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Carcinoembryonic antigen cell adhesion molecule 1 inhibits the antitumor effect of neutrophils in tongue squamous cell carcinoma

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Carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), a transmembrane glycoprotein, has multiple functions. In tongue squamous cell carcinoma (TSCC), CEACAM1 overexpression is correlated with neutrophil infiltration, and both are associated with poor clinical outcomes. However, the mechanism underlying CEACAM1's effect on neutrophil function in TSCC remains unclear. We cocultured tongue carcinoma cells overexpressing CEACAM1-4L, CEACAM1-4S and differentiated HL-60 cells. This significantly upregulated the expression of MMP-9, interleukin 8, and VEGF-A in the differentiated HL-60 cells and downregulated the expression of TNF-α, relative to vector and blank control groups ($P < 0.05$). Additionally, CEACAM1 overexpression in tongue carcinoma cells weakened the cytotoxicity of differentiated HL-60 cells in the coculture system ($P < 0.05$). Thus, CEACAM1 expression in TSCC may induce an antitumor to protumor transformation of neutrophils. We performed qRT-PCR and ELISA to evaluate the underlying mechanism, and found that CEACAM1 expression in tongue carcinoma cells upregulated transforming growth factor β1 (TGF-β1) expression, while blocking of TGF-β1 inhibited the neutrophils' changes in the coculture system. Immunohistochemical analysis of clinical specimens revealed strong expression of TGF-β1 protein in TSCC. TGF-β1 expression was positively correlated with CEACAM1 expression, lymph node metastasis, and tumor recurrence. Double immunofluorescence results revealed colocalization of CEACAM1 and TGF-β1 protein in TSCC. A xenograft nude mouse model revealed that CEACAM1 overexpression in TSCC promoted tumor formation and growth, and was associated with more neutrophils infiltration. Our results indicate that CEACAM1 overexpression in TSCC may induce transformation of neutrophils from antitumor to protumor type via TGF-β1, which may further promote tumor progression.

KEYWORDS
CEACAM1, coculture, neutrophils, tongue squamous cell carcinoma, xenograft

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the ten most frequently diagnosed cancers, with the most common type being tongue squamous cell carcinoma (TSCC). Overall, the clinical outcomes of OSCC remain relatively poor, largely as a consequence of late detection, local recurrence, and metastatic disease. Although the underlying mechanisms remain unclear, accumulating evidence indicates

that inflammation and the immune system are closely related to tumorigenesis and tumor progression. For example, inflammatory cells may be influenced by the tumor microenvironment to promote tumor growth and metastasis,[1,2] and the role of neutrophils in cancer has recently attracted great interest.[3,4] This is because neutrophils participate in multiple stages of the oncogenic process, such as tumor initiation, growth, proliferation, and/or metastatic spread.[5,6] Houghton and colleagues have also reported that neutrophils secrete elastase, which directly induces neoplastic hyperplasia in human and mouse pulmonary adenocarcinoma.[7] In addition, neutrophils can simultaneously promote tumor progression by engaging factors that promote invasion, angiogenesis, and metastasis,[8] although mechanisms that regulate neutrophil function remain incompletely understood.

Carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) is a transmembrane glycoprotein that belongs to the carcinoembryonic antigen family, which includes several proteins encoded on chromosome 19 that have different biochemical properties. A total of 11 different CEACAM1 slice variants have been found in humans, which differ with respect to the number of extracellular immunoglobulin-like domains, membrane anchorage and/or the length of their cytoplasmic tail (long or short).[9] Previous studies have revealed that CEACAM1 is differentially expressed in various neoplasms, where it plays important roles in tumor progression and heterogeneity. For example, CEACAM1 can promote anchorage-independent growth in melanoma[10] and enhance invasion and migration of melanocytic and melanoma cells.[11] In addition, CEACAM1 is associated with the invasion and migration of colorectal cancer,[12] and is associated with increased angiogenic potential in non-small cell lung cancer[13] and hepatocellular carcinoma.[14] Furthermore, CEACAM1 has a close relationship with immune cells,[9,15-18] as CEACAM1 is expressed on T-cells, NK cells, B-cells, monocytes, and granulocytes. Interestingly, CEACAM1 helps prolong neutrophil lifespan and mediates delayed apoptosis in granulocytes.[17,19] Moreover, tumor cell-associated CEACAM1 can downregulate the expression of NKG2D ligand on tumor cells, which inhibits antitumor immunity.[16] Thus, CEACAM1 expression on tumor cells appears to play important roles in immune surveillance.

Our previous study revealed higher CEACAM1 expression and neutrophil infiltration in TSCC tissues than in peritumor tissues. In addition, abundant neutrophil infiltration was correlated with CEACAM1 expression on tumor cells, and both factors were associated with poorer clinical outcomes and shortened cancer-related survival among patients with TSCC.[20] Therefore, the present study aimed to determine whether CEACAM1 expression in TSCC affected neutrophil function and if so to identify the underlying mechanism. Our goal was to help improve the current understanding of crosstalk between tumor cells and the surrounding microenvironment.

2 | MATERIALS AND METHODS

2.1 | Cell culture and differentiation

Human tongue squamous cell carcinoma cell lines Cal-27 and SCC-6 were routinely cultured in Dulbecco's modified Eagle's medium

(DMEM; Gibco, Waltham, MA, USA) containing 10% FBS (Gibco) as described in our previous report.[20] Human promyelocytic leukemia cells (HL-60 cells) were purchased from the Culture Collection of Chinese Academy of Science (Shanghai, China) and cultured in endotoxin-free Iscove's Modified Eagle's Medium (IMEM, Gibco) that contained 10% FBS (Gibco). The cultures were performed at 37°C in a 5% CO_2 atmosphere. The HL-60 cell line, established from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia,[21] has long been used as a model of granulocyte differentiation.[22] HL-60 cells can express CD66a, CD66c and CD66d under differentiating conditions.[23-25] To differentiate the HL-60 cells into neutrophil-like cells (dHL-60), 1 μm/L trans-retinoic acid (dissolved in DMSO) was added to the medium for 3 days, as previously reported.[22,25-27] Flow cytometry was used to detect the total CEACAM expression on HL-60 cells (Figure S1).

2.2 | Construction of CEACAM1-4L and CEACAM1-4S overexpression lentivirus vectors and their transfection into Cal-27 and SCC-6 cells

CEACAM1-4L and CEACAM1-4S are common isoforms in human tumor tissues, which has 4 extracellular immunoglobulin-like domains with a long or short cytoplasmic tail.[11,25] The cDNA sequences of CEACAM1-4L and CEACAM1-4S were a kind gift from Professor John E. Shively.[28] The lentivirus transfection vectors were constructed and transfected into carcinoma cells as our previous study.[20] Cal-27 and SCC-6 cells were divided into four groups for transfection: CEACAM1-4L, CEACAM1-4S, vector and blank. Real-time quantitative RT-PCR and western blot were performed to examine the expressions of CEACAM1-4L and CEACAM1-4S in each group.[20]

2.3 | Indirect coculture of tongue squamous cell carcinoma cells and dHL-60 cells

The four groups of Cal-27 and SCC-6 cells (CEACAM1-4L-Lv, CEACAM1-4S-Lv, vector, and blank) were seeded in a 6-well plate at the same cell density (1×10^6 cells/mL). After 24 hours, the supernatant from each group was collected using high-speed centrifugation and filtration and was added into four dHL-60 groups for indirect coculture, and the fifth dHL-60 group served as the non-coculture control group.

The dHL-60 cells were subsequently cultured for 24 hours. Total RNA was extracted from the five groups of dHL-60 cells,[20] and then subjected to triplicate quantitative RT-PCR (qRT-PCR) reactions. Primers for VEGF-A, MMP-9, IL-8, and TNF-α were synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co. (Table 1).

2.4 | Direct coculture of tongue carcinoma cells and dHL-60 cells and MTT analysis

One day before direct coculture, the four groups of Cal-27 and SCC-6 cells were seeded in 96-well plates at the same cell density

$(2 \times 10^4$ cells/mL). On the second day, dHL-60 cells were added to the wells at a ratio of 5:1 (dHL-60 to tumor cells). Each group was tested in triplicate, and four groups of tongue carcinoma cells without the dHL-60 cells served as the corresponding control groups. After 24 hours, any suspended dHL-60 cells were completely removed from the coculture system using PBS. Next, 150 μL of new all nutrition culture medium and 20 μL of MTT (5 mg/mL) were added to each well, including the control and blank wells. After 4 hours of culture at 37°C, the culture process was terminated and all fluid was removed from each well before 150 μL of DMSO was added to each well. The plates were then shaken on an MPP oscillator for 10 minutes to completely dissolve the purple crystals. Finally, the 96-well plates were placed on a microplate reader and the absorption value was read at 490 nm (OD_{490}). After subtracting the OD_{490} value from the blank control well, the mean OD_{490} value from the triplicate samples was recorded for each group. The kill rate for each group was calculated as (control group OD_{490} - each experimental group OD_{490})/(control group OD_{490}) × 100%.

2.5 | qRT-PCR analysis of TGF-β1 and IFN-β1 expression in tongue carcinoma cells

The total RNA extraction and qRT-PCR processes were performed using the same method as for the indirect coculture assay. The primers for TGF-β1 and IFN-β1 were also synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co. (Table 1).

2.6 | ELISA analysis of TGF-β1 secretion in tongue carcinoma cells

The four groups of tongue carcinoma cells were seeded in 6-well plates at the same cell density (1×10^6 cells/mL). After culture for 24 hours, the supernatant for each group was collected and the debris was removed using high-speed centrifugation. Based on the specifications from the

TABLE 1 Primer sequences and size of PCR products

	Sequences	Product size
β-actin	5′ - TTGCCGACAGGATGCAGA - 3′	100 bp
	5′ - GCCGATCCACACGGAGTACT - 3′	
IL-8	5′ - CTTGGCAGCCTTCCTGATTTCT - 3′	223 bp
	5′ - GTTTTCCTTGGGGTCCAGACAG - 3′	
VEGF-A	5′ - GTCCTGGAGCGTGTACGTTG - 3′	182 bp
	5′ - CGTTTAACTCAAGCTGCCTCG - 3′	
MMP-9	5′ - TTGACAGCGACAAGAAGTGG - 3′	178 bp
	5′ - GCCATTCACGTCGTCCTTAT - 3′	
TNF-α	5′ - ATGATCCGGGACGTGGAGCT - 3′	210 bp
	5′ - CTGGGCCAGAGGGCTGATTA - 3′	
IFN-β1	5′ - GGCACAACAGGTAGTAGGCG - 3′	91 bp
	5′ - AGTGGAGAAGCACAACAGGAG - 3′	
TGF-β1	5′ - CTAATGGTGGAAACCCACAACG - 3′	208 bp
	5′ - TATCGCCAGGAATTGTTGCTG - 3′	

Human TGF-beta 1 Quantikine ELISA Kit (DB100B; R&D Systems, Minneapolis, NJ, USA), the culture supernatant was diluted before the TGF-β1 in the supernatant was activated using HEPES buffer (1N HCL and 1.2N NaOH/O.5 M). The activated supernatant was subsequently added to the corresponding wells, and the concentrations of TGF-β1 for each group was evaluated using the microplate reader.

2.7 | Indirect and direct coculture experiments with TGF-β1-neutralizing antibodies

The role of TGF-β1 in neutrophil transformation was tested by repeating the indirect and direct coculture experiments with TGF-β1-neutralizing antibodies (ab27969; Abcam, Cambridge, UK). The dilutions were selected based on the TGF-β1 concentrations from the ELISA experiments.

2.8 | Patients and specimens

Seventy-four patients with primary tongue squamous cell carcinoma participated in this study after undergoing surgery at the Affiliated Hospital of Qingdao University between 2005 and 2010. All diagnoses were made following the Pathology and Genetics of Head and Neck Tumors of World Health Organization Classification of Tumors. None of the patients received chemotherapy or radiation therapy before surgery. Conventional clinicopathologic parameters have been summarized in Table 2. This study was reviewed and approved by the Institutional Medical Ethics Committee of the Affiliated Hospital of Qingdao University, and written informed consent was acquired from each patient. The study conforms with the Declaration of Helsinki, as revised in October 2013.

2.9 | Immunohistochemistry for TGF-β1 and CEACAM1 expression in TSCC

The detailed immunohistochemistry procedures and evaluation criteria have been described in our previous report.[20] The TGF-β1 antibody was a rabbit anti-TGF-β1 antibody (1:100; ab27937; Abcam). The colocalization of CEACAM1 and TGF-β1 protein in TSCC was evaluated using double immunofluorescence histochemistry. After deparaffinization, rehydration, and epitope retrieval using Tris-EDTA buffer, the endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Heterogenic antigens were blocked using TBSTx containing 5% BSA, and then mouse anti-CEACAM1 antibodies (1:50; 29H2; Abcam) were added to the sections, which were stored overnight at 4°C. On the second day, the sections were washed using PBS-T and incubated for 1 hours at 37°C with fluorescein isothiocyanate (FITC)-labeled anti-mouse secondary antibodies (ZSGB, CHINA). Finally, the rabbit anti-TGF-β1 antibodies (1:100) were added and incubated at 37°C for 2 hours before the FITC-labeled anti-rabbit secondary antibodies were added and incubated for 1 hours. The results were observed under a fluorescence microscope after adding a drop of antifade mounting medium.

TABLE 2 The expression of TGF-β1 in tongue squamous cell carcinoma and its relationship with clinicopathologic feartures

Clinicopathologic variables	n	Mean ± SD	P value
Sex			
Male	51	4.982 ± 1.423	0.504*
Female	23	4.798 ± 1.106	
Age			
≤60 years	41	5.003 ± 1.372	0.689*
>60 years	33	4.828 ± 0.797	
Clinical stage			
I, II	36	4.711 ± 1.276	0.095*
III, IV	38	5.128 ± 1.461	
Grade			
G1	40	4.962 ± 0.782	0.895**
G2	29	4.867 ± 1.264	
G3	5	4.964 ± 0.813	
Lymph node metastasis			
Yes	34	5.719 ± 1.579	0.001*,***
No	40	4.250 ± 1.192	
Tumor extension			
<4 cm(T1-T2)	56	4.857 ± 1.348	0.207*
≥4 cm (T3-T4)	18	5.136 ± 1.243	
Tumor recurrence			
Yes	19	5.539 ± 0.961	0.011*,***
No	55	4.712 ± 1.372	
Carcinoma	74	4.925 ± 1.526	0.002*,***
Adjacent tissues	17	3.587 ± 1.147	

*P value was estimated by the Mann-Whitney test.
**P value was estimated by the Kruskal-Wallis test.
***P < 0.05 was considered statistically significant.

2.10 | Xenograft assays in nude mice and IHC of CD11b+ neutrophils in xenograft tumors

In the in-vitro experiment, we observed that Cal-27 and SCC-6 had the similar tendency on the function of neutrophils. So only one cell line (Cal-27) was choosen to perform the in-vivo experiment. To assess the effect of CEACAM1 expression on cells' in vivo tumor-formation capacity and the roles of neutrophils in it, the three Cal-27 cell groups (CEACAM1-4L-Lv, CEACAM1-4S-Lv and vector) were inoculated subcutaneously (1×10^6 cells) into the bilateral under-arm of 6-week-old BALB/c nude mice (n = 6 for each group). Tumor growth was recorded once per week for 7 weeks starting on the 7th day after implantation. Tumor volume (mm^3) was calculated as ($width^2$ × length)/2. Tumor weights were determined using the bilateral nodules for each mouse. To assess the roles of neutrophils in this tumor-formation process, IHC was used to evaluate the infiltration of CD11b+ (1:200, ab133357; Abcam) neutrophils in the xenograft tumors. Five high power fields in each specimen were selected for neutropihls counting. The neutrophils infiltration was the mean result of all fields in each group. All procedures were approved by the

Animal Ethics Committee of Qingdao University, and all efforts were made to minimize pain and suffering.

2.11 | Statistical analysis

All experiments were repeated at least three times and statistical analyses were performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL). Data were reported as mean ± standard deviation. The Mann-Whitney test and Kruskal-Wallis test were used to evaluate the immunohistochemistry results. The correlation of CEACAM1 expression with TGF-β1 was analyzed using Spearman's rho coefficient. The results from the qRT-PCR, MTT, ELISA, and xenograft model tests were analyzed using Student's t test. Differences were considered statistically significant at P-values of < 0.05.

3 | RESULTS

3.1 | Elevated CEACAM1-4L and CEACAM1-4S expression in Cal-27 and SCC 6 cellls after transfection

The lentivirus vector was used to induce CEACAM1-4L and CEACAM1-4S overexpression in Cal-27 and SCC-6 cells, and the transfection efficiency of both cells was found to be >90% in each group (Figure 1A, SCC-6). Based on the results of qRT-PCR and western blot analyses, CEACAM1-4L and CEACAM1-4S expression of SCC-6 were clearly elevated in CEACAM1-4L and -4S transfection group respectively, relative to the vector or blank control group (Figure 1B,C). The transfection efficiency of Cal-27 was as our previously reported.[20]

3.2 | Supernatant from Cal-27 and SCC-6 cells overexpressing CEACAM1-4L and CEACAM1-4S upregulated VEGF-A, MMP9, and IL-8 but downregulated TNF-α mRNA in dHL-60 cells

The indirect coculture experiment with tongue carcinoma cells and dHL-60 cells revealed that the supernatant from both tongue carcinoma cells upregulated mRNA expressions of VEGF-A, MMP9, and IL-8, but downregulated TNF-α mRNA expression in dHL-60 cells, relative to the non-coculture group (P < 0.05, respectvely) (Figure 2). Overexpression of CEACAM1-4L and CEACAM1-4S in Cal-27 cells both additionally upregulated the mRNA expressions of VEGF-A, MMP9, and IL-8, and further downregulated TNF-α mRNA expression relative to the vector and blank control groups (P < 0.05, respectively) (Figure 2). Over expression of CEACAM1 in SCC-6 cells had the same tendency with Cal-27 cells (P < 0.05, respectively).

3.3 | CEACAM1 overexpression in Cal-27 and SCC-6 weakens the cytotoxicity of dHL-60 cells in the coculture system

After the direct coculture, the MTT results revealed that cell vitality was clearly lower in the CEACAM1-4L and CEACAM1-4S
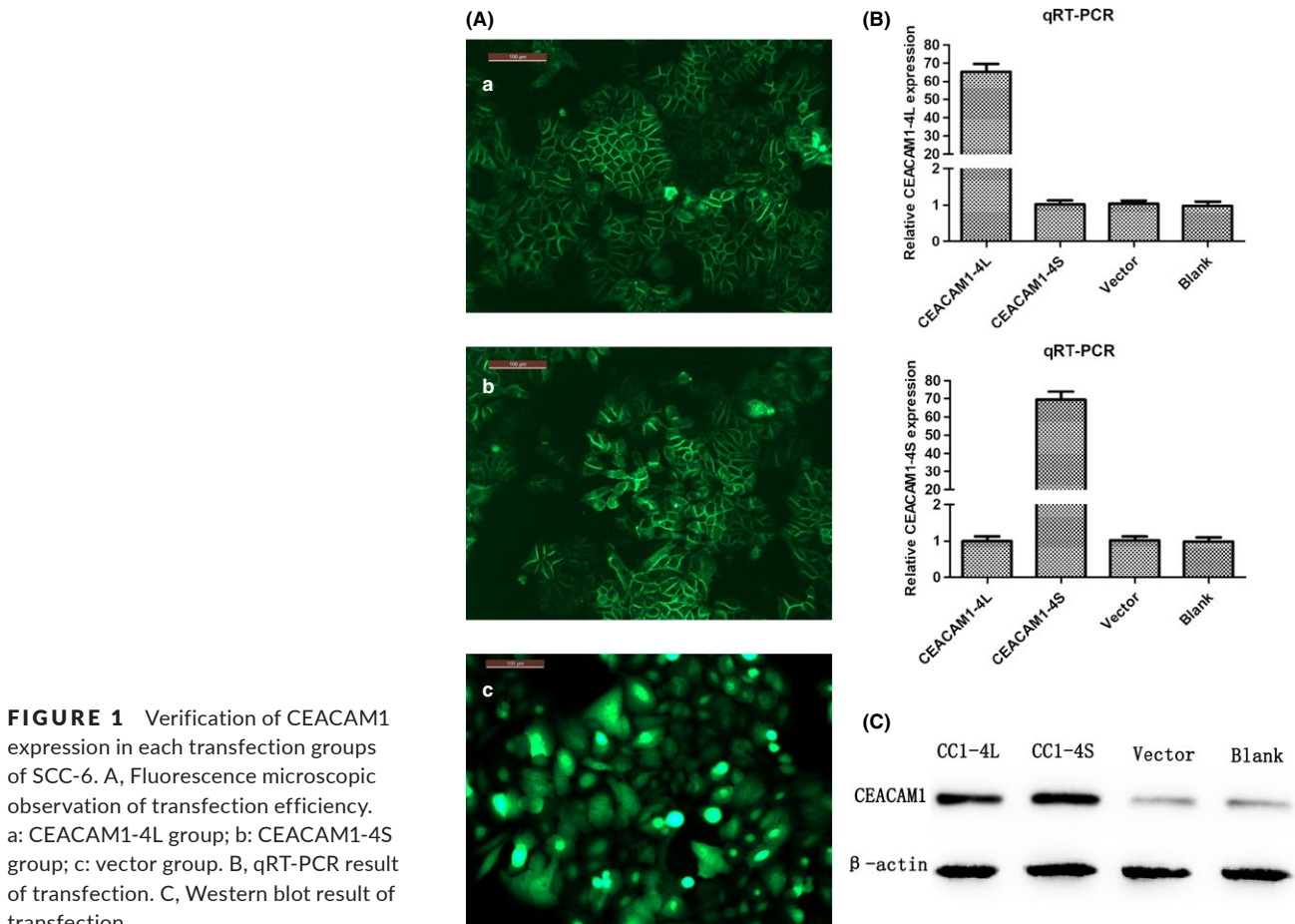
FIGURE 1 Verification of CEACAM1 expression in each transfection groups of SCC-6. A, Fluorescence microscopic observation of transfection efficiency. a: CEACAM1-4L group; b: CEACAM1-4S group; c: vector group. B, qRT-PCR result of transfection. C, Western blot result of transfection

overexpression groups, relative to the vector or blank control groups. The kill rates of Cal-27 cells from this assay were 22.87 ± 1.49% for the CEACAM1-4L overexpression group, 19.53 ± 1.13% for the CEACAM1-4S overexpression group, 40.08 ± 1.27% for the vector group and 41.36 ± 1.89% for the blank group (Figure 3). The kill rates of SCC-6 were 24.91 ± 1.05, 19.87 ± 1.61, 39.37 ± 2.06 and 38.83 ± 1.72 in each corresponding CEACAM1-4L group, CEACAM1-4S group, vector group and blank group (Figure 3). The differences between the CEACAM1-4L/-4S group and the vector/ blank group were significant ($P < 0.05$, respectvely) in both cell lines, which indicates that tumor cell overexpression of CEACAM1 weaken the cytotoxicity of neutrophils.

3.4 | Overexpression of CEACAM1 upregulates TGF-β1 expression in tongue carcinoma cells

The mRNA expressions of TGF-β1 and IFN-β1 in each group of Cal-27 cells were analyzed using qRT-PCR, which revealed that both CEACAM1-4L and CEACAM1-4S significantly upregulated TGF-β1 mRNA expression ($P < 0.05$, respectively) compared with vector and blank group (Figure 4), but has no significant effect on IFN-β1 expression (data not shown). The result from SCC-6 cell groups had the same tendency of TGF-β1 (Figure 4) and didn't significantly affect IFN-β1 expression as well (data not shown). The secretion of TGF-β1 from

each tongue carcinoma cell group was also tested using ELISA, which revealed significant higher mean concentrations of TGF-β1 protein in the CEACAM1-4L and CEACAM1-4S overexpression groups than in the vector and blank groups ($P < 0.05$, respectively) in both Cal-27 and SCC-6 cells (Figure 4). Thus, overexpression of CEACAM1-4L and CEACAM1-4S can promote both expression and secretion of TGF-β1 protein in tongue carcinoma cells.

## 3.5 | CEACAM1 expression in TSCC promotes dHL-60 antitumor to protumor transformation through TGF-β1

Adding the TGF-β1-neutralizing antibodies into the indirect coculture system revealed that blocking TGF-β1 led to clear downregulation of VEGF-A, MMP9, and IL-8, but upregulation of TNF-α in dHL-60 cells, relative to the corresponding groups without the TGF-β1-neutralizing antibodies ($P < 0.05$, respectively) in both Cal-27 and SCC-6 cells (Figure 2). The direct coculture system also showed that the kill rates of Cal-27 were 57.93 ± 1.74% for the CEACAM1-4L overexpression group, 59.17 ± 2.27% for the CEACAM1-4S overexpression group, 62.53 ± 1.65% for the vector group, and 60.71 ± 1.83% for the blank group, while the killing rates of SCC-6 were 58.67 ± 2.08, 57.01 ± 1.96, 61.67 ± 1.34 and 59.56 ± 1.71 in each corresponding group, which confirmed that TGF-β1 blockade

Cal-27

(A)

(B)

(C)

(D)

SCC-6

(A)

(B)


(C)

(D)
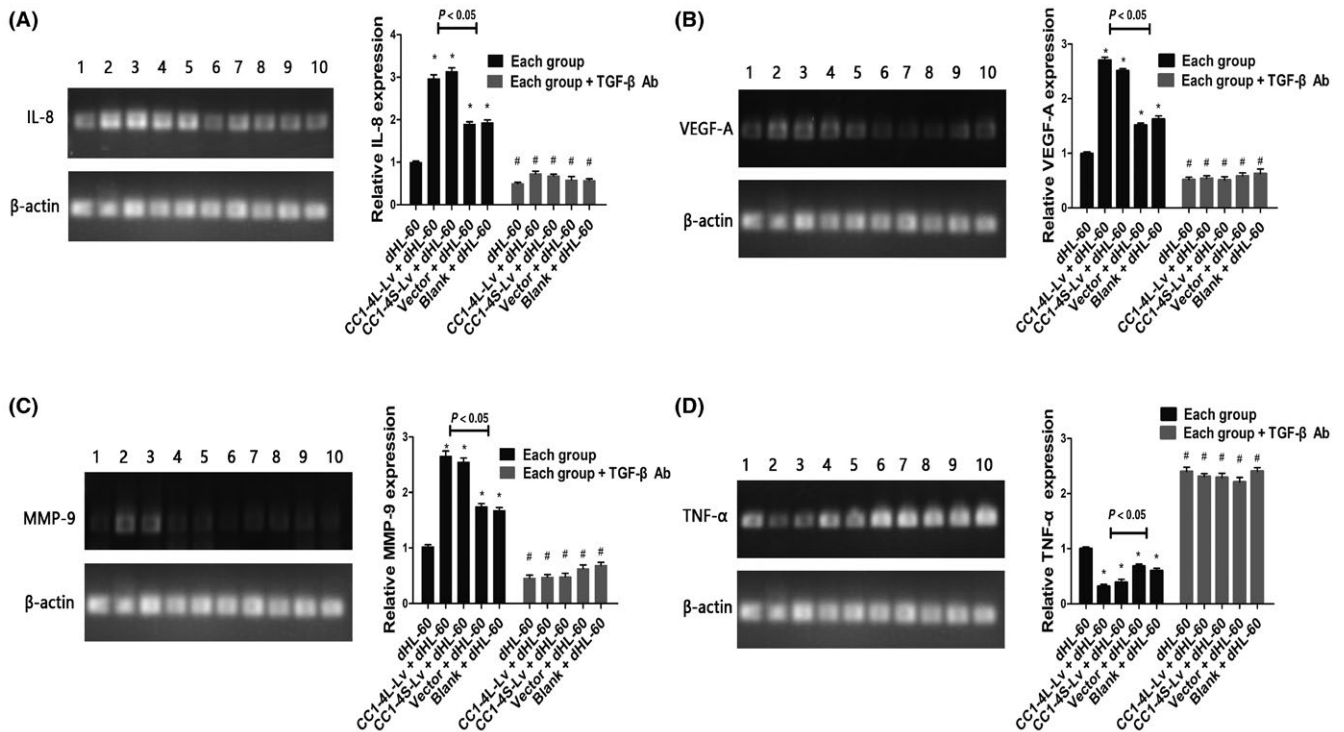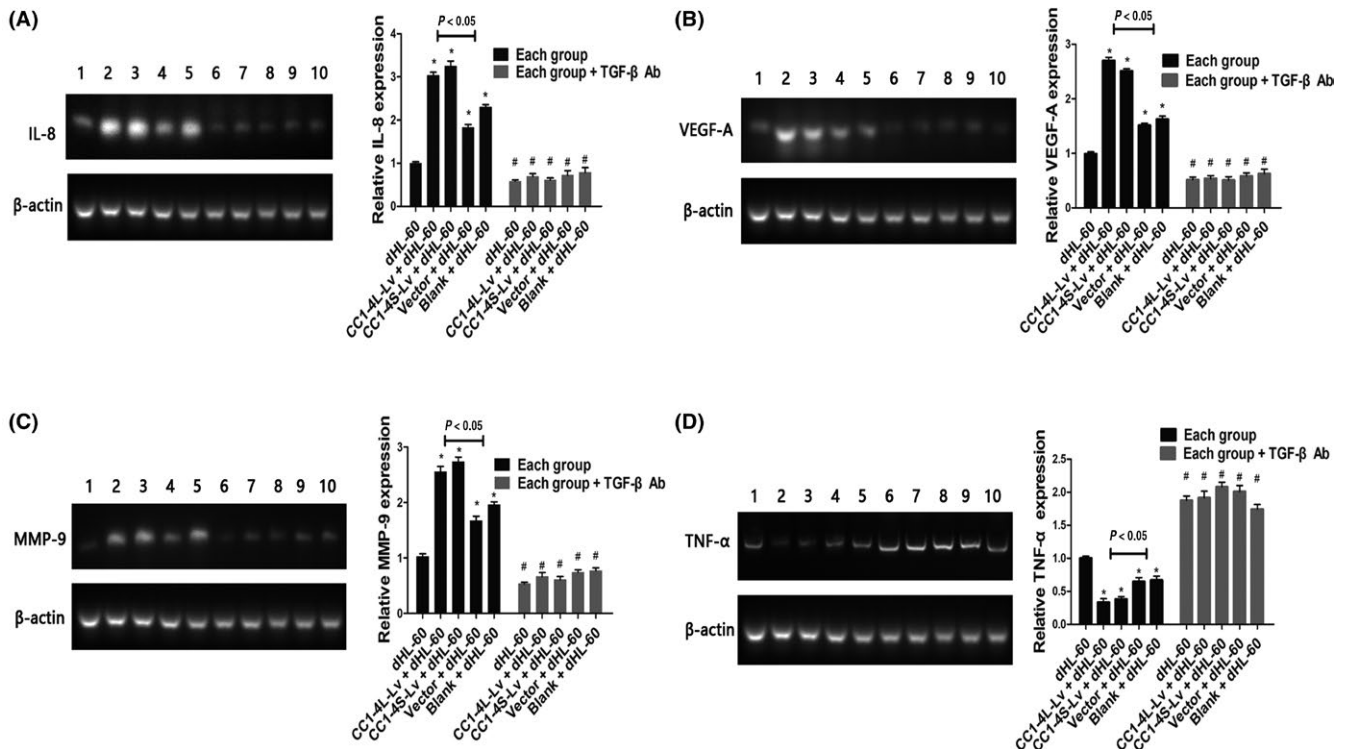
FIGURE 2 The qRT-PCR results for IL-8 (A), VEGF-A (B), MMP-9 (C), and TNF-α (D) in dHL-60 cells before and after coculture with Cal-27 cells and SCC-6 cells. The differences between the coculture groups (2-4) and the non-coculture group (1) were significant (*$P < 0.05$, respectively). There were also significant differences between the experimental groups (2, 3) and the control groups (4, 5) in both tongue carcinoma cells. When TGF-β1-neutralizing antibodies were added to the coculture system (6-10), there were no prominent differences between the groups, although remarkable differences were observed between the same groups with and without the added antibodies to TGF-β1 (1-5) (#$P < 0.05$, respectively). The amplification products of the qRT-PCR were separated and visualized on ethidium bromide-stained agarose gels (corresponding left panels)

Cal-27
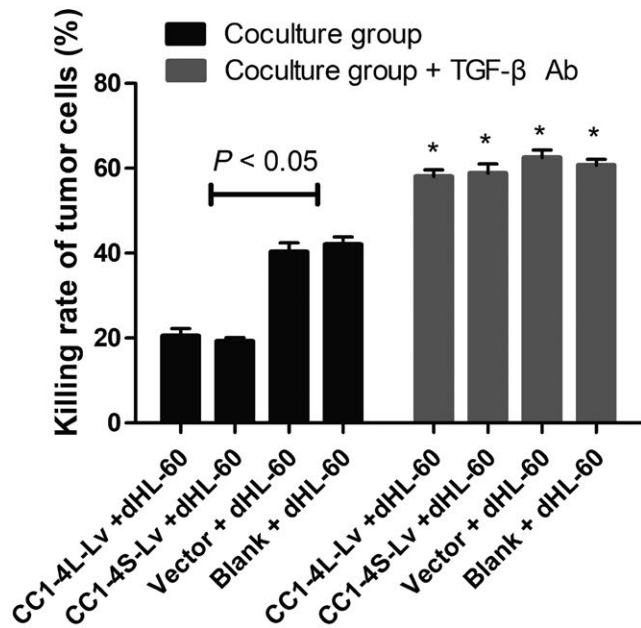
SCC-6
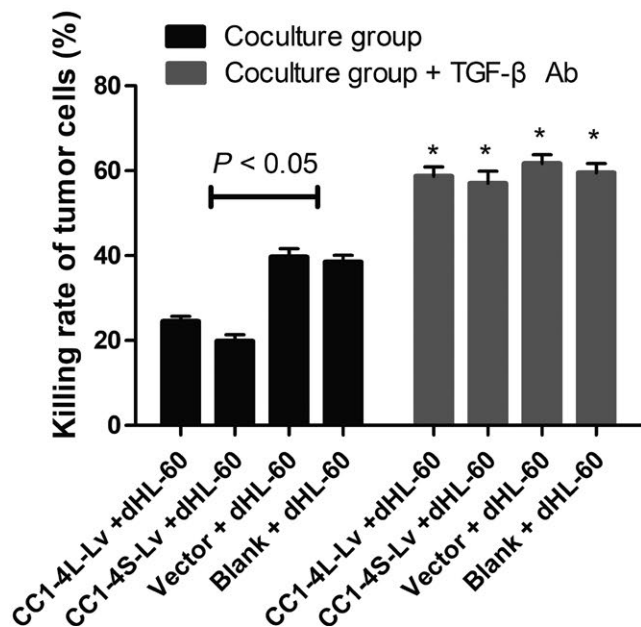
FIGURE 3 The cytotoxicity results for dHL-60 cells in each coculture group. After the coculture, the kill rates of both tumor cells in the CEACAM1-4L and CEACAM1-4S group were clearly decreased relative to the vector and blank control groups (P < 0.05, respectively). When the TGF-β 1 neutralizing antibodies were added to the coculture system, each group's kill rate was higher than that of the corresponding group without the antibodies to TGF-β1 (*P < 0.05, respectively), while there was no obvious difference between the various group

strengthened the cytotoxicity ability of dHL-60 cells relative to the corresponding groups without the TGF-β1-neutralizing antibodies (P < 0.05, respectively) (Figure 3).

3.6 | TGF-β1 expression on TSCC and its correlation with CEACAM1 expression

Double immunofluorescence results revealed colocalization of CEACAM1 and TGF-β1 protein in clinical TSCC specimens (Figure 5). Furthermore, the immunohistochemistry results revealed stronger TGF-β1 protein expression in TSCC tissues than in peritumor tissues (Figure 5), with expression predominantly observed in the nucleus and weaker expression observed in the cytoplasm. In the tumor tissues, TGF-β1 expression was associated with lymph node metastasis and tumor recurrence, but was not associated with other clinical factors (Table 2). Spearman's rho coefficient revealed that TGF-β1 expression was positively correlated with CEACAM1 expression in TSCC (Table 3).

3.7 | Overexpression of CEACAM1 was associated with more neutrophils infiltration and promote tumors growth in nude mice

The nude mouse xenograft model of Cal-27 cells revealed significantly larger tumor sizes in the CEACAM1-4L group (1257.49 ± 78.37 mm^3) and the CEACAM1-4S group (1167.53 ± 122.84 mm^3) than in the vector group (589.25 ± 114.36 mm^3, P < 0.05) (Figure 6A,B). Similarly, significantly heavier tumors were observed in the CEACAM1-4L group (786.30 ± 80.43 mg) and the CEACAM1-4S group (705.36 ± 102.94 mg) than in the vector group (380.67 ± 75.47 mg, P < 0.05) (Figure 6C). The IHC results showed that there were more CD11b (+) neutrophils infiltration in the nude mice tumor of CEACAM1-4L and CECAM1-4S group than in vector group (P < 0.05, Figure 6D). Thus, CEACAM1 overexpression in TSCC could promote tumor formation and growth in nude mice.

4 | DISCUSSION

Accumulating evidence suggests that the tumor microenvironment can influence tumor progression, with tumor-associated neutrophils (TANs) playing important roles.[5,6] For example, TANs may be induced by the tumor cells or tissues to acquire a protumor phenotype,[29] although the related regulatory mechanisms remain incompletely understood.[4] Our previous study[20] revealed that TSCC had abundant neutrophil infiltration and overexpression of CEACAM1, relative to peritumor tissues, with both factors being associated with poor clinical outcomes. Thus, the present study explored the association of tumor cell CEACAM1 expression with neutrophil function.

Previous research has indicated that protumor neutrophils accelerate tumor progression mainly by promoting tumor invasion and angiogenesis, and also by inhibiting neutrophil cytotoxicity. In this context, MMP-9 takes part in the tumor invasion and metastasis processes by degrading and remodeling the surrounding extracellular matrix. Neutrophils are important source of MMP-9 in the tumor microenvironment,[30] and the MMP-9-related degradation

Cal-27

(A)

SCC-6

(A)
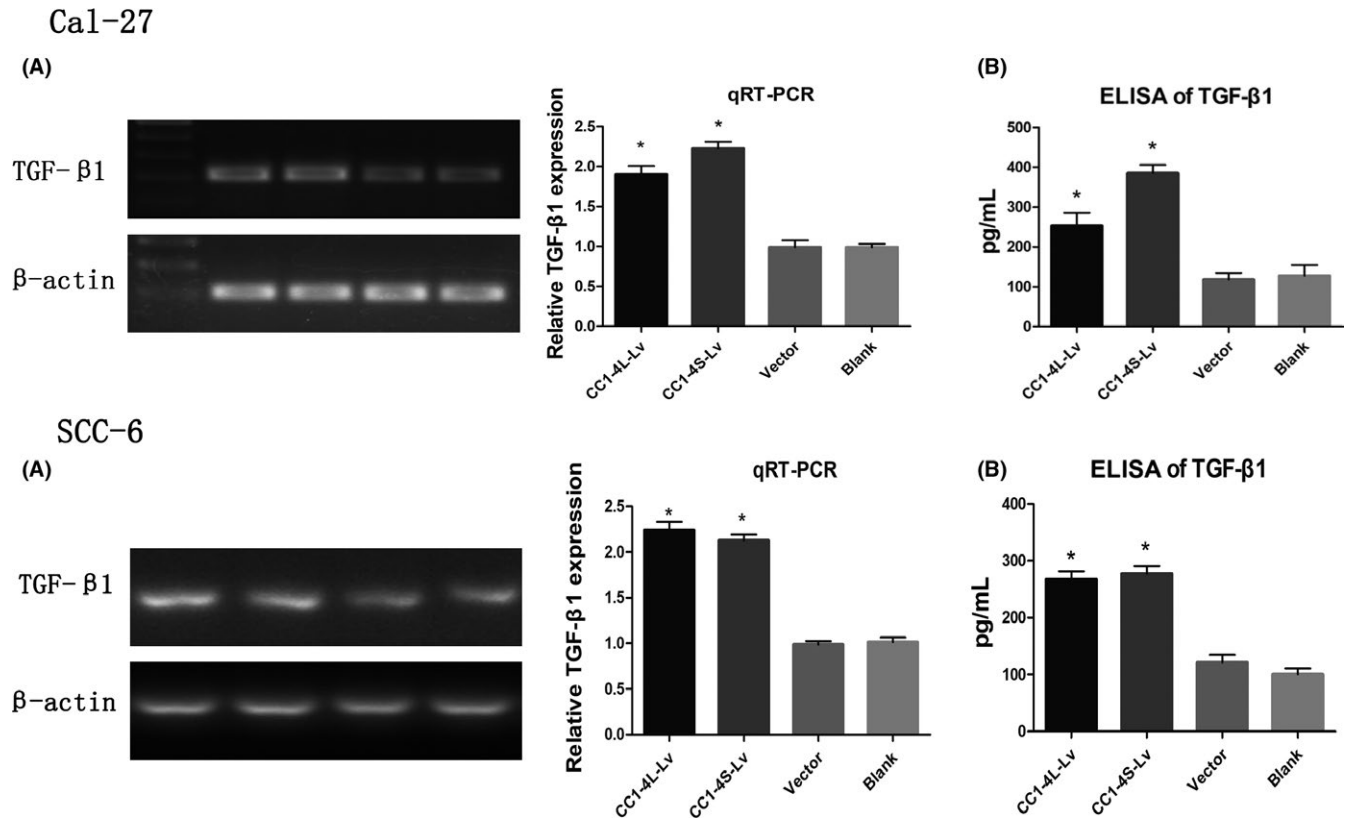
FIGURE 4 The qRT-PCR (A) and ELISA (B) results for TGF-β1 in the four Cal-27 and SCC-6 groups. The mRNA expression and protein secretion of TGF-β1 in the CEACAM1-4L and CEACAM1-4S group were clearly higher than in the vector and blank control groups (*P < 0.05, respectivley). The amplification products of the qRT-PCR were separated and visualized on ethidium bromide-stained agarose gels (corresponding left panels)

and remodeling of the extracellular matrix helps drive angiogenesis.[31] Ardi et al[32] found that human neutrophils are unique in that they release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. Another important and widely recognized angiogenic factor is VEGF-A, while IL-8 is a powerful chemotactic factor[33-35] that attracts neutrophils to the tumor and generates a positive feedback loop. Furthermore, TNF-α can cause tumor cells to undergo necrosis and apoptosis.[36] Thus, we examined the changes in the expressions of these important factors using our coculture experiment, which revealed that coculture of tongue carcinoma cells and dHL-60 cells upregulated the mRNA expressions of VEGF-A, MMP9, and IL-8, but downregulate the expression of TNF-α mRNA in dHL-60 cells, relative to the non-coculture control group. Furthermore, overexpression of CEACAM1-4L and CEACAM1-4S further upregulated the mRNA expressions of VEGF-A, MMP9, and IL-8, and further downregulated TNF-α mRNA expression in transfected dHL-60 cells, relative to the vector and blank groups (P < 0.05) (Figure 2).

Recent studies have demonstrated that neutrophils can be induced to transform from an antitumor type (N1) to a protumor type (N2) based on the tumor microenvironment, similar to the M1 and M2 types of macrophages. This transformation is mainly determined by the quantity of related molecules in the microenvironment, rather than their quality.[37] Similarly, our results indicate that CEACAM1

overexpression in tongue carcinoma cells weakened the cytotoxicity ability of dHL-60 cells in the coculture system (Figure 3). This result also agrees with the findings of Yan et al,[38] who reported that neutrophils from patients with lung cancer had poorer poor cancer-killing activity compared with neutrophils from healthy donors. These results imply that neutrophils in TSCC may be induced to transform from the N1 type to the N2 type when tumor cells express CEACAM1.

Fridlender et al[39] reported that TGF-β within the tumor microenvironment induced a population of TANs to assume the protumor phenotype, while TGF-β blockade resulted in the recruitment and activation of antitumor TANs. In addition, Jablonska et al[40] reported that IFN-β inhibited tumor angiogenesis through the repression of genes encoding proangiogenic and homing factors in N1 tumor-infiltrating neutrophils, while IFN-β deficiency led to higher CXCR4, VEGF, and MMP-9 levels in N2 tumor-infiltrating neutrophils. Thus, we evaluated whether CEACAM1 expression affected the expression of TGF-β1 or INF-β1 in TSCC, and whether any change affected neutrophil function. Our initial qRT-PCR results revealed that CEACAM1 overexpression in tongue carcimoma cells upregulated the mRNA expression of TGF-β1 (Figure 4) but did not affect IFN-β1. In addition, the ELISA results revealed that CEACAM1 overexpression in tongue carcinoma cells led to increased TGF-β1 expression. Finally, we used TGF-β1-neutralizing antibodies in the coculture
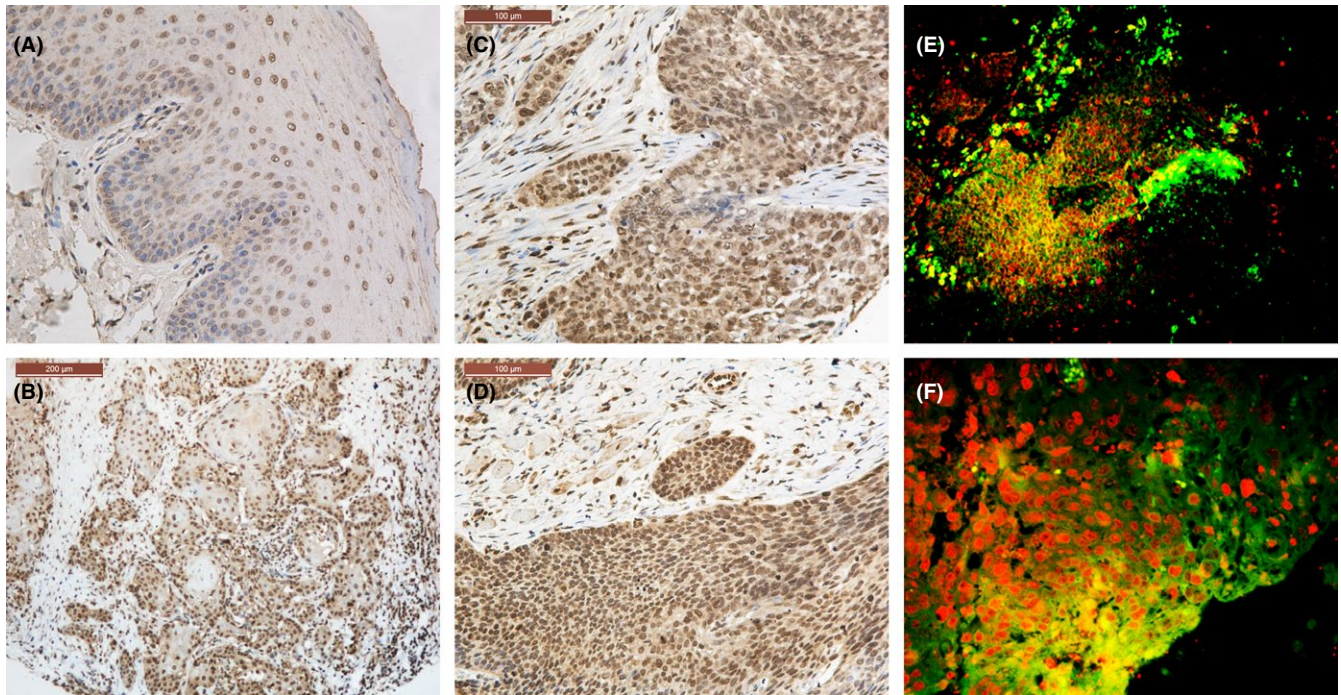
FIGURE 5 Immunohistochemistry results for TGF-β1 and double immunofluorescence results for TGF-β1 and CEACAM1 in tongue squamous cell carcinoma and peritumor tissues. A, The expression of TGF-β1 was negative or weak in the peritumor tissues and was clearly higher in the squamous cell carcinoma tissues (B-D), with the TGF-β1 protein mainly being expressed in the nucleus. E, F, The colocalization of CEACAM1 (green) and TGF-β1 protein (red) in the squamous cell carcinoma tissues (B, E: 100×; A, C, D, F: 200×)

TABLE 3 The relationship of CEACAM1 and TGF-β1 expression in tongue squamous cell carcinoma tissues

| CEACAM1 expression	TGF-β1 expression			Spearman's rho coefficient test	
	Weak	Moderate	Strong	r	P
Negative/weak	14	9	7	0.661	0.014*
Moderate	9	10	12		
Strong	3	4	6		

*P < 0.05 was considered statistically significant.

system and observed that they downregulated the mRNA expressions of VEGF-A, MMP-9, and IL-8, but upregulated the expression of TNF-α (P < 0.05) (Figure 2). Our results also revealed a strengthened cytotoxic ability for neutrophils that were exposed to TGF-β1 blockade (Figure 3).

The in vivo relationship of CEACAM1 and TGF-β1 was evaluated using double immunofluorescence and immunohistochemistry, which revealed colocalization of CEACAM1 and TGF-β1 in TSCC (Figure 5). Furthermore, TGF-β1 was strongly expressed in TSCC, where it is associated with lymph node metastasis and tumor recurrence, and there was a positive correlation between the expressions of CEACAM1 and TGF-β1 protein. Similar results have been observed in research on gastric carcinoma, in which neoplastic lesions had higher co-expression of CEACAM1 and TGF-β1 than non-neoplastic lesions. This may be related to tumor progression via increased angiogenesis.[41] In addition, our xenograft model results revealed that CEACAM1 overexpression in TSCC was associated

with more neutrophils infiltration, which is consistent with our previous research using human specimen,[20] and promoted tumor formation and growth in nude mice (Figure 6). This aggressive growth pattern may be related to MMP-9-mediated degradation of the surrounding extracellular matrix and/or increased nutritional supply that is provided through angiogenesis induced by VEGF-A and MMP-9.

When considered together, our results indicate that CEACAM1 overexpression in TSCC may upregulate TGF-β1 and subsequently induce neutrophil transformation from the N1 type to the N2 type. These results may explain our previous immunohistochemical findings, which revealed that both CEACAM1 expression and neutrophil infiltration were associated with poor clinical outcomes.[20] Therefore, although neutrophil transformation in tumor tissues may be influenced by many aspects of the tumor microenvironment, it appears that this process is at least partially related to overexpression of CEACAM1 on tumor cells in TSCC tissues.
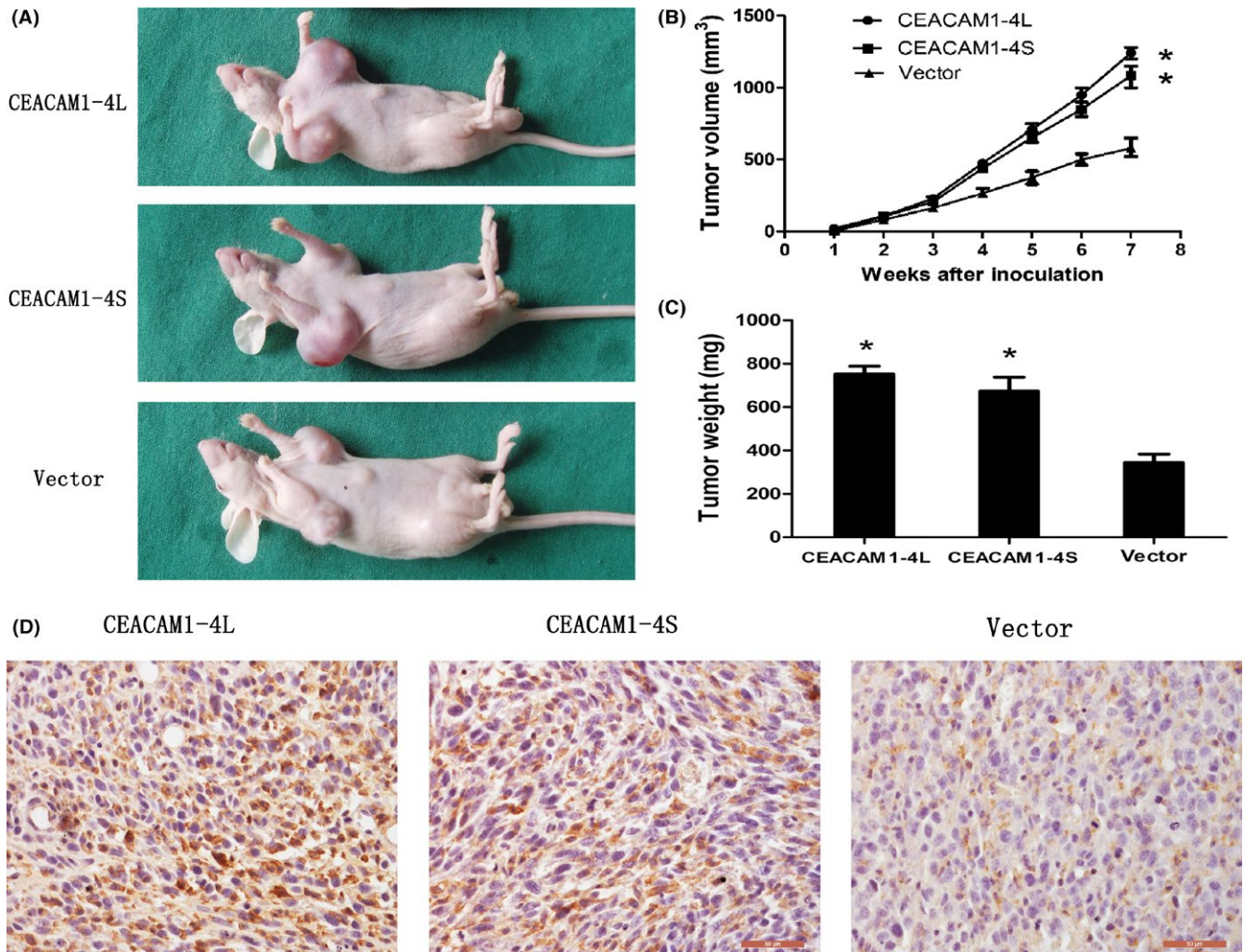
FIGURE 6 The effect of CEACAM1 overexpression on tongue squamous cell carcinoma xenograft formation and growth, and its correlation with neutrophils infltration. Representative pictures are shown for the CEACAM1-4L, CEACAM1-4S, and vector groups' xenograft tumors (A). In addition, the tumor growth curve (B) and average weight (C) were recorded and calculated for each group (*$P < 0.05$). D, representative pictures of neutrophils infiltration in different groups (200×)

CONFLICT OF INTEREST

There are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.