

Original Article

Upregulation of CSF-1 is correlated with elevated TAM infiltration and poor prognosis in oral squamous cell carcinoma

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Received June 29, 2020; Accepted September 21, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: Mounting lines of evidence indicated that the “colony stimulating factor-1 (CSF-1)/tumor-associated macrophage (TAM)” signature plays an important role in the progression, invasion and metastasis of multiple tumors. However, the potential role of CSF-1/TAM in oral squamous cell carcinoma (OSCC) remains largely unknown. In the present study, the expression of CSF-1 from 99 OSCC specimens and its correlation with clinicopathological features and patient outcomes were investigated. Meanwhile, the correlation between CSF-1 expression and TAM infiltration was also explored. To investigate the potential effect of CSF-1 on tumor growth, nude mice were subcutaneously injected with Cal27 cell line and a small molecule inhibitor of CSF-1 (BZL945). The results showed that the high expression rate of CSF-1 (52%) was found in OSCC, and the upregulation of CSF-1 was closely correlated with lymph node metastasis and clinical stage. Additionally, there was a positive correlation between a high CSF-1 level and elevated TAM infiltration. The xenograft model study showed that CSF-1 signal blockade inhibited tumor growth, with a significant synchronous decrease in CSF-1 expression and TAM infiltration. Overall, our findings indicated that CSF-1 plays a crucial role in TAMs-mediated OSCC tumor progression and invasion. The “CSF-1/TAM” signaling axis may serve as a prospective target for anti-tumor therapy of OSCC.

Keywords: Macrophage colony stimulating factor-1, tumor-associated macrophages, oral squamous cell carcinoma, tumor microenvironment

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a progressive and invasive epithelial carcinoma that ranks fifth in 5-year prevalence and seventh in both incidence and mortality [1]. Oral squamous cell carcinoma (OSCC), the most common malignant tumor in the head and neck region, represents up to 90% of HNSCC cases [2]. Traditional OSCC treatment methods mainly rely on surgery, radiotherapy, chemotherapy and immunotherapy [3]. However, current treatment strategies have not met expectations in improving the survival rate of patients. The development of OSCC is a chronic and complex multistage process in which the involved signaling pathways, cytokines and reg-

ulatory mechanisms are still unclear [4]. Elucidating the mechanisms of potential signaling axes and establishing an experimental foundation for clinical anti-tumor targeted therapy may provide a new approach for treatment of OSCC.

Numerous epidemiological and molecular biological studies have shown that the malignant behavior of tumor is regulated by tumor microenvironment (TME). TME refers to the local tumor internal environment, which is composed of stromal cells, immune cells, bioactive mediators, blood vessels and lymphatics [5]. Infiltrating macrophages, which are located in or in close proximity to a tumor, are defined as tumor-associated macrophages (TAMs) [6], and account for 30%-50% of the tumor mass [7].

Notably, TAMs have plasticity and can be regulated by the microenvironment to differentiate into phenotypes with different structures and functions [8]. In vitro, TAMs can be divided into two phenotypes: classically activated macrophages (M1) and alternatively activated macrophages (M2) [9]. More specifically, the M1 phenotype primarily participates in antigen presentation and tumoricidal immune reactions [10], whereas the M2 phenotype is involved in tumor growth, metastasis, angiogenesis and therapy resistance [11]. In solid tumors, TAMs primarily show characteristics and functions related to M2 protumoral macrophages [12].

As mentioned above, TAMs differentiate differently in response to different stimuli and acquire a dynamic equilibrium. Considering the regulation of TAMs, macrophage colony stimulating factor-1 (CSF-1/M-CSF), one of the main cytokines involved in differentiation, proliferation, and functional regulation of macrophages, has gained incremental attention in recent decades [13]. Purified CSF-1 is a homodimeric, 45-90 kD glycoprotein generated by a variety of epithelial or mesenchymal derived cells [14]. Recently, its expression in tumor cells has been documented by in situ hybridization experiments [15]. CSF-1 functions by binding to the macrophage colony-stimulating factor-1 receptor (CSF-1R), which is a member of the type III tyrosine protein kinase receptor family [16] and is mainly confined to the mono-myelocyte lineage [17]. Among studies of different malignancies, although a few have linked high CSF-1 expression levels to good prognosis, the majority have correlated it with invasive and metastatic potential and reduced patient survival, including in liver cancer [18], breast cancer [19], ovarian cancer [20], endometrial cancer [21], and Hodgkin's lymphoma [22]. Therefore, CSF-1 acts as an important molecular contributor to cancer malignancy. Numerous studies have found that the TAM content in primary tumor decreases after CSF-1 signal blockade with neutralizing antibodies or small molecule inhibitors, but the effect of TAM attenuation on tumor growth is still controversial. Thus, it is essential to understand the heterogeneity of the "CSF-1/TAM" signature in different malignant tumors.

Despite reports demonstrating the presence of TAMs in OSCC, with M2 macrophages being endowed with immunosuppression and vascu-

larization functions to promote tumor progression [23], the role of the cytokine CSF-1 is not yet understood. To clarify these questions, in the present study, the expression level of CSF-1 in OSCC and its correlation with TAM infiltration and patient prognosis were investigated. Furthermore, the effects of CSF-1 signal blockade on tumor growth and TAM population were analyzed.

Materials and methods

Patients and specimens

Ninety-nine OSCC specimens were collected from patients hospitalized in the Department of Oral and Maxillofacial Surgery of the Affiliated Hospital of Stomatology, Nanjing Medical University from June 2011 to January 2017. None of the patients had received any preoperative radiation-chemotherapy. Patients with primary OSCC had undergone wide excision, with simultaneous elective dissection of the regional lymph nodes or classical radical neck dissection (56 males and 43 females; average age, 62 years; range, 35-91 years). The primary tumor sites were buccal mucosa (n=32), tongue (n=27), gingiva (n=19), jaw (n=4), palate (n=3), sublabial region (n=2), and other sites, such as the soft palate, oropharynx or mouth floor. Pathological classification was based on the World Health Organization (WHO) criterion, whereas TNM classification and clinical stage were based on the International Union against Cancer (UICC). The follow-up period ranged from 2 to 60 months (average: 42.8 months; median: 44 months). The H&E staining results for each case were reviewed to reconfirm the pathological diagnosis. This study protocol was approved by the Ethics Committee of the Affiliated Hospital of Stomatology, Nanjing Medical University.

Immunohistochemical staining

The specimens were fixed with 10% formalin and prepared in 4 µm-thick paraffin-embedded sections for immunohistochemical (IHC) staining. The sections were incubated separately with human reactive antibodies against CSF-1 (1:500; Abcam, Cambridge, MA, USA), CD68 (1:500; Abcam) and CD206 (1:500; Abcam) overnight at 4°C. All sections were then washed and incubated with secondary antibodies for 15 minutes. In addition, the reactants were

incubated with 3,3'-diaminobenzidine and counterstained with hematoxylin. A negative control was prepared for each staining.

Evaluation of immunoreactivity

CSF-1 evaluation: According to our previous study [24], the immunoreactivity was semi-quantitatively evaluated based on the staining intensity and the proportion of positive staining. The immunoreactive score (IS) = intensity score × proportion score. The intensity score was divided according to the dyeing intensity, ranging from 0 to 3, while the proportion score was divided according to the distribution range, varying from 0 to 4. The final IS ranged from 0 to 12, and immunoreactivity was used to divide the specimens into two groups: low expression (IS≤4) and high expression (IS>4) groups. The immunoreactivity was evaluated by a researcher who was not aware of the patient's clinical condition using a microscope independently at 100× magnification.

CD68 and CD206 evaluation: Anti-CD68 antibody was selected to mark all TAMs, regardless of their phenotype, while CD206 was specifically chosen to mark M2-type TAMs due to its high expression. Each specimen was evaluated using a microscope at low magnification (100×) to determine TAM clusters, and then, 10 high-power visual fields (HPFs; 400×) in the TAM-enriched regions were randomly selected and imaged. ImageJ software was used to calculate the percentage of positively stained areas in each image. After removal of the maximum and minimum values, the remaining 8 values were averaged as the final score for each specimen. Accordingly, the specimens were divided into low and high CD68 and CD206 expression groups according to cut-off values of 13.795%/HPF and 9.677%/HPF, respectively. The specimens were imaged and measured quantitatively by an experimentalist unaware of the clinical characteristics of the patients.

Xenograft tumor study

A total of thirty-two BALB/C nude mice (4-6 weeks, female) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and raised in the Animal Core Facility of Nanjing Medical University under specific-pathogen-free conditions. All the experimental procedures were approved by the Animal Eth-

ics and Welfare Committee of Nanjing Medical University. Cal27 cells (5×10^6) were s.c. injected into the right armpit of the nude mice. When the tumors were palpable, the mice were randomly divided into 4 groups: Vehicle, BLZ945, Cisplatin, and BLZ945+Cisplatin, with eight mice in each group. BLZ945 (Selleck, S7725), a highly selective small molecule inhibitor of the tyrosine kinase of CSF-1R, was dissolved in 1% CMC (Leagene Biotech Co. Ltd, Beijing, China) and delivered via daily oral gavage at a dose of 200 mg/kg body weight. Cisplatin (MCE, HY-17394), an anti-tumor platinum complex that nonspecifically acts on the cell cycle, was dissolved in saline and injected intraperitoneally once a week at a dose of 4 mg/kg body weight. In the BLZ945+Cisplatin group, the BLZ945 and Cisplatin solutions were given simultaneously. For the Vehicle group, 1% CMC was used to replace BLZ945 for gavage.

During the experimental intervention, the body weight and tumor size of the nude mice were measured every 3 days. The tumor volumes were calculated according to the following formula: $V = (ab^2)/2$, where a is the longest diameter, and b is the perpendicular height. The tumors were dissected and weighed 21 days later. IHC staining was carried out, and the sections were incubated with mouse reactive antibodies against CSF-1 (1:500; Abcam) and CD206 (1:250; Abcam). The immunoreactivity was analyzed via ImageJ software by an experimentalist unaware of the mouse disposal. To ensure the reliability of the results, the experiment was repeated once due to the occasional death of mice.

Statistical analysis

SPSS software (version 19.0, IBM, USA) was used for all statistical analyses. Correlations between the expression level of CSF-1, CD68 or CD206 and the clinicopathological parameters were analyzed with a χ^2 test, and Fisher's exact test or a Mann-Whitney U test was selected if necessary. In addition, correlations between CSF-1 and the CD68 or CD206 expression level were evaluated with a χ^2 test, and correlations between CD68 and CD206 were evaluated with a Pearson correlation test. The overall survival rate was analyzed using the Kaplan-Meier method and compared with a log-rank test. All values in the xenograft tumor

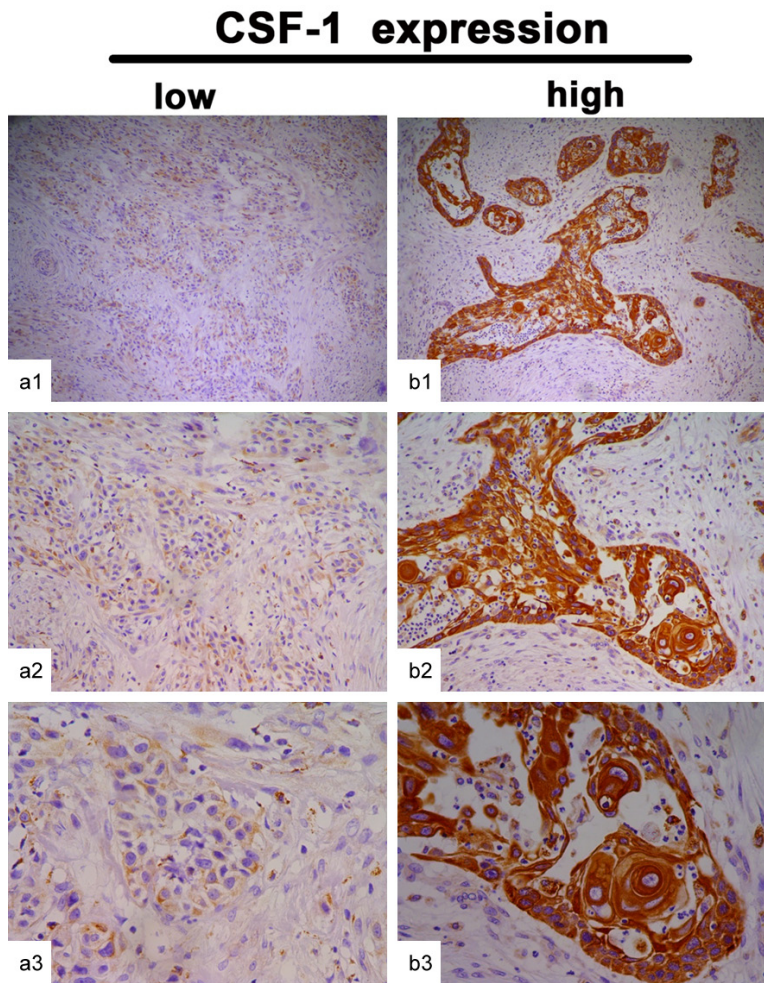


Figure 1. The CSF-1 expression pattern in OSCC was detected via immunohistochemistry. CSF-1 was mainly expressed in the tumor nest, and immunoreactive staining could be observed in the cell membrane and cytoplasm. Representative images of low (a1-a3) and high (b1-b3) expression levels are shown under different magnifications. (a1-b1, 100×; a2-b2, 200×; a3-b3, 400×).

experiment were recorded as the mean $\pm$ SEM, and one-way ANOVA followed by multiple T test was used for data analysis. $P < 0.05$ was considered statistically significant.

Results

Expression level of CSF-1 in OSCC and its relationship with clinicopathological parameters

In this study, the CSF-1 expression pattern in OSCC was first detected. The results showed that CSF-1 was primarily expressed in tumor nests, and immunoreactive staining could be observed in the cell membrane and cytoplasm. Representative images of low and high expres-

sion levels are shown in **Figure 1**.

Statistical analysis indicated that the rates of low and high CSF-1 expression were 48% (48 out of 99) and 52% (51 out of 99), respectively. Correlations between CSF-1 expression and clinicopathological characteristics in OSCC are shown in **Table 1**, and the results suggest that the expression level of CSF-1 was closely correlated with lymph node metastasis ($P = 0.007$) and clinical stage ($P = 0.017$).

Expression level of CD68 and CD206 in OSCC and their relationship with clinicopathological parameters

Immunoreactive staining of CD68 and CD206 can be observed in both “intratumoral” (tumor nests) and “peritumoral” (tumor stroma) regions, primarily in the cell membrane and cytoplasm of the TAMs but occasionally in tumor cells. Representative images of low and high CD68 and CD206 expression are shown in **Figure 2**.

In keeping with the study of Bagul et al [25], we did not distinguish between intratu-

moral and peritumoral areas when calculating the percentage of positive staining per HPF. The rates of low and high CD68 expression were 67% (66 out of 99) and 33% (33 out of 99), respectively. For CD206, the low and high expression levels were 88% (87 out of 99) and 12% (12 out of 99), respectively. Correlations between the expression of CD68 or CD206 and clinicopathological features in OSCC are shown in **Table 2**. Remarkably, the study showed that both CD68 and CD206 expression was significantly associated with lymph node metastasis ($P = 0.027$ and $P = 0.027$, respectively) and mortality ($P = 0.011$ and $P = 0.005$, respectively). Impressively, the expression level of CD206 was

Table 1. The correlations between the expression of CSF-1 and clinicopathological features in OSCC

Variable	No.	CSF-1 expression		P
		Low	High	
Sex				0.640
Male	56	26	30	
Female	43	22	21	
Age (years)				0.929
≤50	21	10	11	
>50	78	38	40	
Tumor location				0.444
Tongue	27	16	11	
Gingiva	19	5	14	
Buccal mucosa	32	16	16	
Palate	3	2	1	
Sublabial	2	1	1	
Jaw	4	2	2	
Others	12	6	6	
Tumor size				0.172
T1	16	11	5	
T2	46	20	26	
T3	21	12	9	
T4	16	5	11	
Lymph node metastasis				0.007*
N0	63	37	26	
N (+)	36	11	25	
Metastasis				0.495
M0	97	48	49	
M (+)	2	0	2	
Clinical stage				0.017*
I	14	10	4	
II	28	15	13	
III	29	14	15	
IV	28	9	19	
Pathological grade				0.359
I	87	44	43	
II	11	3	8	
III	1	1	0	
Local infiltration				0.371
Yes	78	36	42	
No	21	12	9	
Recurrence				0.084
No	82	43	39	
Yes	17	5	12	
Mortality				0.118
Live	95	48	47	
Dead	4	0	4	

No., Number of patients; N0, no lymph node metastasis; N (+), node metastasis. M0, no metastasis; M (+), metastasis. * and bold values signify P<0.05.

also closely correlated with tumor size (P=0.019).

Associations among the expression levels of CSF-1, CD68 and CD206 in OSCC

To investigate associations among the three indexes in OSCC, the expression levels of these proteins in each specimen were compared. Representative images of the immunoreactivity of CSF-1, CD68 and CD206 in adjacent sections of the same specimen are shown in **Figure 3**.

Notably, data analysis demonstrated that the expression level of CSF-1 was closely related to that of CD68 and CD206 (P=0.010 and P=0.029, respectively), and the Pearson correlation coefficients were 0.257 and 0.236, respectively (**Table 3**), suggesting that the expression of CSF-1 was positively correlated with the infiltration of TAMs in OSCC patients. In addition, the Pearson correlation test showed a significant positive correlation between the expression levels of CD68 and CD206, with a correlation coefficient of 0.336 (P=0.001, **Figure 4**).

Survival analysis

The overall survival rate was analyzed using the Kaplan-Meier method and compared with a log-rank test (**Figure 5**). This study showed that high expression of CSF-1 (P=0.049, **Figure 5B**), CD68 (P=0.006, **Figure 5C**), and CD206 (P=0.000, **Figure 5D**) had negative prognostic effects on overall survival. However, univariate and multivariate Cox regression analyses are not reported because the results were invalid, probably due to the occurrence of only 4 deaths among the ninety-nine patients in this study.

Blocking CSF-1 signal with BLZ945 suppressed tumor growth in xenograft tumor

The schedule of the mouse xenograft tumor study was summarized in **Figure 6A**. However, there was no significant difference in body weight among the different groups (**Figure 6B**). Images of the stripped solid tumors are presented in **Figure 6C**, which show that the tumor volumes are significantly reduced after blocking CSF-1 signal with BLZ945. In addition, BLZ945 combined with Cisplatin have a synergistic effect, resulting in the smallest tumor volumes. As shown in **Figure 6D**, the mean

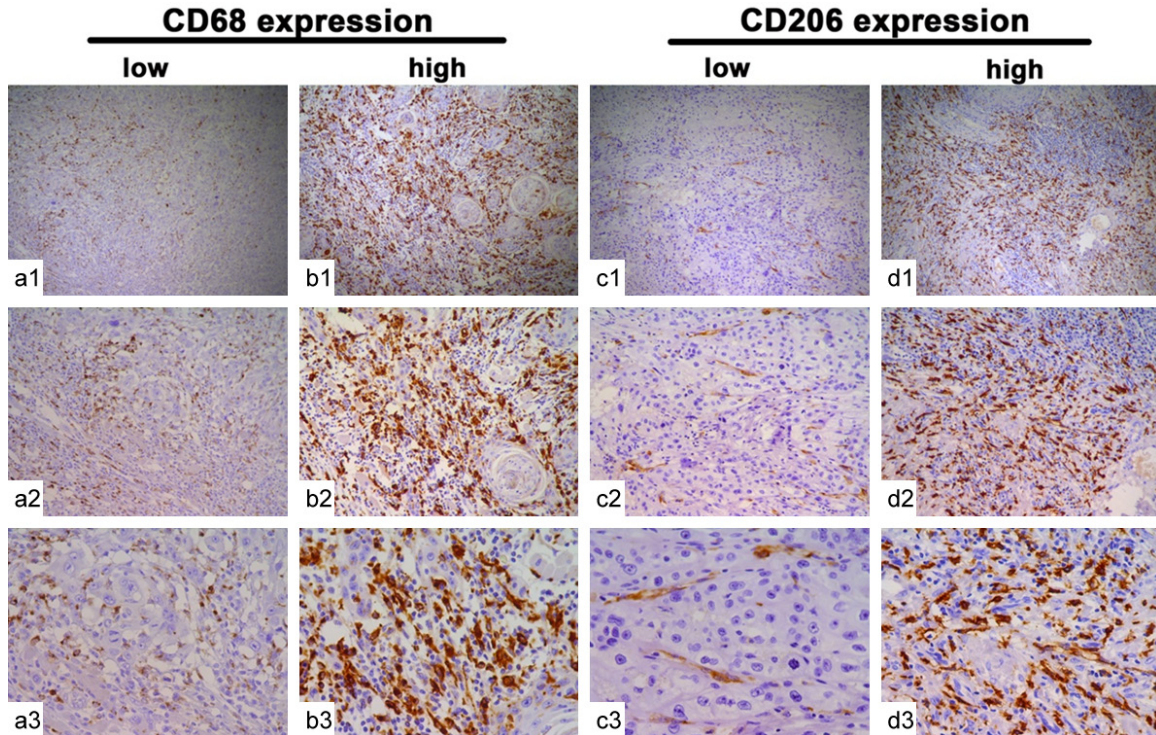


Figure 2. The CD68 and CD206 expression pattern in OSCC. Immunoreactive staining was observed in both “intra-tumoral” (tumor nest) and “peritumoral” (tumor stroma) areas, mainly in the cell membrane and cytoplasm. Representative images of low (a1-a3; c1-c3) and high (b1-b3; d1-d3) expression levels of CD68 and CD206 are shown under different magnifications. (a1-d1, 100×; a2-d2, 200×; a3-d3, 400×).

tumor volume was significantly lower in the BLZ945 group than in the Vehicle group [(112.7±6.9) mm³ vs (167.8±19.7) mm³; P=0.030], and a synergistic effect was achieved in the BLZ945+Cisplatin group [(105.8±6.8) mm³ vs (167.8±19.7) mm³; P=0.018]. Data analysis showed that the tumor weight in the BLZ945 group was significantly reduced compared with that in the Vehicle group [(0.0860±0.0064) g vs (0.1341±0.0175) g; P=0.033]. Meanwhile, tumor growth in the BLZ945+Cisplatin group was synergistically inhibited [(0.0789±0.0043) g vs (0.1341±0.0175) g; P=0.032] (**Figure 6D**); however, there was no significant difference in the Cisplatin group [(0.1316±0.0164) g vs (0.1341±0.0175) g; P=0.920]. Indeed, blocking CSF-1 signal with BLZ945 inhibited tumor growth by 36%, while BLZ945 combined with Cisplatin inhibited tumor growth by 41%.

Blocking CSF-1 signal with BLZ945 decreased the expression level of CSF-1 and the infiltration of M2-type TAMs

After blocking CSF-1 signal, tumor growth was significantly inhibited, and IHC was further car-

ried out to analyze the expression of CSF-1 and the TAM biomarker CD206. Representative images of CSF-1 and CD206 expression levels in different groups are shown in **Figure 7A** and **7B**, respectively. Data analysis demonstrated that the expression level of CSF-1 in the BLZ945 group was significantly lower than that in the Vehicle group [(3.647±0.455)% vs (7.071±1.205)%; P=0.029] (**Figure 7C**). Similarly, the expression level of CD206 in the BLZ945 group was significantly lower than that in the Vehicle group [(2.947±0.554)% vs (4.955±0.612)%; P=0.041] (**Figure 7D**). Moreover, the study also found that the expression levels of both CSF-1 and CD206 were significantly reduced in the BLZ945+Cisplatin group (P=0.022 and P=0.024, respectively). Nevertheless, there was no significant difference in the expression of CSF-1 and CD206 after Cisplatin monotherapy. Overall, CSF-1 signal inhibition with BLZ945 decreased the expression level of CSF-1 and the infiltration of M2-type TAMs. Thus, we speculate that the inhibition effect of BLZ945 on tumor growth may be related to the consumption of tumor-promoting M2-type TAMs.

Table 2. The relationships between the expression of CD68 or CD206 and clinicopathological features in OSCC

Variable	No.	CD68 expression			CD206 expression		
		Low	High	P	Low	High	P
Sex				0.886			0.625
Male	56	37	19		50	6	
Female	43	29	14		37	6	
Age (years)				0.602			0.275
≤50	21	15	6		17	4	
>50	78	51	27		70	8	
Tumor location				0.733			0.442
Tongue	27	18	9		22	5	
Gingiva	19	14	5		18	1	
Buccal mucosa	32	19	13		29	3	
Palate	3	3	0		3	0	
Underlip	2	1	1		1	1	
Jaw bones	4	2	2		4	0	
Others	12	9	3		10	2	
Tumor size				0.492			0.019*
T1	16	9	7		15	1	
T2	46	35	11		43	3	
T3	21	14	7		18	3	
T4	16	8	8		11	5	
Lymph node metastasis				0.027*			0.027*
N0	63	47	16		59	4	
N (+)	36	19	17		28	8	
Metastasis				0.551			1.000
M0	97	64	33		85	12	
M (+)	2	2	0		2	0	
Clinical stage				0.141			0.203
I	14	8	6		13	1	
II	28	25	3		26	2	
III	29	17	12		25	4	
IV	28	16	12		23	5	
Pathological grade				0.691			0.398
I	87	59	28		77	10	
II	11	6	5		10	1	
III	1	1	0		0	1	
Local infiltration				0.297			0.065
Yes	78	50	28		66	12	
No	21	16	5		21	0	
Recurrence				0.706			0.212
No	82	54	28		74	8	
Yes	17	12	5		13	4	
Mortality				0.011*			0.005*
Live	95	66	29		86	9	
Dead	4	0	4		1	3	

No., Number of patients; N0, no lymph node metastasis; N (+), node metastasis; M0, no metastasis; M (+), metastasis; * and bold values signify P<0.05.

Discussion

This study revealed that the upregulation of CSF-1 was closely related to lymph node metastasis, clinical stage, and poor prognosis in OSCC. Furthermore, there was a positive correlation between CSF-1 expression and TAM infiltration. Additionally, in the xenograft model, BLZ945 alone inhibited tumor growth by 36%, and BLZ945 combined with Cisplatin inhibited tumor growth by 41%. The inhibition effect of BLZ945 on tumor growth was speculated to be closely related to the consumption of M2-type TAMs.

Initially, it should be noted that the presence of CSF-1/CSF-1R signal is not a uniform feature in all cancer patients, and the expression level of CSF-1 in different tumor types ranges from 17% to 74% [8, 19]. In this study, the high rate of CSF-1 expression in OSCC patients was 52%. Consistent with our study, high CSF-1 expression levels have also been reported to correlate with higher pathological grade, more frequent metastasis, and worse prognosis in breast cancer [19], endometrioid carcinoma [21], ovarian epithelial tumor [20], leiomyosarcoma [26], and papillary renal cell carcinoma [27]. To verify the crucial role of CSF-1 in promoting tumor progression, accumulating evidence has suggested that in CSF-1-deficient homozygous mutant op/op mice, the development of invasive and metastatic carcinomas are delayed; once the CSF-1 signal was restored via transgene expression, the progression to invasive carcinoma was recovered [28]. Ne-

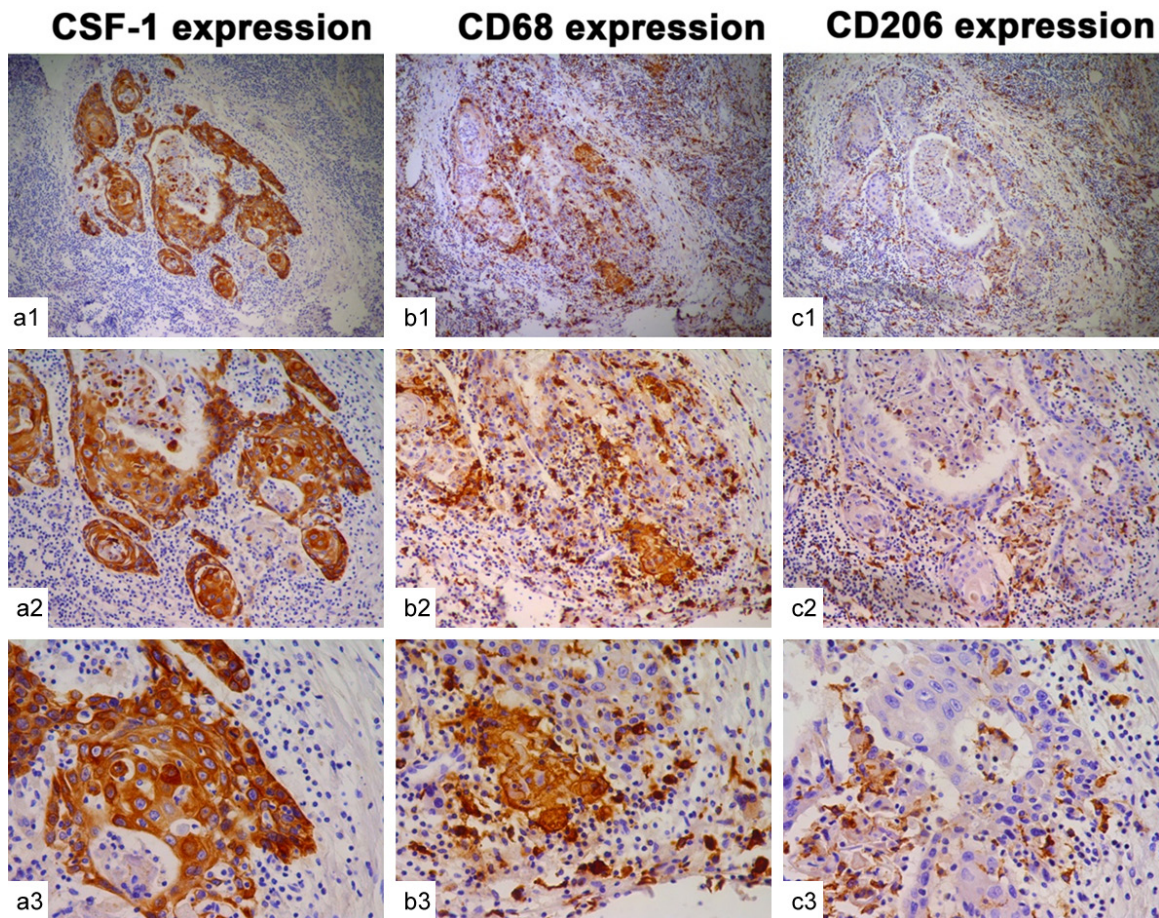


Figure 3. Representative images of the different staining levels of CSF-1 (a1-a3), CD68 (b1-b3) and CD206 (c1-c3) in adjacent sections of the same specimen are shown under different magnifications. (a1-c1, 100×; a2-c2, 200×; a3-c3, 400×).

Table 3. The relationships among CD68, CD206 and CSF-1 expression in OSCC

Variable	No.	CSF-1 expression		
		Low	High	P
CD68 expression				0.010*
Low	66	38	28	
High	33	10	23	
CD206 expression				0.029*
Low	87	46	41	
High	12	2	10	

No., Number of patients; * and bold values signify $P < 0.05$.

vertheless, Beck et al [29] analyzed the expression level of CSF-1 in eight published breast cancer data sets (n=982) and found that the CSF-1 level was associated with poor prognosis among low-grade tumors, but associated with improved prognosis among estrogen-

receptor-negative tumors and TP53 mutated tumors. Hence, to identify patients who might benefit the most from targeted therapy, it is necessary to reasonably stratify tumor patients and determine the patient groups in which CSF-1 is associated with poor prognosis.

To elucidate the specific mechanism underlying the interaction between CSF-1 and tumor cells in OSCC, we detected the expression of TAM biomarkers. Consistent with our findings, 80% of cancer studies have shown that elevated TAM infiltration is correlated with a poor prognosis, including in breast cancer [30], bladder cancer [31], prostate cancer [32], endometrial carcinoma [33], renal cell carcinoma [34], malignant uveal melanoma [35] and follicular lymphoma [36]. Indeed, the potential tumor-promoting effects of TAMs include promoting proliferation, invasion, migration and colonization of tumor cells; enhancing angiogenesis

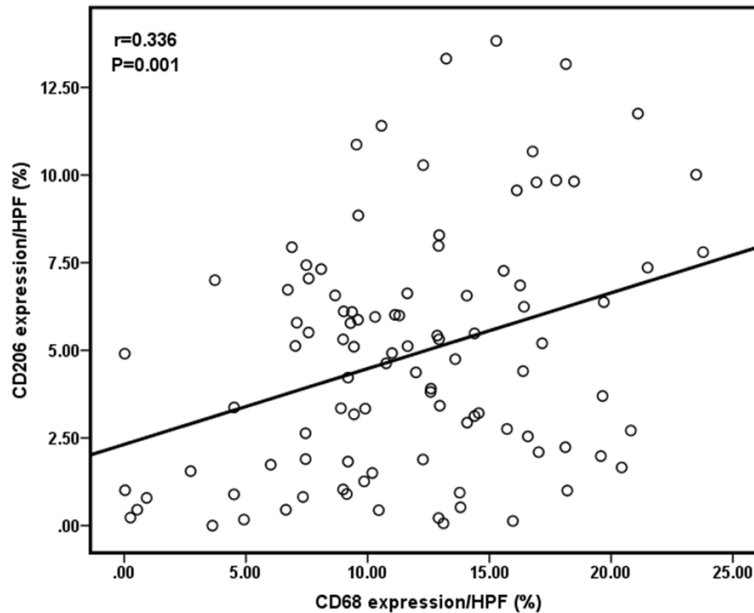


Figure 4. The correlation between CD68 and CD206 was examined. The expression level of CD68 in tumor tissues was positively associated with that of CD206 ($P=0.001$), and the correlation coefficient was 0.336. HPF, high-power visual field.

and providing nutritional function by releasing vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9); inhibiting the activity of cytotoxic T lymphocytes; and promoting tumor recurrence after anti-tumor therapies [8]. However, exceptions to this general trend have been found in gastric cancer [37] and colorectal cancer [38], in which the anti-tumor effect of TAMs can be achieved via the production of TAM-mediated reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS). Additionally, TAMs can also recruit and induce primitive T cells to differentiate into immunosuppressive phenotypes to inhibit tumor progression [8].

Impressively, in the present study, we found a positive correlation between the CSF-1 expression level and TAM infiltration in OSCC. Consistently, the principal role of CSF-1 in TAM reversal was previously reflected in the drastic decrease of macrophage frequency in the primary tumor at different stages of tumor progression towards malignancy in op/op mice; conversely, TAM infiltration increased significantly after recovery of CSF-1 signaling via transgenic expression [28]. It has been reported that CSF-1 can recruit monocyte-macrophage lineages to extravasate from peripheral cir-

ulation into tumor tissues; regulate the differentiation, proliferation and survival of TAMs [39]; and induce TAMs to polarize from the M1 type to the M2 type [40]. In addition to the above evidence, recent studies have suggested that the release of CSF-1 by tumor cells might also enhance the cytotoxicity of TAMs [39]. Overall, in solid tumors, CSF-1 can regulate the survival, proliferation, differentiation, migration and metabolism of the TAM population. Indeed, TAMs and tumor cells are interdependent in tumor migration and invasion, and therein CSF-1 acts as an important bioactive mediator: CSF-1 can promote the expression of epidermal growth factor (EGF) in TAMs, which can then enhance the invasive po-

tential of tumor cells; in turn, EGF can act on tumor cells by promoting CSF-1 expression, thus forming a positive feedback loop [41]. Disruption of this paracrine loop by blocking the “CSF-1/TAM” signal was found to be sufficient to inhibit the malignant behavior of tumor cells [42].

In the xenograft model, blocking CSF-1 signal with BLZ945 inhibited tumor growth by 36%. Considering the expression levels of CSF-1 and CD206 in different groups, we speculated that the inhibition effect of BLZ945 on tumor growth was closely related to the consumption of M2-type TAMs. In studies of multiple xenograft tumor models, including an AX xenograft osteosarcoma model [43], MDA-MB231 breast cancer model, and EL4 lymphoma model [44], it was found that the use of the monoclonal antibody AFS98 to block CSF-1 signal transduction significantly reduced TAM infiltration and the growth of primary tumors. In addition to monoclonal antibodies, it was found that the application of a small molecule inhibitor, BLZ945, could result in a large consumption of TAMs, and inhibit the growth of cervical and mammary carcinoma [45]. Similarly, it was reported that Ki20227 reduced the total TAM content in osteosarcoma and delayed the pro-

CSF-1/TAM signature in OSCC

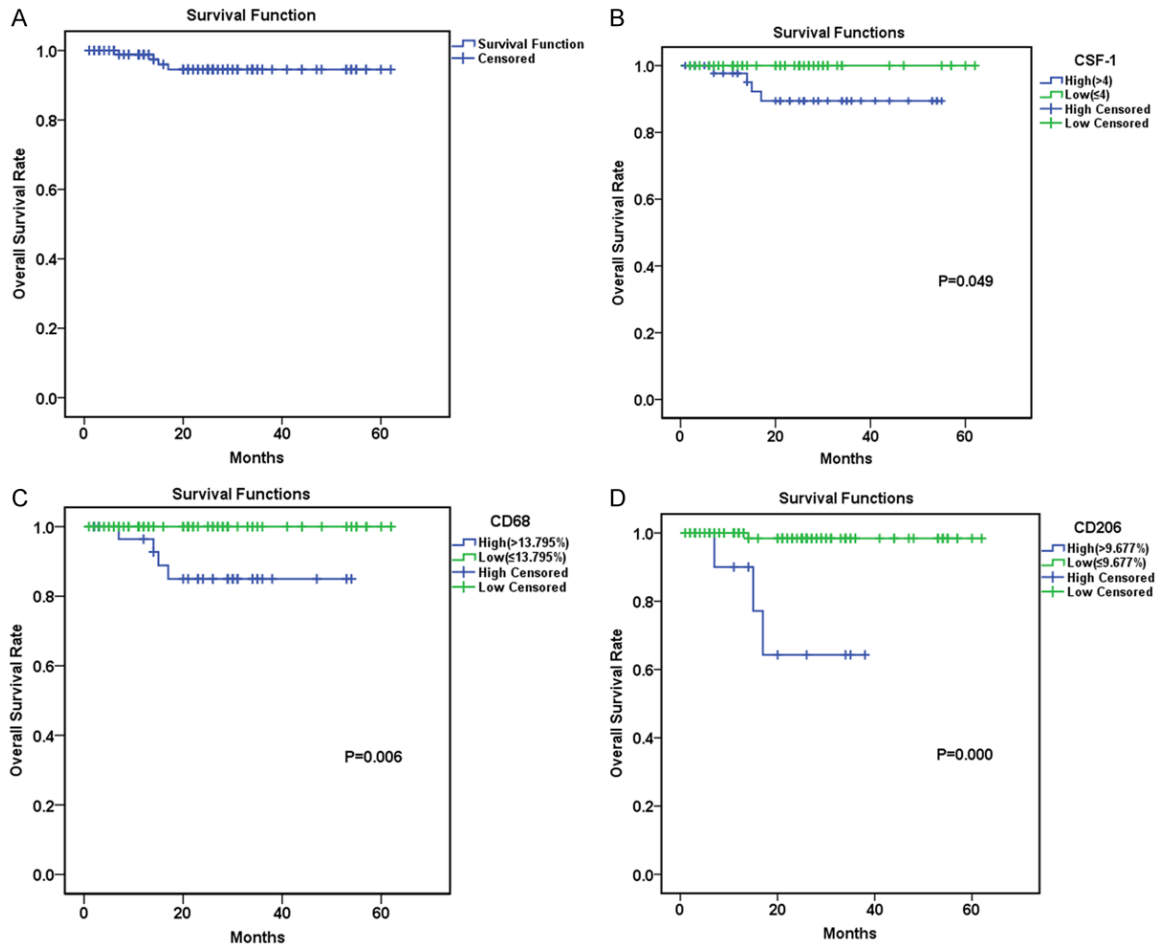


Figure 5. Kaplan-Meier plots with a log-rank test for overall survival (OS) of patients with OSCC. A. The overall survival curve for patients; B-D. High expression levels of CSF-1, CD68, and CD206 had negative prognostic impacts on the overall survival rate.

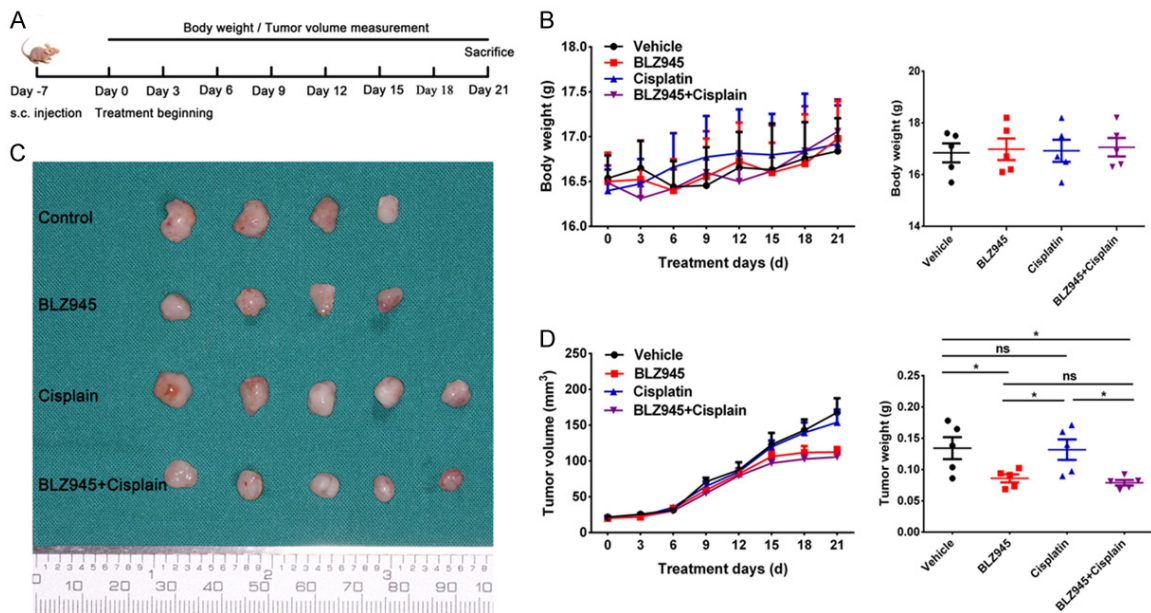


Figure 6. Blocking CSF-1 signal with BLZ945 suppressed tumor growth in xenograft tumor. A. The schedule for anti-tumor treatment, body weight and tumor volume measurement. B. Body weight measurements of mice in each group. C. Images of stripped xenograft tumor tissues after treatment with Vehicle, BLZ945, Cisplatin, or BLZ945+Cisplatin. D. Tumor volumes in each group during the treatment and tumor weight after 21 d. Data are presented as the means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

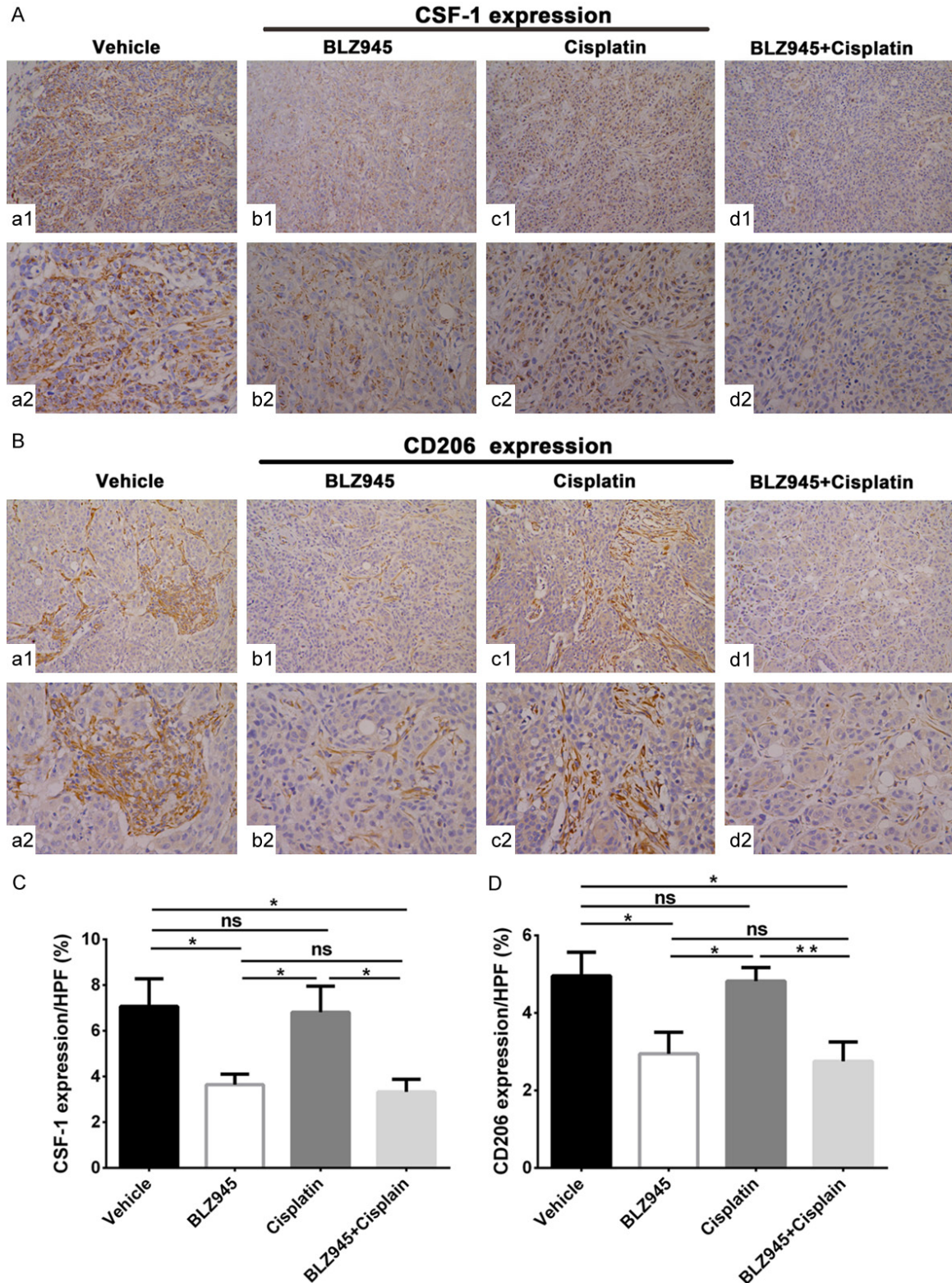


Figure 7. Blocking CSF-1 signal with BLZ945 decreased the expression level of CSF-1 and the infiltration of M2-type TAMs. A. Representative immunoreactive staining of CSF-1 in each group (a1-d1, 200×; a2-d2, 400×). B. Representative immunoreactive staining of CD206 in each group (a1-d1, 200×; a2-d2, 400×). C and D. The expression levels of CSF-1 and CD206 in each group were quantitatively analyzed. HPF, high-power visual field. Data are presented as the means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

liferation of tumor cells [43]. Moreover, blocking CSF-1 signal with PLX3397 resulted in depletion of M2-type TAMs and inhibited tumor growth in a melanoma mouse model [46]. In a spontaneous mammary tumor model, Tymoszuk et al [47] found that the application of another inhibitor, GW2580, resulted in a significant decrease in the number of MHC-II^{low} M2-type TAMs, but had no effect on MHC-II^{high} TAM phenotypes, indicating that CSF-1 signaling plays an important role in the maintenance and expansion of M2-type TAMs.

The possible effects of CSF-1 signal blockade on tumor progression include enhancing the phagocytosis/killing function of cancer cells; reducing the density of proliferating endothelial cells and delaying angiogenesis [48]; inhibiting the migration and invasion potency of TAMs and tumor cells [42]; promoting the activation of other tumor infiltrating lymphocytes in the microenvironment [49]; and reducing the occurrence of metastasis. Nevertheless, TAM consumption does not always inhibit tumor growth. In mesothelioma or lung cancer models, the application of M279 significantly reduced the number of TAMs but had no impact on tumor growth [50]. Additionally, the use of PLX-3397 or GW2580 as monotherapy resulted in significant consumption of TAMs in a prostate cancer model but had little effect on tumor growth [8]. Thus, the role of the “CSF-1/TAM” signaling axis in different tumor types is heterogeneous. Moreover, the synergistic effect of CSF-1 signal blockade combined with chemotherapy has been confirmed in the present study: BLZ945 combined with Cisplatin inhibited 41% of the tumor growth. The reason may be that chemotherapy drugs can increase the level of CSF-1, thereby enhancing the infiltration of immunosuppressive TAMs, when combined with CSF-1 signal blockade, this effect can be greatly weakened, thus antagonizing the chemoresistance of tumor [49]. In the present study, Cisplatin did not significantly inhibit tumor growth compared with the Vehicle group, possibly due to species heterogeneity, inappropriate dosage, or side effects, such as nephrotoxicity and hepatotoxicity.

In conclusion, CSF-1 plays important roles in OSCC progression and invasion, and TAMs might act as crucial cell mediators. This study showed that blocking CSF-1 signal to target TAMs may provide a promising breakthrough in clinical treatment of OSCC. However, this study did not analyze the effects of blocking therapy on the induction of different TAM activation states or on other tumor infiltrating lymphocytes, which were also meaningful.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (NSFC, no. 81972536), Key Project of Health Commission of Jiangsu Province (no. K2019-013), Natural Science Foundation of Jiangsu Province of China (no. BK20171483), the Jiangsu Provincial Medical Innovation Team (no. CXTD2017036), and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD, 2018-87). Thanks to Dr. Fang Shao for assisting us with statistical data presentation.

Disclosure of conflict of interest

None.

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