TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Diminished CD68⁺ Cancer-Associated Fibroblast Subset Induces Regulatory T-Cell (Treg) Infiltration and Predicts Poor Prognosis of Oral Squamous Cell Carcinoma Patients

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Although cancer-associated fibroblasts (CAFs) are crucial stromal cells, characterizing their heterogeneity is far from complete. This study reports a novel subset of CAFs in oral squamous cell carcinoma (OSCC), which positively expressed CD68, the classic marker of macrophages. The spatial and temporal distribution of the CD68⁺ CAF subset of OSCC ($n = 104$) was determined by CD68/actin alpha 2, smooth muscle (ACTA2+; α -SMA) immunohistochemistry of serial sections. The CD68⁺ α -SMA⁺ CAF subset was elevated from dysplasia to OSCC. Moreover, although both the tumor center and invasive front harbor an abundant CD68⁺ CAF subset, patients with low-CD68⁺ CAFs in the tumor center showed more recurrence after operation and shorter survival time, indicating the different function of CD68⁺ CAFs in tumor initiation and progression. Functional analysis in the OSCC–CAF co-culture system found knockdown of CD68 did not change the phenotype of CAFs, tumor growth, or migration. Unexpectedly, low-CD68⁺ CAFs were associated with aberrant immune balance. A high proportion of tumor-supportive Tregs was found in patients with low-CD68⁺ CAFs. Mechanistically, knockdown of CD68 in CAFs contributed to the up-regulation of chemokine CCL17 and CCL22 of tumor cells to enhance Treg recruitment. Thus, up-regulated CD68⁺ fibroblasts participate in tumor initiation, but the low-CD68⁺ CAF subset in OSCC is conducive to regulatory T-cell (Treg) recruitment in the tumor microenvironment and contribute to poor prognosis of OSCC patients. (*Am J Pathol* 2020, 190: 886–899; <https://doi.org/10.1016/j.ajpath.2019.12.007>)

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the head and neck. Local or regional recurrence of OSCC after operation is a major clinical difficulty^{1–3} and the main reason affecting the 5-year survival rate.^{2–4} Tumor heterogeneity manifests as tumor cell heterogeneity and stroma cell heterogeneity, which contribute to the inconsistent findings of the prognostic or diagnostic value of single biomarkers. Previous research found that immunity-regulated molecules TLR-3/7/9 had a distinct expression pattern in tumor cells and CAFs in OSCC. Low expression of TLR-3/7 in tumor cells or abundant expression of TLR-3/7 in CAFs predicts a good prognosis for OSCC patients.^{5,6} Despite extensive studies showing the tumorigenic role of

CAFs, recent studies have suggested an unexpected tumor-suppressing role of CAFs, because depletion of CAFs by targeting myofibroblasts accelerates pancreatic cancer in mouse pancreas cancer models.^{7,8} Thus, understanding the unique pathophysiological functions of different CAFs

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subsets and their roles in tumorigenesis would be helpful to develop a more accurate antineoplastic strategy targeting a specific CAF subset. Indeed, it was previously reported that IL-33⁺ CAF subsets showed an enhanced ability to promote tumor growth of OSCC.⁹ However, the heterogeneity of CAFs in OSCC remains to be further elucidated.

CD68 is a type I transmembrane glycoprotein with advanced glycation and belongs to the lysosome-associated membrane glycoprotein family, which is mainly associated with endosomal/lysosomal compartment.¹⁰ CD68 is a myeloid-specific surface marker abundantly expressed by macrophages.¹¹ Overexpressed CD68 and other macrophage markers in the OSCC stroma usually correlate with higher tumor grade and higher rate of lymph node metastasis.¹² Overexpression of CD68 in tumor tissue may indicate a prometastatic state and may be associated with poor prognosis.¹³ Thus, tumor-associated macrophage (TAM)-associated CD68 expression in the tumor center or stroma could be used as a potential predictive marker for prognosis of patients with cancer as shown in previous studies.¹²

Of note, accumulating studies have demonstrated a lesser extent of CD68 expression in nonmyeloid cells than myeloid cells.¹¹ CD68 was found to exist in several non-hematopoietic cell types including umbilical cord mesenchymal stem cells,¹⁴ fibroblasts,^{15,16} CD31⁺ endothelial cells, several tumor cell lines, and intimal smooth muscle cells of human arteries¹⁷ to different extents, whereas the functions of CD68 in these cell types are totally unknown. Interestingly, the CD68 level was remarkably elevated during the transformation from normal fibroblasts (NFs) to CAFs by transcriptome sequencing,⁹ but the clinical significance of CD68⁺ fibroblasts in the development of OSCC is unclear.

This study aimed to identify the clinical significance of CD68⁺ CAFs in OSCC by analyzing the spatial and temporal distribution of CD68 in stroma and evaluating the correlations between distinct expression of CD68⁺ CAFs with the clinicopathologic parameters and clinical outcomes. Furthermore, we investigated the function of CD68⁺ CAF in tumor growth, metastasis, and immune escape *in vitro* and *in vivo*.

Materials and Methods

Isolation and Culture of CAFs

CAFs used in this study were within five passages and were isolated from nine fresh OSCC tumor tissues immediately after surgical resection. NFs were isolated from normal tissues from the negative margin. Tissues were obtained and placed in PBS with 10% 100× penicillin/streptomycin solution for 2 hours for thorough sterilization. Minced tissues 0.2 × 0.2 × 0.2 cm³ were digested in tissue digestive enzyme containing collagenase and hyaluronidase at room temperature for 20 to 30 minutes. Afterward, the tissues were washed and then were plated into 25-cm² cell culture

flasks and cultured in 5 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum in an incubator (37°C, 5% CO₂). All fibroblasts were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The isolation and culture of primary cells were performed as described previously.^{18–20} Reliable phenotypic identification of primary cells has been conducted by immunofluorescence analysis, as described in our previous articles.⁹

Transient Transfection

According to the manufacturer's instructions, CD68 was overexpressed by plasmid pcDNA3.1-CD68 (GenePharma Biotechnology, Shanghai, China) in NFs and was knocked down by siRNA in CAFs using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA).

mRNA Isolation and Quantitative Real-Time PCR

Total mRNA was isolated from cultured CAFs and NFs using the TRIzol reagent method (Invitrogen; Thermo Fisher Scientific). RNA precipitates were dissolved in diethyl pyrocarbonate water, and the purity and concentration of RNA were measured by UV spectrophotometer. Thereafter, mRNA was transcribed into cDNA using RT-PCR kit (TaKaRa Bio, Kusatsu, Japan) following the manufacturer's instructions. The relative quantitative expression of CD68 (*CD68*), α -smooth muscle actin (actin α 2, smooth muscle; α -SMA), fibroblast-specific protein-1 (*S100A4*), N-cadherin (*CDH2*), and vimentin (*VIM*) were detected by quantitative real-time PCR using the SYBR Green I Master kit (Roche Applied Science, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a house-keeping gene for mRNA analysis. The sequences of the primers are as follows: *GAPDH*, 5'-GGAGCGA-GATCCCTCCAAAT-3' (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse); *CD68*, 5'-GGAAATGCCACGGTTCATCCA-3' (forward) and 5'-TGGGGTTCAGTACAGAGATGC-3' (reverse); *ACTA2*, 5'-AAAAGACAGCTACGTGGGTGA-3' (forward) and 5'-GCCATGTTCTATCGGGTACTTC-3' (reverse); *S100A4*, 5'-GATGAGCAACTTGGACAGCAA-3' (forward) and 5'-CTGGGCTGCTTATCTGGGAAG-3' (reverse); *VIM*, 5'-GACGCCATCAACACCGAGTT-3' (forward) and 5'-CTTTGTCGTTGGTTAGCTGGT-3' (reverse); *CDH2*, 5'-TCAGGCGTCTGTAGAGGCTT-3' (forward) and 5'-ATGCACATCCTTCGATAAGACTG-3' (reverse). All samples were tested strictly in accordance with three replicates. According to the threshold cycle (C_T) value of the specimen to be measured, using *GAPDH* as an internal reference, relative quantification method was used to express the relative expression of *CD68* by $2^{-\Delta\Delta C_T}$, wherein $\Delta\Delta C_T = (C_{T-CD68} - C_{T-GAPDH})$.

Western Blot Analysis

Total proteins from cultured fibroblasts were treated by radioimmunoprecipitation assay lysis buffer (Beyotime Biotechnology, Shanghai, China), supplemented with complete EDTA-free protease inhibitor (Beyotime Biotechnology). Lysates were cleared by centrifugation at $16,099.2 \times g$, and protein samples were loaded onto SDS/polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked for an hour at room temperature in 5% fetal bovine serum in Tris-buffered saline, 0.1% Tween 20. Samples were incubated with the corresponding dilution of GAPDH (1:10,000; Bioworld Technology, St. Louis Park, MN), CD68 (1:500; Abcam, Cambridge, UK), α -SMA (1:10,000; Abcam), N-cadherin (1:1000; Proteintech, Rosemont, IL), and vimentin (1:1000; Proteintech) overnight at 4°C. A second incubation was performed using horseradish peroxidase-linked secondary anti-rabbit or anti-mouse antibody (1:1000; Cell Signaling Technology, Danvers, MA), and enhanced chemiluminescence was detected by chemiluminescent reagent kit (Invitrogen).

Immunofluorescence

Cultured primary fibroblasts were plated into six-well plates at 70% confluence and then washed with PBS and fixed with 4% paraformaldehyde, after which they were incubated in 0.1% Triton X-100 to permeabilize the membranes. Frozen sections of *in situ* tissues were fixed with -20°C pre-cooling acetone and permeabilized with methanol. Cells and sections were blocked with nonspecific protein–protein interactions and then were incubated with primary antibody CD68 (1:200; Santa Cruz Biotechnology, Dallas, TX), α -SMA (1:200; Abcam), and cytokeratin (1:400; Dako, Glostrup, Denmark) at 4°C overnight. After washing three times, the samples were stained by Alexa Fluor 647–conjugated (1:100; Abcam), Alexa Fluor 555–conjugated (1:200; Abcam), and Alexa Fluor 488–conjugated (1:400; Abcam) secondary antibodies at room temperature for 30 minutes and then DAPI staining for 5 minutes. The stained samples were assessed by fluorescence microscopy.

Tissue Specimens and Tumor Cell Line

Formalin-fixed, paraffin-embedded tissues were collected from 104 patients with primary OSCC who had undergone curative resection between 2003 and 2013 at the Department of Pathology at Nanjing Stomatology Hospital. Clinical follow-up (median 58 months; range, 3 to 135 months) was complete for these patients. The research ethics committee of Nanjing Stomatology Hospital approved the study. All of the tissues of OSCC were evaluated according to World Health Organization classifications by two pathologists (Y.Y. and S.C.). The study had strict entry criteria: the patients must have primary OSCC without anti-cancer therapies including

chemotherapy and/or radiotherapy before surgery, and no distant metastasis prior to surgery. Lactating individuals, pregnant women, and patients who were diagnosed with other malignant diseases or certain autoimmune diseases were excluded. All patients were followed up until July 2018.

The experimental study groups included 30 oral leukoplakia patients with various histologic grades of oral epithelial dysplasia diagnosed by experienced pathologists (X.H., Y.Y., and S.C.). The 10 cases of normal oral tissues in this study were acquired from the excision margin that was at least 2 cm away from the tumor nest. The contralateral site of the tumor is not commonly available according to the ethics in OSCC. All normal and epithelial dysplasia tissues were diagnosed by hematoxylin and eosin staining. The OSCC tumor cell line Cal27 was obtained from Prof. Yvonne L. Kapila (Michigan University, MI) and cultured in DMEM (Sigma-Aldrich) containing 10% fetal bovine serum in an incubator (37°C , 5% CO_2). These cells were characterized by *Mycoplasma* detection, DNA fingerprinting, isozyme detection, and cell viability by the provider.

Immunohistochemistry

All immunohistochemistry was performed using the formalin-fixed, paraffin-embedded tissue sections. Tissue sections were deparaffinized and rehydrated. After antigen retrieval, the slides were incubated overnight at 4°C with the primary antibody directed against human CD68 (1:200; Santa Cruz Biotechnology), anti-CD3 (1:50; Abcam), anti-CD4 (1:200; Abcam), anti-CD8 (1:200; Abcam), anti-Ki-67 (1:200; Abcam), and anti-forkhead box protein 3 (FoxP3) (10 $\mu\text{g}/\text{mL}$; Abcam), then diaminobenzidine chromogen and nuclear staining was performed. All sections were then washed in PBS.

Quantification of Immunohistochemistry

All immunohistochemical markers were assessed by NIS-Elements software version 4.2.0 by two experienced pathologists (Y.Y. and S.C.). Three high-power fields were selected in the tumor center and the tumor invasive front, respectively, in each tumor sample. Results were expressed as the ratio of positive fibroblasts to overall fibroblasts per high-power field. The percentage of positive cells was divided into either low or high according to the median value.

CCK8 Assay

pcDNA-NC, pcDNA-CD68, siNC, or siCD68 (all from GenePharma Biotechnology) were transfected into primary NFs and CAFs as indicated. The conditioned medium (CM) was collected and used to treat Cal27 cells seeded in a 96-well plate. Cell proliferation was determined by the optical density at 450 nm at 0, 24, 48, 72, and 96 hours after CM treatment.

Wound Healing Assay

After overnight starvation with serum-free DMEM medium, straight-line scratches were made to Cal27 by a micropipette tip, and the cells were then washed to remove detached cells and debris. At 0 and 24 hours, photographs of the same area were taken, and the closure of the wound was measured after incubation of the serum-free CM from conditional NFs/CAFs.

Transwell Cell Migration and Proliferation Assays

The invasion and proliferation abilities of Cal27 cells were tested in Transwell plates with 8- μ m and 0.4- μ m pore sizes of the polycarbonate membranes, respectively. Details of the procedure are described in previous reports.²¹

Separation of PBMCs and the Migration Assay

Peripheral blood mononuclear cells (PBMCs) were prepared from healthy donor blood, and PBMCs were separated by density gradient centrifugation. Cells were cultured in DMEM for 2 hours at 37°C to separate adherent monocytes, and suspended cells were used for Transwell assay. Before the cell migration assay in 12-well plates, 500 μ L of CAF conditional supernatant was added to Cal27 in the lower chamber, and cultured for 24 or 48 hours. Two million human PBMCs were then added to the upper chamber with a 5- μ m pore size (Cat No. PIMP15R48; MilliporeSigma, Burlington, MA). Cells from the lower compartment were collected after migration for 8 hours, and Tregs were analyzed by flow cytometry.

Flow Cytometry

For the Tregs analysis, fluorescein isothiocyanate anti-human CD4 (BioLegend, San Diego, CA), PE anti-human CD25 (BioLegend), and Alexa Fluor 647 anti-human FoxP3 (BioLegend) were used to label Tregs, and fluorescein isothiocyanate anti-human CD68 (BioLegend) was used to mark CD68⁺ CAFs, which were quantified by flow cytometry on a BD FACSCalibur instrument (BD Biosciences, Franklin Lakes, NJ).

Data Analysis

All statistical analyses were performed with SPSS software version 18.0 (SPSS Inc., Chicago, IL). Subgroups of each immunostaining parameter were divided by median values. *U*-test, Pearson χ^2 test, Fisher exact test, or χ^2 test was used to compare clinicopathologic parameters of the patients. Kaplan-Meier analysis was applied to plot the survival curve. Log-rank test was used to compare patient survival between subgroups. A multivariate Cox proportional hazards regression model was applied to estimate the adjusted hazard ratio (HR) and 95% CIs, and to identify independent prognostic factors. Statistical significance was determined

by analysis of variance. Significance was generally accepted at *P* < 0.05 unless otherwise noted.

Results

CD68⁺ α -SMA⁺ CAF Subset Is Abundantly Present in Tumor Stroma of OSCC

Five paired NFs and CAFs from fresh OSCC tissues with reliable phenotypic identification were successfully isolated and cultured.⁹ Differentially expressed mRNAs with a fold change above 2 and a false discovery rate <0.05 were selected for further analysis. Five paired CAFs/NFs were selected for transcriptome sequencing and bioinformatics analysis, and it was found that expression of CD68 in CAFs was abundant and was significantly elevated during the transformation from NFs (Figure 1, A and B). Moreover, the mRNA relative expression of CD68 in nine paired NFs/CAF and protein expression in four pairs were validated (Figure 1, C–E), which was consistent with the results of the transcriptome sequencing results.

In order to more intuitively demonstrate the expression of CD68 in CAFs, immunofluorescence of the primary fibroblasts was performed. The results showed that α -SMA⁺ CAFs harbored more positive expression of CD68 than NFs (Figure 1F). Furthermore, the expression pattern of CD68 was determined in OSCC *in situ*, whereas the tumor nest was marked by pan-cytokeratin. The results indicated that α -SMA⁺ CAFs in tumor stroma also expressed CD68, but CD68 was absent in α -SMA⁺ fibroblasts in normal epithelium (Figure 1, G and H). These data indicate the existence of CD68⁺ α -SMA⁺ CAFs in tumor stroma of OSCC.

CD68⁺ Fibroblasts Subset Increases from Dysplasia to OSCC for Tumor Initiation

Next, the authors systematically analyzed the spatial and temporal distribution of CD68 during the development of OSCC. The expression pattern of CD68 was determined in normal mucosa, mild dysplasia, moderate dysplasia, severe dysplasia, and OSCC *in situ*, respectively, by immunohistochemistry. CAFs were detected as being dark brown by double staining of CD68 and α -SMA in serial sections accompanied with typical long spindle-shaped fibrocyte-like morphology to distinguish CAFs from other positively stained cells, especially TAMs. The results showed that CD68 stained positive in infiltrated macrophages and stroma fibroblasts, but was absent in epithelial cells. Consistent with previous research,^{22,23} the percentages of CD68⁺ TAMs were up-regulated from dysplasia to OSCC. Of note when compared with the normal tissues, the CD68⁺ CAFs were also up-regulated in stroma of severe dysplasia and OSCC *in situ* (Figure 2, A and B).

Importantly, in serial sections of OSCC, the immunohistochemistry of CD68 and α -SMA also showed that not all α -SMA⁺ CAFs express CD68 (Figure 2C). Consistent

Figure 1 Cluster of differentiation 68-positive (CD68⁺) actin alpha 2, smooth muscle-positive (ACTA2⁺; α -SMA⁺) cancer-associated fibroblasts (CAFs) subset is present in the tumor stroma of oral squamous cell carcinoma (OSCC). **A**: The whole-transcriptome resequencing and bioinformatics analysis in five paired CAFs and normal fibroblasts (NFs). **B**: The abundance of the transcriptome gene reveals the high expression of CD68 in CAFs. **C**: Gene expression of nine paired CAFs and NFs. **D**: Western blot of four paired CAFs/NFs. **E**: Quantitative analysis of gray values demonstrates the up-regulation of CD68 in CAFs. **F**: Immunofluorescence of isolated fibroblasts shows CAFs express higher CD68 and α -SMA than do NFs, whereas not all α -SMA⁺ CAFs express CD68. **G**: Immunofluorescence of resident fibroblasts shows no expression of CD68 in normal epithelium. **H**: Strong CD68 was expressed in α -SMA⁺ CAFs of OSCC *in situ*. **White dashed lines** indicate the junction between tumor nests and stroma. * $P < 0.05$, ** $P < 0.01$. Scale bars: 50 μm (F); 100 μm (G and H). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

with the findings in immunofluorescence, besides the CD68⁺ α -SMA⁺ CAFs, there also exist CD68⁺ macrophages that lack α -SMA expression, as well as α -SMA⁺ CD68⁻ CAFs around the tumor nest (Figure 2D). Thus, stroma fibroblasts present two subsets according to CD68 expression pattern in OSCC microenvironment.

The heterogeneity of the tumor microenvironment also manifests as the distinct localization of one cell type, thus, the authors assessed the clinical significance of CD68⁺ CAFs in the tumor center and invasive front, respectively. A wide distribution of CD68⁺ CAFs can be observed in the tumor center as well as in the tumor invasive front (Figure 2E). Moreover,

the mean proportion of CD68⁺ CAF in the tumor center and invasive front was 0.296 ± 0.011 and 0.300 ± 0.013 , respectively. However, there was no significant difference ($P = 0.990$) in the proportion of CD68⁺ CAFs between the tumor center and invasive front (Figure 2F).

OSCC Patients with Low-CD68⁺ CAFs in the Tumor Center Are Vulnerable to Recurrence

The current study further investigated the correlation between CD68⁺ CAFs and pathologic characteristics of 104 primary OSCC patients. The correlations between the

Figure 2 The spatial and temporal distribution of cluster of differentiation 68-positive (CD68⁺) fibroblasts in oral squamous cell carcinoma (OSCC). **A:** Immunohistochemistry in normal epithelial mucosa and different grades of dysplasia tissues including mild, moderate, and severe dysplasia compared with OSCC tissues in different kind of cells, including epithelial cells (**top row**), macrophages (**middlerow**), and fibroblasts (**bottom row**). **B:** Quantitative analysis of immunohistochemistry. **C:** Immunohistochemistry of CD68 and actin alpha 2, smooth muscle-positive (ACTA2⁺; α -SMA) in serial sections represents two subsets of cancer-associated fibroblasts (CAFs) in consideration of CD68 expression. **D:** Immunofluorescence images show CD68⁺ α -SMA⁺ CAFs, CD68⁻ α -SMA⁺ CAFs, and CD68⁺ α -SMA⁻ tumor-associated macrophages (TAMs). **E:** Immunohistochemistry of distinct expression of CD68⁺ CAFs in the tumor center and tumor invasive front compartments. **Black dashed lines** indicate the invasive front of tumor cells. **F:** Quantitative analysis of CD68 between the tumor center and invasive front revealed no significance between the two groups. * $P < 0.05$, ** $P < 0.01$. Original magnification: $\times 40$ (**A**, and **E**, **bottom row**); $\times 100$ (**C**, **top row**); $\times 400$ (**C**, **bottom row**); $\times 200$ (**D**); $\times 10$ (**E**, **top row**)

proportion of CD68⁺ CAFs in the tumor center and invasive front compartments, and clinicopathologic characteristics were analyzed (Table 1 and Supplemental Table S1). As is shown, CD68⁺ CAF in the tumor center is associated with tumor recurrence ($P = 0.022$) and inflammatory status ($P = 0.000$); patients with high-CD68⁺ CAFs revealed lower recurrence after operation and higher inflammatory infiltration.

Low-CD68⁺ CAFs in the Tumor Center Predict Poor Prognosis in OSCC Patients

All of the patients included in this study underwent complete surgical excision with negative margins. The mean follow-up time of these patients was 58 months with a range of 3 to 135 months. Kaplan-Meier survival analyses were used to determine the correlations between CD68 expression and survival rate including overall survival (OS), recurrence-free survival (RFS), recurrence-free survival (MFS), and disease-free survival (DFS). Statistical results showed that the higher

proportion of CD68⁺ CAFs in the tumor center was significantly correlated with better OS ($P = 0.029$) (Figure 3A), RFS ($P = 0.023$) (Figure 3B), and DFS ($P = 0.024$) (Figure 3D); nevertheless, no significant effect was found in MFS ($P = 0.305$) (Figure 3C). In addition, patients with higher CD68⁺ CAFs in the invasive front tended to have longer DFS ($P = 0.026$) (Figure 3H), whereas there was no influence on OS ($P = 0.069$) (Figure 3E), RFS ($P = 0.084$) (Figure 3F), or MFS ($P = 0.105$) (Figure 3G).

As shown in Table 2 along with Supplemental Tables S2–S4, the prognostic value of each clinicopathologic feature was analyzed in univariate and multivariate analyses. To define the prognostic values of CD68⁺ CAFs in different regions, univariate analyses revealed that age, smoking habit, tumor stage, differentiation, predominant pattern of invasion, worst pattern of invasion, and treatment strategy were not significantly associated with OS/RFS/MFS/DFS ($P > 0.05$), but CD68⁺ CAF infiltration in the tumor center was shown to be a protective factor for the OS ($P = 0.034$), RFS ($P = 0.035$), and DFS ($P = 0.031$) of OSCC patients.

Table 1 Correlations between CD68 Expression in the Tumor Center and Clinicopathologic Characteristics in OSCC Patients

Characteristic	CD68 in tumor invasive front			χ^2	P	OR	95% CI
	Total, n (%)	Low cases, n (%)	High cases, n (%)				
Sex							
Male	52 (50.0)	30 (57.7)	22 (42.3)	2.462	0.117	1.860	0.854–4.048
Female	52 (50.0)	22 (42.3)	30 (57.6)				
Age, years							
<60	58 (55.8)	26 (50.0)	32 (61.5)	1.403	0.236	0.625	0.287–1.363
≥60	46 (44.2)	28 (50.0)	20 (38.5)				
Smoking habit							
No	80 (76.9)	40 (76.9)	40 (76.9)	0.000	1.000	1.000	0.402–2.490
Yes	24 (23.1)	12 (23.1)	12 (23.1)				
Tumor stage							
I–II	62 (59.6)	31 (59.6)	31 (59.6)	0.000	1.000	1.000	0.457–2.189
III–IV	42 (40.4)	21 (40.4)	21 (40.4)				
PPOI							
I–III	79 (76.0)	37 (71.2)	42 (80.8)	1.316	0.251	0.587	0.235–1.465
IV–V	25 (24.0)	15 (28.8)	10 (19.2)				
WPOI							
I–III	60 (57.7)	28 (53.8)	32 (61.5)	0.630	0.427	0.729	0.334–1.592
IV–V	44 (42.3)	24 (46.2)	20 (38.5)				
Inflammatory status							
Mild	60 (57.7)	46 (88.5)	14 (26.9)	40.339	0.000***	20.81	7.293–59.374
Moderate–severe	44 (42.3)	6 (11.5)	38 (73.1)				
Differentiation							
Well	45 (43.3)	20 (38.5)	25 (48.1)	0.979	0.322	0.675	0.309–1.472
Moderate–poor	59 (56.7)	32 (61.5)	27 (51.9)				
After-surgery local recurrence							
No	90 (87.5)	41 (78.8)	49 (94.2)	5.283	0.022*	0.228	0.060–0.874
Yes	14 (12.5)	11 (21.2)	3 (5.8)				
After-surgery distant metastasis							
No	93 (89.4)	45 (86.5)	48 (92.3)	0.915	0.339	0.536	0.147–1.954
Yes	11 (10.6)	7 (13.5)	4 (7.7)				

Total $n = 104$.

* $P < 0.05$, *** $P < 0.001$.

OR, odds ratio; PPOI, predominant pattern of invasion; WPOI, worst pattern of invasion.

Figure 3 Kaplan-Meier survival curves show correlations between cluster of differentiation 68-positive (CD68⁺) cancer-associated fibroblasts (CAFs) and prognosis of oral squamous cell carcinoma (OSCC) patients in the tumor center (**top row**) and invasive front (**bottom row**). Overall survival (OS) (**A** and **E**), recurrence-free survival (RFS) (**B** and **F**), metastasis-free survival (MFS) (**C** and **G**) and disease-free survival (DFS) analysis.

Meanwhile, CD68⁺ CAF infiltration in the invasive front could be a protective factor for the DFS ($P = 0.033$). However, multivariate analyses implicated that expression of CD68⁺ CAFs was not an independent risk factor for OS/RFS/MFS/DFS, either in the tumor center or in the invasive front of OSCC patients.

Univariate analysis revealed that inflammatory status was relevant to OS with a $P = 0.047$ (HR: 0.460; 95% CI, 0.214–0.990). When the inflammatory status was included in the Cox proportional hazards analysis, the P value of CD68⁺ CAFs in the tumor center was 0.655 (HR: 0.794; 95% CI, 0.289–2.182), but when this factor was excluded in multivariate analysis, the value was significantly decreased to 0.464 (HR: 0.702; 95% CI, 0.272–1.812). These findings suggest that inflammatory status might interact with CD68⁺ CAFs. Indeed, the authors found that Tregs could be recruited by CD68⁺ CAFs, which participated in inflammation and immunity regulation.

Inhibition of CD68⁺ CAFs Shows No Effects on Tumor Proliferation and Invasion

CAFs serve as an important component in tumor microenvironment and are mainly derived from resident NFs. The authors explored the function of CD68 during the NF/CAF transformation. Thus, CD68 expression in NFs and CAFs was knocked down separately, and CAF phenotypes were evaluated by determining the expression of CAF classical molecular markers such as α -SMA, FSP-1, N-cadherin, and vimentin. The interference rate of siRNAs was stable up to 70%, and overexpression of CD68 in NFs was also successful. However, overexpression of CD68 in NFs had no effect on the expression of α -SMA, FSP-1, N-cadherin, or vimentin; and CD68 inhibition in CAFs could change the

expression of CAF phenotype markers (Figure 4, A–C), indicating that CD68 showed no relevance to the malignant transition of NFs to CAFs.

Moreover, the authors estimated whether CD68 expression in CAFs changed their tumor supportive functions. The conditioned medium obtained from NFs/CAFs was then added to Cal27 cells in a wound healing assay. Modulation of CD68 in NFs/CAFs did not significantly affect the migration function of Cal27, which was consistent with the result of the Transwell migration assay (Figure 4, D and E). In CCK-8 and Transwell proliferation assays, it was found that CD68 modulation in NFs/CAFs had little effect in inducing Cal27 proliferation (Figure 4, F and G).

Low CD68⁺ CAFs Accompany High Treg Infiltration in OSCC Patients

CAFs have been demonstrated to participate in induction of immunosuppression and promotion of tumor evasion from immune surveillance by producing cytokines and growth factors to directly or indirectly influence the behavior of malignant cells.²⁴ Because CD68 is a pan-macrophage marker that participates in antigen presentation between active immune cells, the authors further estimated the correlation between CD68⁺ CAFs and lymphocyte infiltration, along with Ki-67, in OSCC. The immunohistochemistry analysis indicated that CD68 expression was not related to Ki-67 in tumor tissue, proving that CD68⁺ CAFs was not relevant to OSCC proliferation, but CD68⁺ CAFs were accompanied with more CD3⁺ and CD4⁺ T cells. Moreover, high Foxp3⁺ cells were found in patients with low CD68⁺ CAFs, which implies that diminished CD68⁺ CAFs might participate in immunosuppression (Figure 5, A and B), which is consistent with the finding that patients with high expression of Foxp3 exhibited

Table 2 Cox Proportional Hazards Model of Overall Survival for Clinicopathologic Parameters in OSCC Patients

Parameter	Univariate			Multivariate		
	HR (95% CI)	95% CI	P	HR	95% CI	P
Sex						
Male vs female	1.728	0.861–3.487	0.125	2.048	0.898–4.670	0.088
Age						
<60 vs ≥60 years	1.743	0.863–3.442	0.115	1.037	0.998–1.078	0.063
TNM stage						
I–II vs III–IV	1.372	0.806–1.623	0.363	1.480	0.703–3.115	0.302
Differentiation						
Well vs moderate–poor	1.585	0.834–3.297	0.213	1.448	0.666–3.150	0.350
Smoking habit						
No vs yes	0.778	0.343–1.824	0.557	0.558	0.187–1.666	0.296
PPOI						
I–III vs IV–V	1.175	0.545–2.535	0.681	0.984	0.332–2.916	0.977
WPOI						
I–III vs IV–V	1.099	0.553–2.183	0.788	1.059	0.390–2.875	0.910
Inflammatory status						
Mild vs moderate–severe	0.460	0.214–0.990	0.047*	0.724	0.242–2.169	0.565
Treatment modality						
Sole vs combined	0.556	0.133–2.328	0.422	0.664	0.129–3.426	0.625
CD68 in tumor center						
Low vs high	0.457	0.222–0.943	0.034*	0.794	0.289–2.182	0.655
CD68 in invasive front						
Low vs high	0.525	0.258–1.066	0.075	0.955	0.364–2.504	0.925

**P* < 0.05.
HR, hazard ratio; PPOI, predominant pattern of invasion; WPOI, worst pattern of invasion.

a worse prognosis (*P* = 0.054 by log-rank test and 0.047 by Breslow test) (Figure 5C).

Diminished CD68⁺ CAFs Up-Regulate the CCL17/22–Treg Recruitment Axis in Tumor Cells

CCL17 and CCL22 are essential for the recruitment of Tregs to tumor sites and thereby the inhibition of anti-tumor response.^{25,26} The expression of CCL17 and CCL22 in CAFs with CD68 knockdown was examined in this study. It was found that inhibition of CD68⁺ CAFs did not affect CCL17 or CCL22 levels in CAFs, and CAF-expressed chemokines CCL17 and CCL22 at a much lower level than tumor cell lines (Figure 6, A and D). Isolated PBMCs were added in the upper chamber and co-cultured with Cal27 in the lower chamber (Figure 6B). Flow cytometry of Foxp3⁺ Tregs showed that modulation of CD68 in CAFs does not directly promote Tregs aggregation (Figure 6C). These results lead the authors to investigate the indirect effect of CD68⁺ CAFs in regulating immune microenvironment through reprogramming cancer behavior. As shown in the schematic diagram of the chemotactic assay, before the cell migration assay of PBMCs, 500 μL of CAF conditional supernatant was added to Cal27 in the lower chamber and cultured for 24 or 48 hours. Afterward, PBMCs were added to the upper chamber (Figure 6E). After migration for 8 hours, suspension cells that migrated from the upper chamber in the well plates were collected, and Tregs were

analyzed by flow cytometry. In this research, after incubation with CAF-derived CM, Cal27 exhibited elevated expression of CCL17 and CCL22 compared with negative control supernatants (Figure 6D), and enhanced chemotactic effect on Tregs (Figure 6F). CCL22 was mainly secreted by tumor cells and macrophages.²⁷ CCL17, known as a thymus and activation-regulated chemokine, can be produced by myeloid dendritic cells, endothelial cells, bronchial epithelial cells, and several tumor cells.²⁸ The modulation of CD68 resulted in the increased secretion of inflammatory factors or chemokine in CAFs that would then interact with the tumor cells and lead to a change of secretory phenotypes in tumor cells.

Discussion

CAFs are a heterogeneous population of irreversibly activated fibroblasts with distinct functions.^{7,8,29} Some CAFs are related to myofibroblasts that play an important role in wound healing and are characterized by expression of α-SMA. However, not all CAFs express high α-SMA.^{30,31} It was also found that not all CAFs showed the CD68 positivity in stroma which suggested that CAFs with distinctly different CD68 expression patterns may play different roles in tumor microenvironment. CD68 is reported to be highly expressed in the mononuclear phagocyte lineage including macrophages, microglia, and

Figure 4 Effect of dysregulated cluster of differentiation 68 (CD68) on cancer-associated fibroblasts (CAFs) phenotype transition and tumor progression. **A:** The gene and protein levels confirm the effective interference and overexpression of CD68 in CAFs and normal fibroblasts (NFs), and show the modulation of CD68 levels in CAFs cannot change the expression of CAF phenotype markers. **B** and **C:** Western blot validates that the CAF markers were basically unchanged regardless of the overexpression of CD68 in NFs or interference in CAFs. **D** and **E:** Wound healing and Transwell migration assays of Cal27 cells incubated in the conditioned medium (CM) obtained from NFs/CAFs show that modulation of CD68 in NFs/CAFs does not significantly affect the migration function of Cal27. **F** and **G:** Cck-8 and Transwell proliferation assays confirm that CD68 modulation in NFs/CAFs has little effect in inducing Cal27 proliferation. **White dashed lines** indicate migration front of tumor cells in **D**. ** $P < 0.01$, *** $P < 0.001$. OD 450, optical density are 450 nm. Original magnification, $\times 200$ (**D**, **E**, and **G**).

myeloid dendritic cells.³² A previous study found that high infiltration of CD68⁺ TAMs in tumor stroma, instead of the tumor nest, was associated with high tumor grade, lymph node metastasis, and shorter survival time.¹² Similar results were found with hepatocellular carcinoma in that the abundance of CD68⁺ macrophages that infiltrated peritumoral liver tissue, but not in tumor, was associated with poor prognosis for the patients after curative liver resection.^{33,34} However, no significant difference of CD68⁺ CAFs between the tumor center and the invasive front was found in this study. Thus, TAMs and CAFs showed the distinct expression pattern of CD68 in the tumor microenvironment of OSCC.

In the majority of solid cancers, worse prognosis is associated with a high frequency of CD68⁺ TAMs. For

example, the higher the CD68⁺ TAM infiltrating density in laryngeal squamous cell carcinoma, the worse the prognosis.³⁵ Also, increased numbers of CD68⁺ TAMs indicate an adverse overall outcome in Hodgkin lymphoma.³⁶ However, the prognostic value of CD68⁺ CAF infiltration in tumor has never been revealed, and only a few studies found the CD68 expression in fibroblasts without further research. According to the Kaplan-Meier analysis, the current study discovered that a low infiltration of CD68⁺ CAFs in the tumor center predicted poor OS, RFS, or DFS of OSCC patients. A low proportion of CD68⁺ CAFs in the invasion front is only associated with DFS. Thus, CD68⁺ fibroblasts increased during the process of malignant transformation, but low CD68⁺ CAFs

Figure 5 Correlation between cluster of differentiation 68 (CD68) and infiltration lymphocytes in oral squamous cell carcinoma (OSCC). **A** and **B**: CD68 expression in tumor center was positively associated with CD3⁺ T cells and CD4⁺ T cells, but was negatively related to Foxp3⁺ cells, whereas no significance was found in CD4 and Ki-67. **C**: Ki-67 and CD8 had no significant effect on the five-year survival rate ($P = 0.209$, 0.360 , and 0.796 , respectively). High infiltration of CD3⁺ T cells in tumor tend to be correlated with better prognosis ($P = 0.054$) and patients with high expression of Foxp3 exhibited a worse prognosis ($P = 0.054$). * $P < 0.05$, ** $P < 0.01$. Original magnification, $\times 400$ (**A**).

in OSCC were correlated with poor OS, indicating the different function of CD68⁺ CAFs in tumor initiation and progression.

In terms of the mechanism of low CD68⁺ CAFs in promoting tumor progression, this study evaluated the function of CD68 in phenotypic transformation of NFs to CAFs. However, neither the expression of phenotypic markers in interfered NFs and overexpressed CAFs nor the invasive function of tumor cells exhibited a significant difference. The results indicated that CD68 had no influence on NF to CAF transformation, and invasion and proliferation of tumor cells. However, CD68 was positively associated with CD3⁺ CD4⁺ T cells and was negatively correlated to Foxp3⁺ cells, suggesting the immunomodulatory effect of CD68 in immune microenvironment. CAF-supported tumor progression was attributed to the chemokine and cytokine secretion. For example, CAF-induced IL6 secretion

promotes pancreatic cancer cell migration,³⁷ and CAF-secreted CCL18 could promote tumor invasion in breast and ovarian cancer.^{38,39} Furthermore, CXCL12, CCL22, and CCL17 have been described as critical factors for Treg recruitment in different cancer types,^{40,41} thereby suppressing tumor-specific T-cell immunity. In oral cancer specimens, CCL22 mRNA was up-regulated, and increased CCL22 was not only associated with reduced DFS, but also was strongly correlated with an increase in Foxp3 mRNA,⁴² a master regulator of Treg development and functions. Accumulation of Treg via CAF-secreted chemokines could also be orchestrated to avoid an anti-tumor response. In this study, CCL17 and CCL22 could be induced in tumor cells though the interaction with CD68⁺ CAFs, whereas CAFs hardly secreted CCL17 and CCL22. Down-regulated CD68 in CAFs promoted the ability of tumor cells to recruit Treg through secretion of CCL17 and CCL22.

Figure 6 Diminished cluster of differentiation 68 (CD68) in cancer-associated fibroblasts (CAFs) stimulates regulatory T cells (Tregs) recruitment through tumor-secreted chemokine. **A** and **D**: Gene level reveals distinct expression of CCL17 or CCL22 in CAFs and conditional Cal27. **B** and **E**: Flowchart about migration assay. **C**: Flow cytometry confirms that CD68 modulation in CAFs cannot promote the migration of Tregs. **F**: CD68-siRNA CAF-conditioned medium (CM) was used to stimulate tumor cells and then the conditioned Cal27 was used to perform the Transwell assay. The recruitment of Tregs by tumor cells was assessed by flow cytometry. * $P < 0.05$.

Conclusion

These data support a model that diminished CD68⁺ CAFs foster a pro-tumor environment by promoting CCL17- and CCL22-induced Treg infiltration and promoting the poor clinical outcome of OSCC patients. However, the specific mechanism underlying dysregulated CD68 participating in promoting the chemotactic effect of tumor cells remains to be further elucidated.

Author Contributions

Conceptualization, Q.H. and Y.N.; methodology, X.Z. and L.D.; software, Z.L.; formal analysis, Y.J.; investigation, Y.Y.; resources, Xiaofeng Huang; data curation, S.C.; writing—original draft preparation, X.Z.; writing—review and editing, L.D.; funding acquisition, Q.H. and Y.N.

Supplemental Data

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