⁺ T cell subpopulation constituted the majority of the proliferating cells.

The Treg subset is the major subset that negatively regulates the T-cell subpopulation of CD4⁺ T cells (66,67). It can inhibit the differentiation and activation of CD8⁺ effector T cells (68,69). It also inhibits the immune response via the secretion of IL-10 and TGF- β , and the expression of high levels of IL-2R (70-72). Most studies have shown that the Treg subpopulation significantly increases in the tumor

microenvironment (73,74). In addition, an increase in the Treg subset in the tumor microenvironment is the hallmark of tumor immune escape and microenvironment remodeling (75). The present study demonstrated that HTS promoted antigen-induced Treg differentiation; when 20% HTS was added to the PHA-induced PBMC system, >25% of CD4⁺ T cells were Tregs, which was significantly greater than that in the FBS control group.

The present study revealed that without PHA, the serum of patients had no effect on T-cell proliferation or differentiation; this mechanism will be our major research focus in the future. It is hypothesized that naïve T cells do not proliferate or differentiate, whereas when PHA stimulates and activates T cells, the serum of patients with OSCC drives T cells to differentiate into Treg and T-cell exhaustion subsets. The limited impact of serum alone on T-cell proliferation and differentiation is due mainly to the fact that the activation of T cells requires dual-signal stimulation. In the absence of the first signal provided by antigens or polyclonal activators (such as PHA), it is difficult for components in the serum alone to effectively activate T cells. The relevant molecules in the serum may be able to better regulate the functions of T cells, such as promoting Treg differentiation and inducing T-cell exhaustion, only when T cells are in an activated state. This phenomenon indicates that, to some extent, the effects of serum on T cells depend on the preactivation state of T cells and that when the effects of serum on immune cells are studied, the activation state of T cells should be considered an important factor. In future research, we will further explore the differences in the effects of serum on T cells under different activation conditions and the underlying mechanisms involved.

Exhaustion of T cells is another characteristic and critical problem of tumor immune evasion (76). Targeting exhausted T cells and rescuing their function are the focuses of current tumor biotherapies (77,78). Dysfunction and high expression of immune checkpoint molecules are the major characteristics of exhausted T cells (77,79). It is well known that long-term and repeated stimulation by tumor antigens is one of the mechanisms by which T cells are exhausted (77,80,81). Therefore, total protein from the OSCC cell line Cal-27 was used as a tumor antigen and PBMCs from healthy donors were used as the target cells. PBMCs were cultured in medium supplemented with 20 μ g/ml tumor antigen and 20% HTS for 14 days to simulate long-term and repeated antigen stimulation *in vitro*. Compared with in the control group, HTS induced T cells to express high levels of the exhausted T-cell-related markers PD-L1 and TIM3. By contrast, the CD69⁺ active T-cell subset was significantly decreased. In addition to surface marker expression, the supernatants contained high concentrations of the immune negative regulatory factors TGF- β , IL-4 and IL-10. In addition to the expression of PD-L1 and TIM3, the killing effect of T cells was significantly decreased (Fig. 5). These results indicated that HTS could significantly promote tumor antigen-induced T-cell exhaustion. In subsequent studies, we plan to validate these findings using inhibitors of T-cell exhaustion markers (such as anti-PD-1/PD-L1 or TIM3 blocking antibodies).

The present study revealed that the serum of patients with OSCC can promote remodeling of the tumor immune escape microenvironment via the promotion of antigen-induced Treg

differentiation and T-cell exhaustion. It may be hypothesized that components such as IL-10 and TGF- β in the serum of patients with OSCC drive Treg differentiation and T-cell exhaustion by activating inhibitory signaling pathways and metabolic pathways. To further validate this hypothesis, we aim to conduct Olink Multiplex Assays to screen serum samples from patients with OSCC, combined with flow cytometric analysis of Treg differentiation and T-cell exhaustion. The present findings carry notable clinical implications, demonstrating that serum-mediated immunosuppression in patients with cancer demands greater attention in therapeutic strategies, particularly within cancer immunotherapy. In recent decades, despite the use of omics techniques in research, a number of potent biomarkers have been identified in the saliva and serum of patients with OSCC, but none of these biomarkers have been used clinically for diagnosis or prognostics prediction because of their specificity and sensitivity (82). For example, IL-6 is elevated in both oral inflammatory conditions and OSCC; and CYFRA 21-1 levels are increased in the serum of patients with OSCC, yet this biomarker may demonstrate false-positive in cases of pneumonia. Furthermore, a number of biomarkers fall below detectable limits during stage I OSCC, resulting in elevated false-negative rates (83-85).

The current findings indicated that biomarkers in the serum related to Treg differentiation and T-cell exhaustion have the potential to be used for the diagnosis and prognostic assessment of OSCC. In terms of diagnosis, detecting the levels of these biomarkers may help clinicians identify potential patients with OSCC at an early stage, improving the accuracy and timeliness of diagnosis. In terms of prognostic assessment, the dynamic changes in these biomarkers may reflect the treatment efficacy and disease progression of patients. For example, a significant decrease in biomarker levels after treatment indicates a good response to treatment and a favorable prognosis, whereas an increase may suggest disease recurrence or progression, allowing for clinicians to adjust treatment strategies in a timely manner, and improving the survival rate and quality of life of patients.

The results of the present study were obtained from *in vitro* serum experiments. To further validate the findings, we aim to conduct *in vivo* experiments in the future. Considering that the central mechanism of immune checkpoint blockade therapy involves blocking immune checkpoints and rescuing exhausted T cells, we aim to inject the serum of patients with OSCC and PBMCs into nude mice, and analyze T-cell differentiation and exhaustion in subsequent experiments. Subsequently, we aim to transplant an OSCC cell line, observe the formation of tumors and analyze immune cell subset infiltration to evaluate the tumor immune environment.

It may be hypothesized that serum-mediated immunosuppression represents a pan-cancer phenomenon. To test this hypothesis, we aim to conduct comparative studies using serum samples from patients with diverse tumor types. Serum samples from patients with lung cancer, liver cancer, ovarian cancer and breast cancer are being collected for further study. Using the same experimental methods as those in the present study, their effects on the differentiation of Tregs and the exhaustion of T cells in PBMCs can be observed. By comparing the differences in the effects of serum from patients with different types of cancer, we aim to clarify whether these

immunoregulatory effects are common, providing a theoretical basis for revealing the general mechanisms of tumor immune escape and developing broad-spectrum tumor immunotherapeutic strategies.

The key limitations of the present study include its cross-sectional design and lack of longitudinal serum data, limiting causal inference of OSCC progression dynamics. *In vitro* coculture models may not fully recapitulate the *in vivo* tumor microenvironment. Longitudinal clinical studies analyzing serial serum samples before and after treatment (including chemotherapy, radiotherapy and surgery) will clarify whether serum markers reflect treatment response or disease recurrence, as suggested. Translational validation using *in vivo* models (such as patient-derived xenografts) is necessary to identify the clinical relevance of *in vitro* findings. Multiomics integration (such as serum proteomics with transcriptomics/metabolomics data) to identify the mechanistic pathways involved in OSCC progression should also be conducted.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

PZ and YF conceptualized and designed the study. HC performed experiments. PZ, HC, JC, DL, BC and WH collected data. JC and YF performed data analysis and interpretation. HC and PZ wrote the manuscript. PZ and HC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Committee for Ethics Approval of the West China School of Stomatology, Sichuan University (approval no. WCHSIRB-D-2022-491). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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