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CCL5 at the tumor-bone interface: A lymphocyte-derived gatekeeper against mandibular invasion in oral squamous cell carcinoma

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ABSTRACT

Objectives: Mandibular bone invasion (MBI) in oral squamous cell carcinoma (OSCC) reflects advanced disease and poor prognosis. The role of the tumor immune microenvironment (TME) in MBI remains incompletely defined. We investigated the prognostic relevance of CCL5 and its association with bone invasion.
Materials and methods: CCL5 mRNA expression was analyzed in the TCGA HNSCC cohort (n = 327) across T stages and correlated with overall survival (OS). Single-cell RNA sequencing data were used to identify the cellular sources of CCL5. Protein expression of CCL5 and FoxP3 was assessed by immunohistochemistry in a retrospective cohort of 206 OSCC patients using tissue microarrays, distinguishing tumor and TME compartments. Systemic inflammatory markers (neutrophil-to-lymphocyte ratio [NLR], C-reactive protein–to–albumin ratio [CAR]) were also evaluated.
Results: CCL5 mRNA expression decreased with increasing T stage and was lowest in T4a tumors with bone invasion ($p = 0.038$). High CCL5 levels were associated with improved OS ($p = 0.026$). Single-cell analysis identified $CD8^+$ T cells, regulatory T cells, and natural killer cells as principal sources of CCL5. In the clinical cohort, low CCL5 and FoxP3 expression in the TME correlated with MBI ($p = 0.008$ and $p < 0.001$). Multivariable analysis confirmed high CCL5 expression in the TME as an independent predictor of favorable OS (HR = 0.294, $p = 0.015$), whereas high NLR predicted poor survival (HR = 2.664, $p = 0.044$).
Conclusions: A CCL5- and FoxP3-deficient TME characterizes bone-invasive OSCC and independently predicts poor survival, supporting their value as biomarkers for risk stratification.

Introduction

Oral squamous cell carcinoma (OSCC) affects nearly 390,000 individuals annually and is strongly associated with tobacco and alcohol use [1]. Despite progress in multimodal therapies, the prognosis of advanced oral squamous cell carcinoma (OSCC) remains poor. In advanced stage, mandibular involvement necessitates extensive bone resection and complex reconstruction, significantly reducing patients' quality of life [2,3]. The molecular mechanisms driving bone invasion in OSCC are incompletely understood. Key regulators of bone homeostasis include RANK, RANKL, and OPG, with activation of the RANK–RANKL axis promoting osteoclastogenesis, while OPG acts as a decoy receptor inhibiting this process [4–7].

Traditionally, research on bone invasion has focused on these direct osteoclastogenic pathways; however, it is increasingly recognized that the tumor immune microenvironment (TME) plays a decisive role in modulating this process. Bone represents a unique immunological niche, often described as relatively immunosuppressive, characterized by low $CD8^+$ T cell infiltration and high levels of $CD4^+$ T cells, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [8]. Whether this specific immunological landscape actively promotes the invasion of the mandible by the primary tumor remains a critical open question. Central to the orchestration of the TME are chemokines, which govern the recruitment and activation of immune effector cells. Among these, CCL5

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(RANTES) is a potent proinflammatory chemokine primarily secreted by $CD8^+$ T cells, Tregs and other immune cells.

CCL5 attracts monocytes, dendritic cells and other immune cells to the site of inflammation exerting its effects mainly through the CCR5 receptor [8]. In epithelial cancers, the role of the CCL5/CCR5 axis is highly context-dependent. While it can create an immunosuppressive microenvironment in some cancers [9], in OSCC, upregulated CCL5 has been associated with poorer prognosis and a more aggressive phenotype, promoting invasion and metastasis [10–12]. However, these studies primarily focused on tumor-cell-intrinsic CCL5 signaling or CCL5 derived from cancer-associated fibroblasts (CAFs). In contrast, the role of lymphocyte-derived CCL5 within the tumor immune microenvironment remains poorly defined. We hypothesize that a deficiency in lymphocyte-derived CCL5 might lead to an 'immune-cold' microenvironment, potentially impairing immunosurveillance and facilitating local tumor spread, including bone invasion. Furthermore, emerging evidence suggests that CCL5 might directly interfere with osteoclast differentiation, making its absence a potential driver of bone resorption.

In this retrospective analysis, we assessed the role of CCL5 expression in samples available online from the TCGA cancer database to establish a transcriptomic baseline across tumor stages. Secondly, we analyzed the immunohistochemical expression of CCL5 in primary tumors and the tumor immune microenvironment in a large clinical cohort of patients presenting with or without mandibular bone invasion. Furthermore, we characterized the TME by analyzing the presence of immunological cells (CD4+, CD8+, FoxP3 + T cells as well as CD163 + M2 macrophages). By correlating CCL5 levels with clinical outcomes and bone invasion status, we wanted to clarify whether a CCL5-deficient TME serves as a facilitator for mandibular involvement and a biomarker for poor survival.

Materials and methods

mRNA expression analysis using the cBioPortal database

CBioPortal (https://www.cBioPortal.org, accessed on November 15th, 2025) was used to analyze mRNA expression profiles of CCL5. For this, Head and Neck Squamous Cell Carcinoma TCGA Firehose Legacy data (n = 530) were selected. Primary tumor sites were set as “oral tongue,” “oral cavity,” “floor of mouth,” “buccal mucosa,” “base of tongue,” “alveolar ridge,” “hard palate,” and “lip” to focus specifically on oral cavity subsites. Only primary tumor samples were included (n = 327). mRNA expression levels were normalized (Z-scores) relative to diploid tissue. To assess the prognostic value of transcriptomic levels, the cohort was dichotomized into “CCL5 low” (bottom 50%) and “CCL5 high” (upper 50%) based on the median expression. For the stage-dependent analysis, CCL5 mRNA expression was treated as a continuous variable. For survival analysis, the cohort was dichotomized at the median.

Single-cell RNA sequencing data analysis

To validate the cellular origin of CCL5 and investigate potential immune-stromal interactions at single-cell resolution, we utilized the Tumor Immune Single-cell Hub 2 (TISCH2) database (https://tisch.comp-genomics.org/) [13]. This resource provides pre-processed single-cell RNA sequencing (scRNA-seq) data with standardized cell-type annotations. One dataset was selected for analysis based on tumor entity (OSCC/HNSCC) and the resolution of specific cell populations:

GSE172577 [14]: An OSCC-specific cohort comprising 6 patients and 51,728 cells was used to characterize the expression profiles of CCL5 across malignant and immune cell subsets.

Gene expression levels were visualized using violin plots to assess the distribution of target genes across major and minor cell lineages. The associations between gene expression and specific cell types were evaluated to corroborate the immunohistochemical findings (“immune-cold” phenotype) and to explore potential mechanistic links to bone invasion.

Patients and clinical cohort

Clinical and histopathological data of 206 OSCC patients treated at the Department of Oral and Maxillofacial Surgery between January 2004 and August 2024 were collected. Median follow-up time was 3.8 years (95% CI: 2.9–4.6 years), with a maximum follow-up of 17.5 years. Characteristics are summarized in Table 1. Inclusion criteria were primary OSCC of the floor of the mouth or the lower alveolar gingiva with close contact to the mandible. Malignancies which developed from adjacent anatomic regions such as the maxilla or oropharyngeal carcinomas were excluded. Tumors diagnosed at a very early stage (Tis, T1) were also not included in this study. In addition, preoperative blood samples from tumor patients were used to calculate the neutrophil-to-lymphocyte ratio (NLR) and the C-reactive protein-to-albumin ratio (CAR). NLR data were available for 102 of 206 patients. Based on the median, an NLR > 3.2 was defined as high and an NLR ≤ 3.2 as low. For CAR calculation, data was available in 95 out of 206 patients. The mean CAR was 0.08; values > 0.08 were considered high, and values ≤ 0.08 as low.

Assessment of tumor invasion

Tumor invasion patterns were classified using the Worst Pattern of Invasion (WPOI) scoring system [15]. Mandibular bone invasion (MBI) was confirmed histopathologically by a board-certified pathologist (W. F.) in all T4a cases. Hematoxylin and eosin (H&E) stained slides were analyzed for the extent of invasion into adjacent tissue and the mandible if microscopic bone involvement was observed. WPOI scores of 1–3 indicated non-aggressive behavior, while scores 4–5 indicated aggressive invasion [16].

Immunohistochemical sample preparation and staining protocol

Tissue microarrays (TMAs) were assembled from three 1.5 mm tissue cylinders per patient from different areas of the bone-tumor interface, totaling 618 samples. To ensure representativeness, cylinders were specifically sampled from the invasive front of the tumor. For T4a tumors with confirmed MBI, this corresponded to the bone-tumor interface. For T2/T3 tumors without bone invasion, cylinders were obtained from the deepest point of invasion in the area of closest proximity to the mandible. TMAs were cut into three-micrometer sections and mounted onto Superfrost® Plus Microscope Slides (Thermo Scientific, Waltham, MA, USA) [17]. Slides were deparaffinized overnight at 37°C and rehydrated via serial passages through xylene (three times, 10 min each) and a downward-graded series of alcohol. Antigen retrieval was performed in a Decloaking chamber™ (Biocare Medical, Concord, CA, USA) in TRIS/EDTA buffer for 5 min at 120°C and 15 bars. Endogenous peroxidase was blocked with Peroxidase Blocking Solution (Dako, Glostrup, Denmark). Primary antibody incubation was performed by using the following antibodies: anti-CCL5 polyclonal anti-rabbit IgG antibody (abcam, Cambridge, UK) diluted to a concentration of 1:200; anti-FOXP3 monoclonal anti-mouse IgG antibody (eBioscience, Thermo Fisher Scientific, San Diego, CA, USA), diluted to a concentration of 1:60. Staining was performed using the Dako EnVision+™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark). Slides were counterstained with hematoxylin.

IHC staining assessment

Assessment and analysis of staining was performed by an experienced pathologist and two trained investigators. The scoring system was designed to distinguish between tumor-intrinsic expression and the immunological response in the TME.

Tumor Expression: Positive if > 20% of tumor cells showed

Table 1
Overview of patients' characteristics and association with immunohistochemical CCL5 expression.

Parameter		CCL5 primary tumor expression (N = 206)			CCL5 TME expression (N = 206)			FoxP3 + Treg count (N = 202)		
		Negative	Positive	p-value	Low	High	p-value	Low	High	p-value
Age (mean 63.4 years; 38–89 years)										
< 63.4 years	**113**	64	49		55	58		60	50	
≥ 63.4 years	**93**	46	47	0.304	48	45	0.674	41	51	0.203
Sex										
male	**159**	87	72		78	81		76	79	
female	**47**	23	24	0.433	25	22	0.618	25	22	0.739
Anatomic Site										
Gingiva	**109**	48	61		58	51		52	56	
Floor of mouth	**97**	62	35	**0.004**	45	52	0.329	49	45	0.672
Tobacco Use										
Yes	**145**	83	62		67	78		75	66	
No	**61**	27	34	0.088	36	25	0.093	26	35	0.220
Alcohol Use										
Yes	**120**	69	51		51	69		59	58	
No	**86**	41	45	0.163	52	34	**0.011**	42	43	1.000
T Stage										
T2 + T3 (= no MBI)	**95**	50	45		38	57		33	61	
T4a (= MBI)	**111**	60	51	0.838	65	46	**0.008**	68	40	**<0.001**
N Stage										
N0	**110**	56	54		56	54		51	58	
N+	**96**	54	42	0.443	47	49	0.780	50	43	0.397
Grade										
G1 + G2	**160**	89	71		75	85		82	75	
G3	**46**	21	25	0.232	28	18	0.094	19	26	0.310
Tumor Recurrence										
Yes	**53**	29	24		27	26		29	32	
No	**153**	81	72	0.823	76	77	0.873	72	78	0.421
WPOI Score										
Non-aggressive	**77**	43	34		36	41		40	34	
Aggressive	**129**	67	62	0.548	67	62	0.471	61	67	0.465

membranous/cytoplasmic staining. This cut-off was determined according to Fujimoto et al. [18].

TME Expression: CCL5-positive lymphocytes were quantified and averaged across the three samples. High expression was defined as a count > 78, representing the median value of the clinical cohort.

Immune cells: CD4, CD8, CD163 and FoxP3 were quantified by counting positive cells in high-power fields (HPF) at x400 magnification. Counting was done manually to ensure reproducibility; the area with the highest number of cells of interest was counted in each core of the TMA and a mean value was calculated over three samples.

Statistical analysis

Statistical analysis was performed using SPSS31 software (IBM Germany GmbH, Ehningen, Germany). Correlations were calculated using Pearson's Chi square test or Fisher's exact test. Univariable survival analysis for overall survival (OS), disease free survival (DFS) and disease-specific survival (DSS) was performed using the Kaplan-Meier-method and log-rank tests. OS was defined as time from diagnosis to death by any cause, DFS as time from therapy to tumor recurrence or death, and DSS as time from diagnosis to tumor-related death. Median follow-up was calculated using the reverse Kaplan-Meier-method. Variables with $p \leq 0.1$ in univariable analysis were included in a multivariable Cox proportional hazards model using backward elimination (Wald) to identify independent prognostic factors. Potential collinearity was ruled out using VIF calculation; all VIF coefficients were < 5.

Results

CCL5 mRNA expression in the TCGA cohort

To obtain an initial overview of the relevance of CCL5 in OSCC, transcriptomic CCL5 expression was analyzed in an online TCGA cohort (n = 327). CCL5 mRNA expression showed a significant stage-dependent decline, progressively decreasing from T1 to T4a tumors ($p = 0.038$). Notably, the lowest transcript levels were observed in T4a cases, which are clinically defined by mandibular bone invasion (Fig. 1A).

Kaplan-Meier analysis demonstrated a significant association between CCL5 mRNA expression and overall survival. Patients with high CCL5 mRNA expression showed significantly improved overall survival compared with those with low expression ($p = 0.026$). Median overall survival was 71.2 months in the high CCL5 group versus 32.8 months in the low CCL5 group (Fig. 1B).

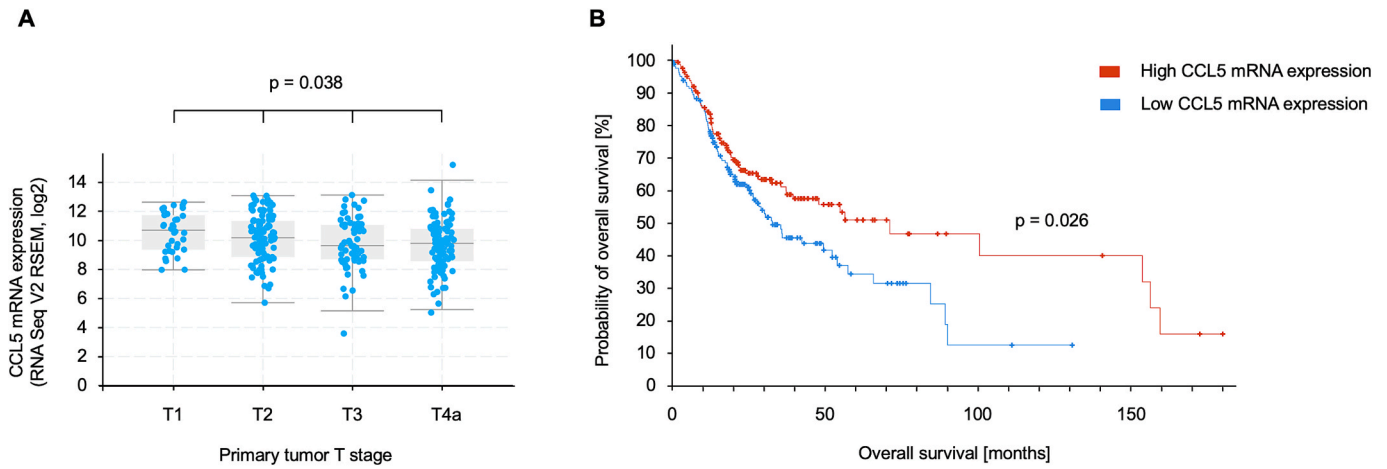

Fig. 1. A CCL5 mRNA expression in stages T1-T4a in the TCGA cohort decreased with advancing T stage (p = 0.038). B Kaplan Meier analysis revealed high CCL5 mRNA expression to be beneficial to overall survival (p = 0.026).

Single-Cell RNA sequencing data analysis

In the GSE172577-cohort, CCL5 was predominantly expressed by CD8 + T cells, Tregs and Natural Killer (NK) cells, but not by other cell types, especially not in tumor cells.

Patient characteristics and association with CCL5 expression

Given the observed association of CCL5 mRNA expression with tumor stage and overall survival, we next investigated CCL5 protein expression and its clinicopathological correlates in our independent clinical cohort (Fig. 2).

Table 1 summarizes the cohort characteristics. The majority of patients were male (77.2%) with a mean age of 62.2 years. Primary tumors were localized in the floor of the mouth (47.1%) or the lower alveolar gingiva (52.9%). Mandibular bone invasion (MBI) was histopathologically confirmed in 53.9% of cases (n = 111).

CCL5 immunohistochemistry was evaluable in all cases. In the primary tumor, 53.4% of cases were classified as CCL5-negative (< 20%). In the TME, low CCL5 expression in lymphocytes was significantly associated with two key factors: alcohol consumption status (p = 0.011) and, most importantly, the presence of MBI (58.6% in MBI + vs. 40.0% in MBI-; p = 0.008). CCL5 expression in the primary tumor was significantly associated with the anatomic site; tumors of the floor of the mouth showed a higher frequency of CCL5-negative cases compared to gingival tumors (p = 0.004; see Table 1). No significant correlations were found between CCL5 expression and N-stage or histological grade.

Expression correlation analysis with CD4+, CD8 + T cells and M2 macrophages was available in 161 cases, FoxP3 evaluation was available in 202 cases. The highest correlation was with CD8 + T cells (Spearman's $\rho = 0.558$, $p < 0.001$), followed by CD4 + T cells (Spearman's $\rho = 0.475$, $p < 0.001$) and M2 macrophages (Spearman's $\rho = 0.178$, $p = 0.024$). Additionally, regulatory T cells (FoxP3 + Tregs) were quantified and correlated with CCL5 TME expression (Spearman's $\rho = 0.300$, $p < 0.001$).

Representative staining patterns are shown in Fig. 3.

Analysis of systemic inflammatory markers (NLR and CAR)

Given the growing interest in systemic inflammatory markers, the prognostic relevance of the neutrophil-to-lymphocyte ratio (NLR) and the C-reactive protein-to-albumin ratio (CAR) has increasingly been investigated in oral squamous cell carcinoma (OSCC). To evaluate the systemic immune status, we analyzed NLR (n = 102) and CAR (n = 95). The median NLR was 3.2 and the median CAR was 0.08.

Data required for NLR calculation were available for 102 patients. Of these, 51 patients were classified as having a low NLR (≤ 3.2), while 51 patients had a high NLR (> 3.2). CAR analysis was performed

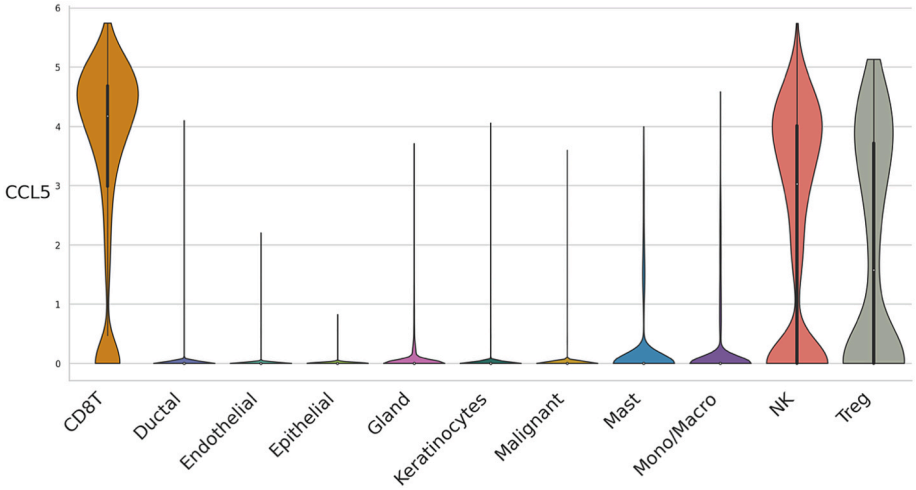

Fig. 2. Violin plot of CCL5 expression in the GSE172577-cohort. CCL5 was predominantly expressed by tumor infiltrating lymphocytes (CD8 + T cells, Tregs and NK cells, respectively). In malignant cells no clinically relevant CCL5 expression was detectable.

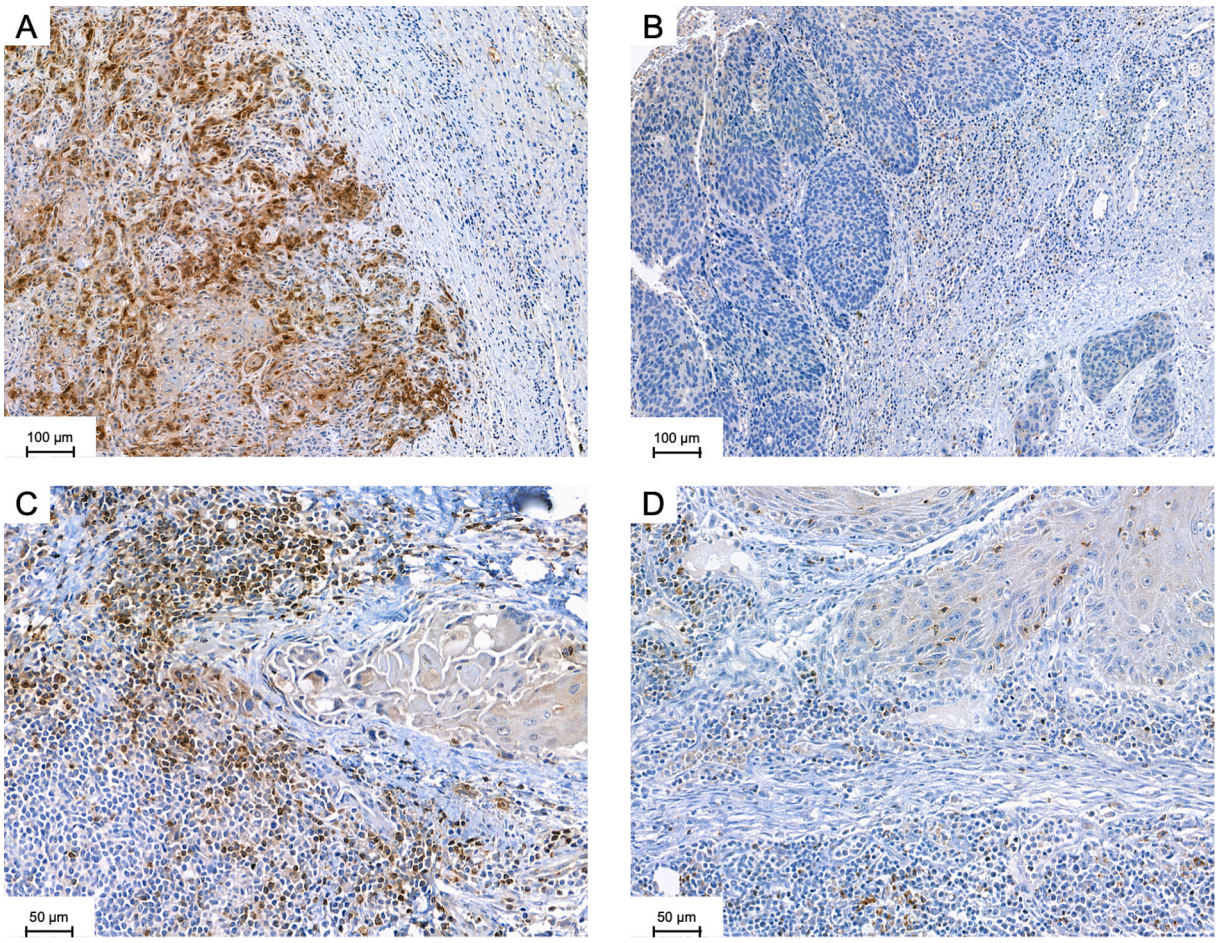

Fig. 3. Staining results for CCL5 in primary tumors and tumor immune microenvironment. **A** Example of a tumor classified as CCL5 positive (100x magnification). **B** Example of a CCL5 negative tumor (100x magnification). **C** Tumor immune microenvironment with a high quantity of CCL5 positive lymphocytes (200x magnification). **D** Tumor immune microenvironment with a low count of CCL5 positive lymphocytes (200x magnification).

analogously. CAR data were available for 95 patients; 48 patients exhibited a high CAR (> 0.08), whereas 47 showed a low CAR (≤ 0.08).

Survival analysis

To assess the prognostic value of CCL5 TME and tumor expression and clinicopathological parameters, survival analysis was carried out as previously described.

Kaplan-Meier analysis revealed that a CCL5-deficient TME was strongly associated with poor prognosis. Patients with low CCL5 expression in the TME showed significantly shorter overall survival ($p = 0.001$). Similarly, negative CCL5 expression in the primary tumor was associated with both poorer OS ($p = 0.038$) and disease-specific survival (DSS; $p = 0.012$; Fig. 4).

Multivariable analysis (Cox proportional hazards model) confirmed the independent prognostic value of these markers. High CCL5 expression in the TME (HR = 0.294; 95% CI: 0.110–0.785; $p = 0.015$) was an independent predictor for better OS. Additionally, high NLR correlated with poor overall survival (HR = 2.664p = 0.044). For disease-specific survival, CCL5 tumor expression remained a significant independent predictor (HR = 0.526; $p = 0.035$). Furthermore, negative CCL5 tumor expression was significantly associated with poorer disease-specific survival ($p = 0.012$), whereas CCL5 expression in the tumor microenvironment was statistically not significant for disease-specific survival ($p = 0.297$). None of our markers were significantly associated with disease-free survival. Other markers relevant for outcome are listed in Tables 2A and B. Positive CCL5 tumor expression also was predictive for better disease-specific survival (HR = 0.526; $p = 0.035$). None of our markers demonstrated a significant predictive value for DFS.

Discussion

This retrospective study provides further insight into the complex role of CCL5 and FoxP3 in cancer and especially OSCC. Our data demonstrate the presence of analogous effects of CCL5 expression in OSCC at the transcriptomic and proteomic levels in two independent cohorts. The central finding is that a deficiency in CCL5, both at the mRNA level and within the tumor immune microenvironment, serves as a facilitator for mandibular bone invasion (MBI) and a potent indicator of poor survival. The biological rationale for these findings likely lies in the recruitment of anti-tumor effector cells.

CCL5 is a classical chemokine that recruits diverse immune effector cells, including T lymphocytes, natural killer (NK) cells, and dendritic cells, via interactions with receptors such as CCR5 to the tumor site [19]. In preclinical models, CCL5 has been shown to mediate chemotaxis of $CD4^+$ T cells and, in some contexts, indirectly support $CD8^+$ T cell recruitment, suggesting that loss of CCL5 may lead to impaired immune infiltration and reduced immunosurveillance in OSCC. In our cohort, the significant association between low CCL5 expression in the TME, missing FoxP3 + cells, and the presence of MBI ($p = 0.008$ or $p < 0.001$, respectively) suggests that a CCL5-deprived environment represents an "immune-cold" niche. In such a microenvironment, the lack of immunosurveillance may allow the tumor to expand unchecked into the adjacent bone tissue. This is also supported by the fact of a low FoxP3 +

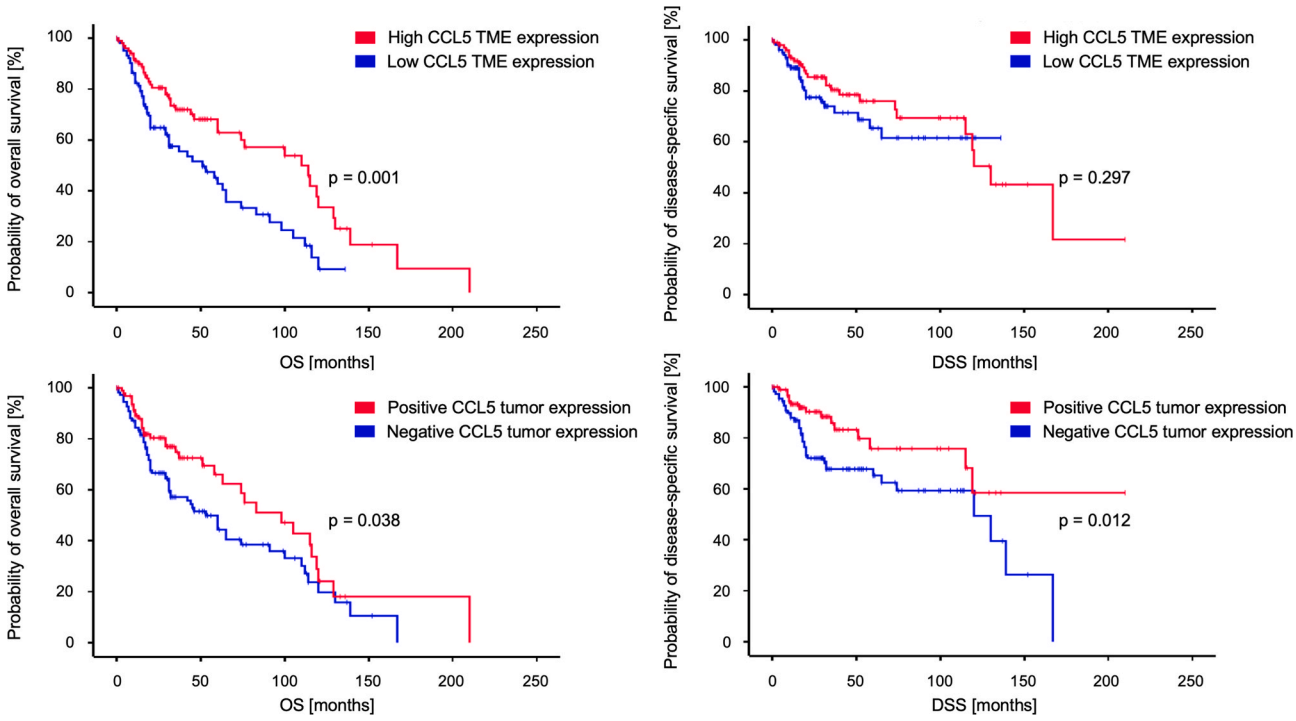

Fig. 4. A and B Kaplan-Meier analysis of CCL5 TME expression revealed a beneficial role for high CCL5 TME expression regarding overall, but not disease-specific survival (p = 0.001 or 0.297, respectively). **C and D** For CCL5 tumor expression, positive CCL5 tumors were associated both with favorable overall and disease-specific survival (p = 0.038 or 0.012, respectively).

Table 2A
Results of univariable and multivariable analysis (backward elimination) for overall survival.

Parameter	Univariable	Multivariable (backward elimination)		HR (95% CI)
	Log-rank	Step 1	Step 2	
CCL5 TME (high vs. low)	**0.001**	**0.015**	–	**0.294 (0.110 – 0.785)**
Neutrophile/Lymphocyte ratio (high vs. low)	**0.019**	**0.044**	–	**2.664 (1.028 – 6.906)**
CCL5 tumor (positive vs. negative)	**0.038**	0.062	–	**0.400 (0.153 – 1.045)**
Relapse (yes vs. no)	**0.033**	0.051	–	2.008 (0.635 – 5.713)
WPOI (non-aggressive vs. aggressive)	**0.016**	0.407	–	1.904 (0.821 – 4.912)

HR: Hazard ratio; *CI:* Confidence interval.

Table 2B
Results of univariable and multivariable analysis (backward elimination) for disease-specific survival.

Parameter	Univariable	Multivariable (backward elimination)		HR (95% CI)
	Log-rank	Step 1	Step 2	
CCL5 tumor (positive vs. negative)	**0.012**	**0.035**	–	**0.526 (0.283 – 0.979)**
Histological Grade (G3 vs. G2 + G1)	**0.004**	0.051	–	1.905 (1.025 – 3.539)
Relapse (yes vs. no)	**0.006**	0.057	–	1.773 (0.996 – 3.157)
WPOI (non-aggressive vs. aggressive)	**0.039**	0.074	–	1.703 (0.930 – 3.092)

HR: Hazard ratio; *CI:* Confidence interval.

Treg count in the TME, which was highly significant for MBI in our cohort.

A CCL5-deprived TME could subsequently lead to enhanced invasional capabilities affecting MBI and survival [20,21]. Furthermore, our results point toward a direct link between immune signaling and bone homeostasis. Our results support the fact that the presence of CCL5 prohibits osteoclastogenesis [22]. Preclinical studies have shown that mice lacking CCL5 exhibit impaired bone formation and increased osteoclast activity [23]. Thus, the loss of CCL5 in the OSCC microenvironment might not only impair the immune response but also "release the brakes" on osteoclast-mediated bone resorption, thereby actively facilitating mandibular invasion. This dual role – as an immune recruiter and an osteoclast inhibitor – makes CCL5 a critical gatekeeper at the tumor-bone interface.

Analyses of CCL5 across different cancer entities have highlighted its context-dependent roles. In colorectal and hepatocellular cancer, elevated CCL5 expression has been linked to poorer prognosis, increased tumor aggressiveness, and enhanced immune evasion. These effects have been attributed, at least in part, to CCL5-mediated stabilization of immunosuppressive proteins such as PD-L1, as well as activation of the CCR5/CCL5 axis, which promotes the recruitment of immunosuppressive Tregs and M2-polarized macrophages into the tumor microenvironment [24–26]. However, our findings align more closely with observations in breast and small cell lung cancer, where CCL5-mediated infiltration of CD8 + T cells suppresses tumor growth [27]. In OSCC, the literature is divided. While some studies suggest CCL5 promotes metastasis [10–12], these often focus on tumor-cell-intrinsic signaling or cancer-associated fibroblasts (CAFs). One larger, comparable study including 217 patients demonstrated that CCL5 activates CAFs within the OSCC tumor microenvironment, thereby promoting protumorigenic effects [28]. Our study distinguishes itself by specifically quantifying CCL5 within the TME (tumor infiltrating lymphocytes). This suggests that the source and localization of CCL5 are decisive: while tumor-derived CCL5 might support EMT and migration, lymphocyte-derived CCL5 in the TME appears to be a protective factor which prevents

local bone invasion.

The absence of FoxP3 + Tregs also supports an immune-cold TME. While their prognostic role remains context-dependent, evidence in OSCC links their presence – particularly at the invasive front – to improved outcomes, in contrast to detrimental associations reported in HNSCC [29–31]. In our cohort, loss of FoxP3 + Tregs was strongly associated with MBI ($p < 0.001$), but did not translate into prognostic relevance. This discrepancy may be explained by the context-dependent and partly contradictory prognostic role of FoxP3 + Tregs in head and neck cancer: while their presence at the invasive front has been associated with improved outcomes in OSCC – possibly reflecting an overall higher immune infiltration – detrimental associations have been reported in HNSCC more broadly [29,30]. It is therefore conceivable that Treg infiltration in our cohort reflects a general marker of immune activity rather than an independent prognostic driver, with CCL5 serving as the more functionally relevant determinant of survival.

Another finding supporting the immune-cold phenotype observed in our cohort was the neutrophil-to-lymphocyte ratio (NLR), which emerged as a significant prognostic marker. A high NLR reflects both an increased presence of neutrophils and a relative depletion of tumor-infiltrating lymphocytes (TILs), collectively undermining the anti-tumor immune response and negatively impacting patient survival. To validate the cellular source of CCL5 within the specific context of OSCC, we analyzed single-cell RNA sequencing data from an independent external cohort [14]. This analysis confirmed that CCL5 expression is predominantly restricted to infiltrating lymphocytes (CD8 + T cells, Tregs) and NK cells, while being virtually absent in malignant epithelial cells. Consequently, the immunohistochemical loss of CCL5 observed in our MBI-positive cohort serves as a robust surrogate for an 'immune-cold' phenotype, characterized by the specific exclusion of cytotoxic effector cells that would otherwise constrain tumor progression. The fact that both low CCL5 in the TME and a high NLR independently predicted poor overall survival (Tables 2) reinforces the hypothesis that systemic and local immune exhaustion are major drivers of OSCC progression.

Consistent with our observations, elevated NLR has been repeatedly associated with poor prognosis in OSCC in previous studies [32–34]. The C-reactive protein-to-albumin ratio (CAR), another potential marker of systemic inflammation, did not emerge as a significant prognostic factor in our analysis, which may be attributable to the limited sample size for this sub-cohort [35]. Further studies with larger patient numbers are warranted to clarify its role in bone-invasive OSCC. Tumor stage was evaluated in univariate survival analysis but did not reach the predefined inclusion threshold of $p \leq 0.1$ for the multivariable Cox model (univariable log-rank $p = 0.119$). This is likely attributable to the restricted T-stage range of our cohort (T2-T4a only) and the inherent overlap between T4a classification and MBI status by definition, which limits the independent prognostic contribution of stage in this specific population.

Despite the clear clinical correlations, this study has limitations. Its retrospective design and the use of TMAs, while efficient, may not capture the full heterogeneity of the tumor. The rigorous selection criteria applied to anatomic site and T-stage could result in a form of selection bias, which could lead to the inclusion of more aggressive tumors. Furthermore, while CD4+, CD8+, CD163 + and FoxP3 + Treg subpopulations were characterized in a subset of patients, a more comprehensive functional characterization of CCL5-recruited effector cells remains an important avenue for future studies. Additionally, an external validation cohort for immunohistochemical cut-off values is missing. Lastly, future functional assays are needed to decipher the exact crosstalk between CCL5-positive lymphocytes and osteoclasts at the invasive front. The extended recruitment period (January 2004 – August 2024) is acknowledged as a limitation. However, standard-of-care for resectable OSCC – surgery followed by risk-adapted adjuvant (chemo-)radiotherapy – has remained essentially unchanged over this period. To our knowledge, no patient in this cohort received immune checkpoint inhibitor therapy, which is currently approved only for recurrent/metastatic HNSCC and not for primary curative-intent treatment of resectable oral cavity tumors.

Conclusion

This study highlights once more the complex role of CCL5 in cancer, and especially bone-invasive OSCC. We demonstrate that a CCL5-deficient TME is a hallmark of aggressive tumors with a high propensity for mandibular bone invasion. The effect of CCL5 seems to depend mainly on the composition of the TME. Furthermore, our findings support the role of NLR as a prognostic biomarker. CCL5 may serve as a valuable tool for identifying high-risk patients who might benefit from more intensive monitoring or targeted immunotherapies.

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Data availability

The data presented in this study are available on reasonable request from the corresponding author.

CRediT authorship contribution statement

Jonas Eichberger: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Hammer:** Writing – review & editing, Investigation, Data curation. **Michael Gerken:** Writing – review & editing, Validation, Software, Formal analysis. **Daniela Schulz:** Writing – review & editing, Resources, Investigation. **Mathias Fiedler:** Investigation, Data curation. **Katja Himmelstoß:** Investigation, Data curation. **Johannes Schuderer:** Visualization, Investigation. **Richard Bauer:** Writing – review & editing, Resources. **Torsten Eugen Reichert:** Writing – review & editing, Supervision. **Florian Weber:** Writing – review & editing, Validation, Resources, Data curation. **Tobias Ettl:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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