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Research Paper
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Regulatory T cells and M2-polarized tumour-associated macrophages are associated with the oncogenesis and progression of oral squamous cell carcinoma

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Abstract. Regulatory T cells (Tregs) and tumour-associated macrophages (TAMs) contribute to the tumour microenvironment by inhibiting anti-tumour immune responses. This study was performed to investigate the roles of Tregs and TAMs in oral squamous cell carcinoma (OSCC) and oral epithelial precursor lesions (OEPL). The expression of Treg markers CD25 and FoxP3 and TAM markers CD163 and CD204 was investigated in 82 OSCC and 45 OEPL specimens, and their associations with clinicopathological parameters were analyzed. Correlations were found among CD25, FoxP3, CD163, and CD204 levels ($P < 0.001$), and these targets were up-regulated in OSCC compared to OEPL ($P < 0.001$). In OSCC, infiltration of Tregs and/or M2 TAMs was associated with sex and clinicopathological features, such as tumour size, nodal metastasis, tissue differentiation, stromal reaction, invasive behaviour, and invasive depth. In OEPL, CD25, FoxP3, CD163, and CD204 immunoreactivities were significantly associated with sex, postoperative recurrence, and cancerization to OSCC. This study is novel in showing that the infiltration of Tregs and M2 TAMs is significantly associated with the progression of premalignant lesions to OSCC. This suggests that these cells represent prognostic biomarkers for premalignant lesion progression and that immunotherapeutic approaches to control Treg/M2 TAM numbers could protect against progression to malignancy.

Key words: regulatory T cell; tumour-associated macrophage; oral cancer; squamous cell carcinoma; oral epithelial precursor lesions.

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Oral squamous cell carcinoma (OSCC), an invasive epithelial neoplasm with varying degrees of squamous differentiation, is the most common malignancy of the oral cavity[1]. Oral epithelial precursor lesions (OEPL) are defined as morphologically altered tissue in which OSCC is more likely to occur than in apparently normal oral mucosa. OSCC may evolve from oral mucosal lesions with OEPL, such as benign epithelial hyperplasia without dysplasia, mild to severe epithelial dysplasia, or carcinoma in situ[2].

The tumour microenvironment, which is formed by multiple cellular and molecular interactions, plays an important role in mediating the biological behaviours of cancer, including OSCC[3]. The host immune response, which is represented by tumour-infiltrating immune/inflammatory cells and secreted molecules, is a key factor shaping the tumour microenvironment. Recent studies have shown that several types of tumour-infiltrating immune/inflammatory cells, such as regulatory T cells (Tregs) and alternatively activated macrophages, support tumour growth and expansion by suppressing host immune responses and accelerating angiogenesis and tissue remodelling[4].

Immunosuppressive Tregs down-regulate the induction of autoreactive T cells and maintain tolerance to self-antigens, thus inhibiting autoimmune reactions. Previous reports have shown that the assembly and multiplication of Tregs are associated with advanced tumour growth and unfavourable clinical characteristics in some types of malignant tumour[3]. Macrophages are important cancer stromal cells involved in immunological self-tolerance, and the infiltration of tumour-associated macrophages (TAMs) is correlated with the prognosis in several types of solid tumour, including oral cancer[5]. There are two different activation states of macrophages, M1 and M2. M1 macrophages are responsible for type I inflammatory responses, which are guided and accompanied by the elimination of pathogens and tissue damage[6]. In contrast, M2 macrophages cause type II responses, including immune reactions involved in wound healing, tissue reconstruction, and angiogenesis, and are associated with carcinogenesis and tumour progression[7].

Although the association of Tregs and M2 TAMs with various types of malignant tumour has been reported[8–14], the roles of these tumour-infiltrating inflammatory cells in OSCC and OEPL have not been investigated fully, and it is unclear how Tregs and M2 TAMs influence the clinicopathological behaviour of these lesions.

This study was performed to examine the correlations between Treg and M2 TAM infiltration in OSCC and OEPL specimens based on the expression of the M2 macrophage markers CD163 and CD204 and the Treg markers CD25 and FoxP3, as determined using immunohistochemistry. Furthermore, the associations between the presence of Tregs and M2 TAMs in tumours and clinicopathological characteristics were investigated in patients with OSCC and OEPL.

Materials and methods

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry. The aims and design of the study were explained to the patients and written informed consent was obtained.

Clinicopathological characteristics

Biopsy specimens were obtained from 82 patients with primary OSCC and 45 patients with OEPL by surgical resection performed in the Department of Oral and Maxillofacial Surgery, Tohoku University Hospital from 2010 through 2013.

No patients with OSCC received chemotherapy or radiotherapy before surgery. Patients with OSCC (36 male and 46 female) ranged in age from 27 to 93 years (mean 67.07 years). Carcinomas were located in the tongue ($n = 46$), buccal mucosa ($n = 10$), lower gingiva ($n = 10$), upper gingiva ($n = 8$), floor of the mouth ($n = 5$), and lower lip ($n = 3$). TNM disease stages obtained from the medical records were classified according to the Union for International Cancer Control system[15]. Stage T1 was detected in 29 patients, T2 in 27 patients, T3 in seven patients, and T4 in 19 patients. Stage N0 was observed in 65 patients, N1 in 10 patients, N2 in six patients, and N3 in one patient. Stage M0 was observed in 78 patients and M1 in four patients. According to overall TNM staging, 27 had stage I OSCC, 21 had stage II, 10 had stage III, and 24 had stage IV. During the follow-up period (37–74 months), 18 patients had local recurrence and 10 had postoperative metastasis; 15 patients died of OSCC and three died of unrelated causes.

No patients with OEPL were diagnosed with OSCC at any site before surgery. Patients with OEPL (21 male and 24 female) ranged in age from 30 to 89 years (mean 67.31 years). Nineteen OEPL were located in the tongue, 15 were located in the lower gingiva, six were located in the buccal mucosa, three were located in the upper gingiva, and two were located in the hard palate. Lesion diameters ranged from 2 mm to 60 mm (mean 19.07 mm). During the follow-up period (37–66 months), two patients with severe dysplasia had local recurrence. Ten patients with severe dysplasia showed cancerization from OEPL to OSCC; while these cases were originally diagnosed with severe dysplasia based on biopsy specimens, they were diagnosed with OSCC based on surgical specimens or additional biopsy specimens taken during the follow-up period (37–66 months). For this study, the 45 patients with OEPL were chosen at random. No selected patient had been diagnosed with OSCC at any site before surgery and the 10 cases with cancerization from OEPL to OSCC did not overlap with any of the 82 OSCC cases. The areas that most noticeably showed the features of OSCC and OEPL were chosen and evaluated as specimens for this study.

Sample preparation

Tissue samples were fixed in 10% buffered formalin for several days, embedded in paraffin, and sliced into 3-μm-thick sections for routine histological examination and subsequent immunohistochemical detection of CD25, FoxP3, CD163, and CD204. The 82 OSCC samples were classified into well-differentiated (60 cases), moderately differentiated (20 cases), and poorly differentiated (two cases) types according to the World Health Organization (WHO) classification of tumours of the oral cavity and oropharynx[1]. The degree of stromal lymphocytic reaction in OSCC was classified as slight for 11 samples, moderate for 69 samples, and severe for two samples. The mode of OSCC invasion was classified as described previously[1]; carcinoma was grade 2 in 12 specimens, grade 3 in 47 specimens, grade 4C in 13 specimens, and grade 4D in 10 specimens. OSCC invasion depth was classified as micro-invasion in six tumours, mucosal invasion in 34 tumours, and submucosal invasion in 42 tumours.

The pathological diagnosis of OEPL was made according to the WHO classification of tumours of the oral cavity and oropharynx[2]. Fifteen patients had leukoplakia without epithelial dysplasia (hyperkeratosis or hyperplasia (HP)), 15 had low-grade epithelial dysplasia (mild to

moderate dysplasia (LD)), and 15 had high-grade epithelial dysplasia (severe dysplasia or carcinoma in situ (HD)).

Immunohistochemistry

Data on the specificity and sensitivity of antibodies against CD25 (interleukin 2 receptor alpha chain), FoxP3 (forkhead box P3), CD163 (macrophage scavenger receptor cysteine-rich type I), and CD204 (macrophage mannose receptor, C type I) in immunohistochemistry are controversial[8–14]. Therefore, the staining patterns of several commercially available antibodies were tested: those against CD25 and FoxP3 in human non-small cell lung carcinoma and those against CD163 and CD204 in lymphoma. The following antibodies were selected: anti-CD25 (clone 4C9) and anti-CD163 (clone 10D6), both from Novocastra Leica Biosystems (Newcastle Upon Tyne, UK); anti-FoxP3 (clone 236 A/E7; Abcam, Cambridge, UK); and CD204 (clone 10D6; Transgenic, Kumamoto, Japan). Slides were processed using an automated immunohistochemistry/in situ hybridization slide staining system (Ventana Benchmark ULTRA; Roche, Basel, Switzerland).

As Tregs have been reported to infiltrate the lamina propria and epithelium and TAMs have been shown to infiltrate tumour-neighbouring connective tissue[9–14], the expression of cell-specific biomarkers was analyzed in these tissues. CD25, CD163, and CD204 staining was evaluated based on the number of lymphocytes or macrophages exhibiting strong immunolabelling of cell membranes and/or cytoplasm, in accordance with previous studies[9,13,14]. FoxP3 expression was assessed based on the number of lymphocytes with nuclear staining, as described previously[10–12,14]. Areas into which Tregs and TAMs were recruited based on secondary tumour-dependent effects, such as stomatitis and tissue necrosis or ulceration, were not analyzed. Pathological examinations were performed, and immunolabelled lymphocytes and macrophages were counted under a microscope at 200× magnification by pathologists blinded to the clinical information associated with the samples. Average numbers were calculated based on the observation of five areas in each sample preparation.

Statistical analysis

All statistical analyses were performed using JMP Pro software (version 12.0; SAS Institute, Inc., Cary, NC, USA). Immunohistochemistry results and clinicopathological characteristics were analyzed using the Mann–Whitney *U*-test for differences between two groups or the Kruskal–Wallis *H*-test for differences among three or more groups. Pairwise comparisons of immunohistochemical expression levels were performed by determining the Pearson correlation coefficient (r). Analyses of various pairs of groups or subgroups were adjusted for multiple comparisons with the Bonferroni correction. Differences with P-values of less than 0.05 were considered statistically significant.

The required sample size was calculated after first examining a small number of samples in a pilot study, as follows: (1) Based on the results of the pilot study, the sample size was most critical when CD204 was tested. (2) When the effect size was 0.60, the ratio of OEPL to OSCC individuals was expected to be 1:2. (3) Under these conditions and a power of 0.8, the required sample size was determined to be 35 and 71 individuals in the OEPL and OSCC groups, respectively. (4) The time period of the experiment needed to reach and collect the required number of individuals was set. The Youden index, which maximizes 'sensitivity − (1 − specificity)', was used to determine cut-off values for biomarkers for discriminating among clinicopathological characteristics.

Results

Immunoreactivity for CD25, FoxP3, CD163, and CD204 in OEPL and OSCC

Typical staining results indicating the presence of Tregs and M2 TAMs in OEPL and OSCC samples are shown in Fig. 1. Tregs were detected among epithelial and tumour cells and neighbouring lymphocytes based on plasma membrane staining for CD25 and nuclear staining for FoxP3. TAMs were detected based on CD163 and CD204 expression mainly in the membrane and cytoplasm of macrophages around epithelial and tumour tissues. The results indicated that the expression of CD25, FoxP3, CD163, and CD204 tended to increase as the malignancy of the oral epithelial lesions increased (Fig. 2).

The quantitative analysis of immunohistochemical reactivity for CD25, FoxP3, CD163, and CD204 in OEPL and OSCC is presented in Table 1. The expression levels of CD25, FoxP3, CD163, and CD204 differed significantly between OEPL and OSCC ($P < 0.001$); among HP, LD, and HD groups in OEPL; and among HP, LD, and HD groups and OSCC. In particular, significant differences in the expression of all tested biomarkers were observed between HP and epithelial lesions with dysplastic changes.

Correlation analysis indicated that immunohistochemical staining for CD25, FoxP3, CD163, and CD204 was significantly associated both with OSCC ($P < 0.001$) and OEPL ($P < 0.001$) for examining pairwise correlations among CD25+ T cells, FoxP3+ T cells, CD163 + macrophages, and CD204+ macrophages in the 82 OSCC cases and 45 OEPL cases (Figs. 3 and 4).

Associations between clinical and pathological characteristics and immunoreactivity for CD25, FoxP3, CD163, and CD204 in OSCC

The results of the association analysis showed that the expression of CD25, FoxP3, CD163, and CD204 in the 82 OSCC specimens was not associated with some clinical variables, including tumour site, M classification, staging, recurrence, postoperative metastasis, and survival (Table 2). At the same time, CD204 expression was significantly associated with sex ($P < 0.01$). With regard to clinical characteristics, CD25 levels were significantly correlated with the size and/or expansion of the primary tumour (T classification; $P < 0.05$), while CD163 immunoreactivity was associated with the degree of spread to regional lymph nodes (N classification; $P < 0.05$).

Associations between pathological variables and CD25, FoxP3, CD163, and CD204 expression in OSCC are summarized in Table 3. High CD163 and CD204 expression was frequently observed in OSCC with poor differentiation ($P < 0.05$). Significant correlations were also found between CD25, FoxP3, and CD204 levels and stromal lymphocytic reaction ($P < 0.05$). Moreover, significant associations of all four markers with OSCC invasion depth ($P < 0.01$) and of CD25, FoxP3, and CD163 expression with OSCC mode of invasion ($P < 0.01$) were observed.

Associations between clinical characteristics and CD25, FoxP3, CD163, and CD204 expression in OEPL

The results of the association analysis are summarized in Table 4. CD25, FoxP3, CD163, and CD204 immunoreactivity in the 45 OEPL specimens did not significantly correlate with demographic and clinical variables such as age and tumour site or size. However, the expression of CD25 and FoxP3 was significantly asso-

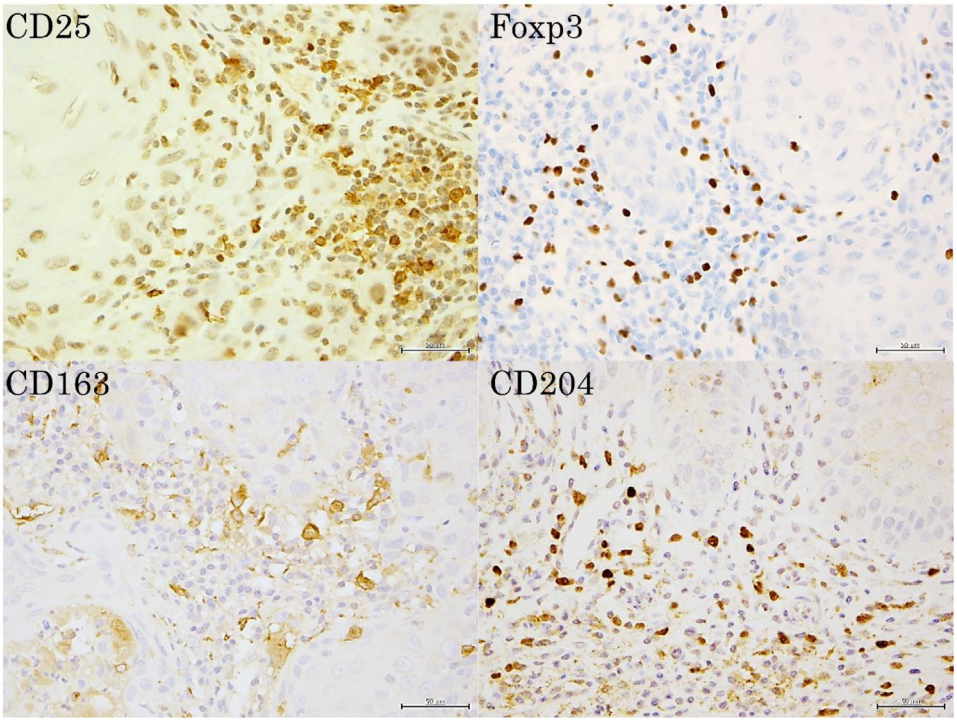

Fig. 1. Expression of CD25, FoxP3, CD163, and CD204. The expression of CD25, FoxP3, CD163, and CD204 was analyzed in oral squamous cell carcinoma and epithelial precursor lesion tissues by immunohistochemistry. Tregs were detected among epithelial and tumour cells and neighbouring lymphocytes based on membrane staining for CD25 and nuclear staining for FoxP3. Macrophages were detected in neighbouring epithelial and tumour tissues by CD163 and CD204 expression, observed mainly in the cell membrane and cytoplasm. Magnification, 200 × .

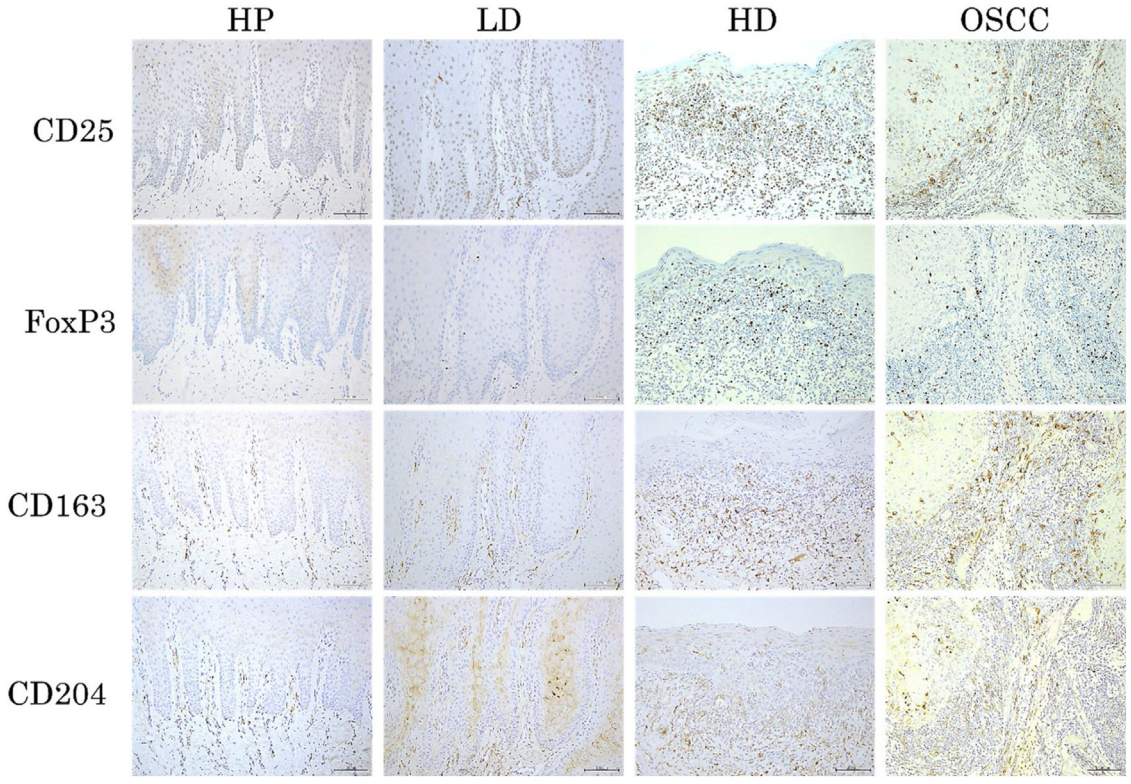

Fig. 2. Representative images of CD25, FoxP3, CD163, and CD204 expression in oral epithelial precursor lesions and oral squamous cell carcinoma. The expression of CD25, FoxP3, CD163, and CD204 tended to increase with the malignancy of oral epithelial legions. OSCC, oral squamous cell carcinoma; HP, hyperplasia; LD, low-grade epithelial dysplasia; HD, high-grade epithelial dysplasia. Magnification, 100 × .

Table 1. Immunohistochemical reactivities of CD25, FoxP3, CD163, and CD204 in OEPL and OSCC.

	Number of cases	Tregs		TAMs	
		$CD25^+$ T cell count	$FoxP3^+$ T cell count	$CD163^+$ macrophage count	$CD204^+$ macrophage count
OEPL	45	20.69 ± 17.46	21.56 ± 20.96	24.38 ± 22.08	20.84 ± 28.94
Leukoplakia without epithelial dysplasia (hyperkeratosis or hyperplasia)	15	4.59 ± 5.69	5.84 ± 7.96	10.57 ± 11.80	3.87 ± 5.60
Low-grade epithelial dysplasia (mild to moderate dysplasia)	15	20.68 ± 13.32	19.55 ± 14.44	21.81 ± 13.83	18.73 ± 18.57
High-grade epithelial dysplasia (severe dysplasia or CIS)	15	36.80 ± 13.88	30.08 ± 23.41	40.76 ± 26.51	39.92 ± 39.41
OSCC	82	49.76 ± 28.07	78.29 ± 63.43	42.92 ± 18.97	36.57 ± 17.45

OEPL, oral epithelial precancerous lesion; CIS, carcinoma in situ; OSCC, oral squamous cell carcinoma; Tregs, regulatory T cells; TAMs, tumour-associated macrophages. Cell count: mean ± standard deviation. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

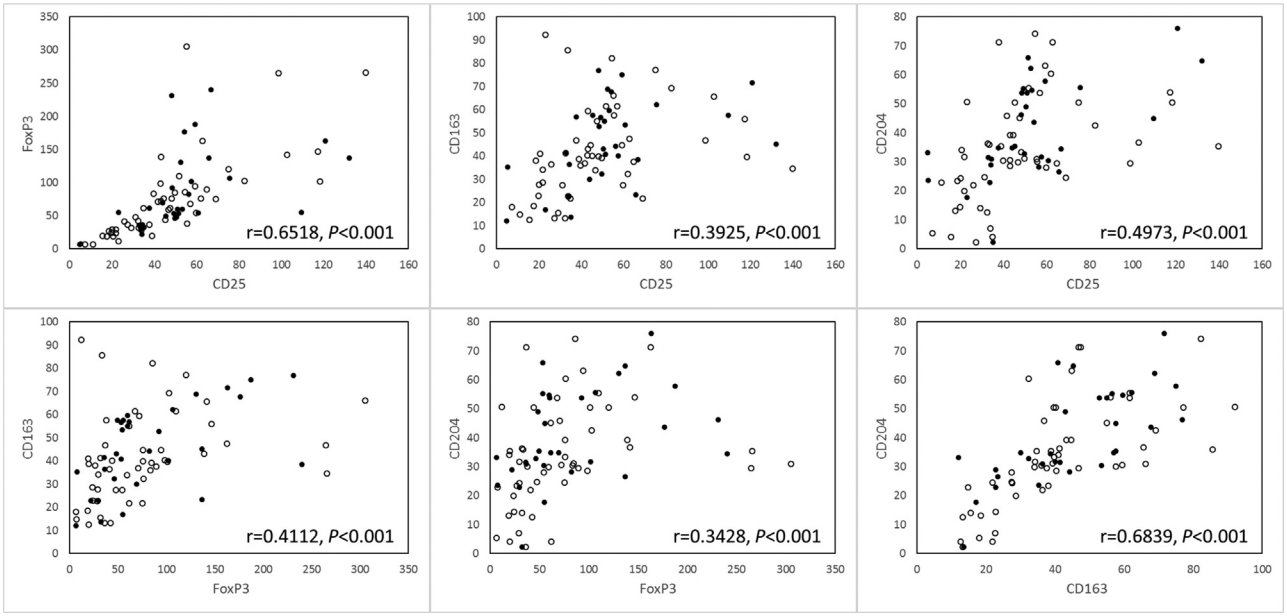

Fig. 3. Pairwise correlations among CD25 + T cells, FoxP3+ T cells, CD163+ macrophages, and CD204+ macrophages in 82 cases of oral squamous cell carcinoma (OSCC) (open circle: successful case; closed circle: poor prognosis case with recurrence, metastasis, or death). Significant positive correlations were found among CD25, FoxP3, CD163, and CD204 levels in OSCC ($P < 0.001$).

ciated with sex ($P < 0.05$), while the expression of CD25, FoxP3, and CD204 were correlated with OEPL recurrence ($P < 0.05$). Furthermore, the expression levels of all four biomarkers were significantly associated with cancerization of OEPL to OSCC ($P < 0.001$, $P < 0.001$, $P < 0.05$, and $P < 0.01$ for CD25, FoxP3, CD163, and CD204, respectively). For cancerization from OEPL to OSCC, the relevant cut-off values were calculated, as follows: CD25: 26.9, FoxP3: 26.0, CD163: 35.6, and CD204: 31.8 (/200× magnification).

Discussion

Host immune responses to neoplastic tissues have been studied extensively in recent years[4]. In the field of cancer immunotherapy, antibodies against T-lymphocyte antigens, such as nivolumab and ipilimumab, antibodies against programmed cell death protein 1 (PD-1) and cytotoxic T cell antigen 4, respectively, have been shown to have efficacy in some solid tumours[16]. Immune cells expressing these antigens do not attack neoplastic cells; therefore, specific antibodies exert anti-tumour effects by blocking negative regulators of T cell activation, thereby triggering immune responses against tumour cells. In OEPL and OSCC, positive correlations have been shown between PD-1 and programmed cell death 1 ligand 1 (PD-L1), which is involved in PD-1-dependent inactivation of T cells[17].

Among T-lymphocytes present in the tumour microenvironment, Tregs play critical roles in the maintenance of immunological tolerance and homeostasis[18]. Tregs represent 5–10% of all peripheral CD4 + T cells; they are positive for CD4, CD25, and FoxP3 expression and are required to prevent autoimmune reactivity and lymphoproliferative diseases[19,20]. The transcription factor FoxP3, a member of the forkhead and winged helix family, is a master regulator of CD4+CD25 + Treg development and function[21]; thus, FoxP3+ Tregs are known to modulate anti-tumour immune responses and suppress the activity of cytotoxic T cells[22]. Moreover, FoxP3 is considered a specific biomarker of Tregs[18] and is frequently used to assess Treg function in neoplastic diseases[23]. In solid tumours of the lung, gastrointestinal tract, and ovary, increased Treg numbers are correlated with a poor prognosis[11,12,14], implying that cancer cells attract Tregs for protection against anti-tumour immune responses.

M2 TAMs produce many immunomodulatory molecules, including checkpoint inhibitors and factors promoting angiogenesis and tissue reconstruction, thus exerting trophic effects that facilitate stromal angiogenesis, matrix breakdown, and cancer cell motility[24]. The surface antigens CD163 and CD204 are used as biomarkers of M2-polarized macrophages[6], and recent studies have shown that infiltration of CD163+CD204+ TAMs correlates with tumour progression and poor patient outcomes in lung, renal, breast, and oral cancers[5,25,26,27].

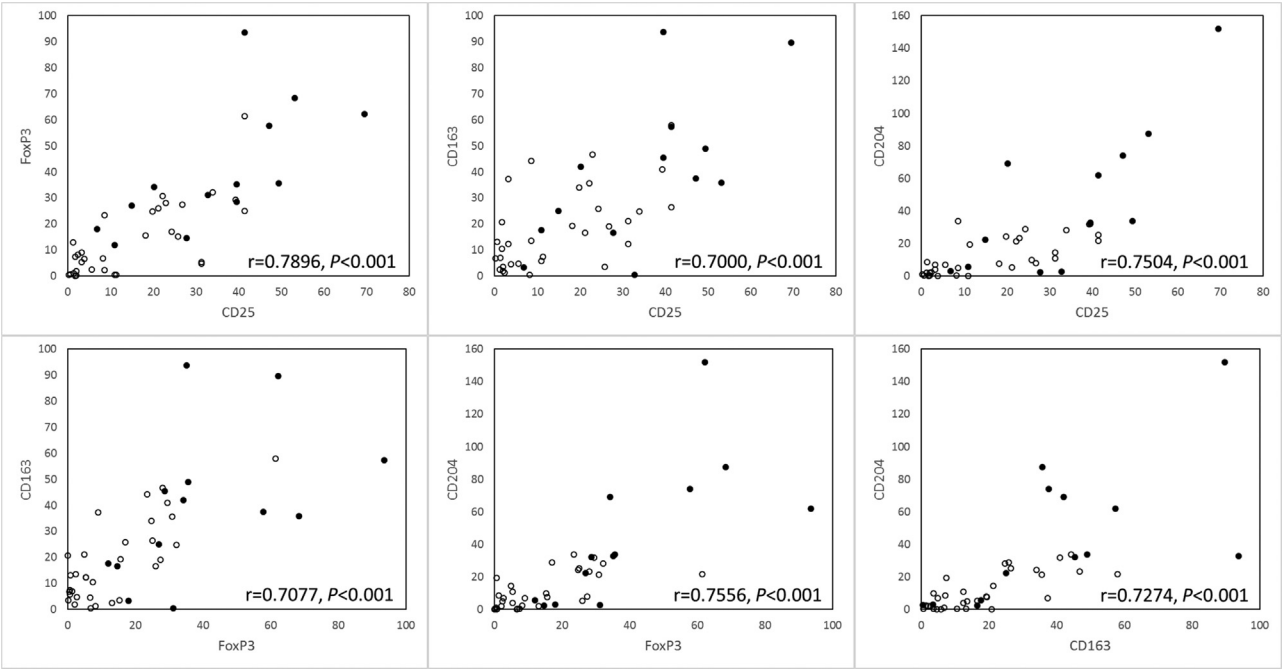

Fig. 4. Pairwise correlations among CD25 + T cells, FoxP3+ T cells, CD163+ macrophages, and CD204+ macrophages in 45 cases of oral epithelial precursor lesion (OEPL) (open circle: successful case; closed circle: poor prognosis case with recurrence or cancerization). Significant positive correlations were found among CD25, FoxP3, CD163, and CD204 levels in OEPL ($P < 0.001$).

In this study, the profiles of infiltrating Tregs and TAMs were investigated in 82 OSCC and 45 OEPL samples. In both lesion types, CD25+FoxP3+ Tregs were detected among epithelial nests and neighbouring lymphocytes, whereas CD163 +CD204+ M2 macrophages were observed in close proximity to epithelial nests, a pattern consistent with previous findings in malignant tumours[8–14]. These data suggest that infiltrating Tregs and M2 TAMs may be involved in the regulation of epithelial cell behaviours. It was also found that the presence of Tregs and M2 TAMs was positively correlated with both malignant OSCC and premalignant OEPL. The study findings are consistent with those of previous studies showing the associations between CD25, FoxP3, CD163, and CD204 expression and tumour progression in pancreatic carcinoma, large B-cell lymphoma, and oesophageal carcinoma[9,13]. However, it appears that the association of Tregs and M2 TAMs with precancerous lesions has not been reported previously.

Furthermore, it was found that CD25, FoxP3, CD163, and CD204 levels correlated with the malignancy of oral epithelial lesions, as demonstrated by significant differences in expression levels between OEPL and OSCC. These results indicate that the expression of CD25, FoxP3, CD163, and CD204 may be used as a diagnostic and disease-specific marker for tumour malignant potential and invasiveness.

The prevalence of Tregs and M2 macrophages in OSCC and OEPL was higher in females than in males, which may be related to the predominance of autoimmune diseases in the female population[28]. Current reports describing the effect of sex differences on Tregs and M2 macrophages and the contributions of these cells to the prevalence of autoimmune diseases in females are limited. However, a report by Nie et al. showing that Tregs are regulated by hormonal fluctuations suggests that these may disrupt the balance between T helper cells and Tregs, thus inducing autoimmune disease[29]. The present study data suggest that cancerization and progression of cancers characterized by aberrant immune reactivity may be affected by sex-related differences and enhanced autoimmunity. Moreover, it was found that CD25 expression was significantly up-regulated in patients with large tumours, whereas CD163 expression was associated with lymph node metastasis, and the levels of other Treg and M2 TAM biomarkers tended to correlate with TNM staging. A study of pancreatic cancer revealed the significant association of CD163 with patient survival and of CD204 with tumour size, lymphatic invasion, and survival, indicating the effects of Treg infiltration on clinical outcomes[9]. Another study showed significant associations of FoxP3 + Tregs with tumour staging and survival in patients with lung adenocarcinoma and primary cutaneous large B-cell lymphoma[11]. Consistent with these findings, our data indicated that the presence of Tregs and M2 TAMs in OEPL was significantly increased in patients with recurrent tumours and cancerization to OSCC, suggesting that Treg and M2 TAM infiltration into oral premalignant lesions correlated with tumour progression and patient prognosis.

Histological analyses have shown that most OSCC are well differentiated[1]. In the present study, it was found that Treg markers did not correlate with tumour differentiation; however, the immunoreactivity of TAM-specific CD163 and CD204 was significantly higher in poorly differentiated tumours, suggesting that M2 TAMs may negatively affect cell differentiation in OSCC. These results are consistent with those of a study by Ino et al.[9], which showed increased levels of CD163 in high-grade pancreatic cancer.

Stromal lymphocytic reactions play important roles in immune responses, limiting tumour invasion and promoting positive clinical outcomes[30]. In the present

Table 2. Associations between clinical variables and immunoreactivities of CD25, FoxP3, CD163, and CD204 in 82 OSCC cases.

			Tregs				TAMs			
		Number of cases	$CD25^+$ T cell count	*P*-value	$FoxP3^+$ T cell count	*P*-value	$CD163^+$ macrophage count	*P*-value	$CD204^+$ macrophage count	*P*-value
All cases		82	49.76 ± 28.07	–	78.29 ± 63.43	–	42.92 ± 18.97	–	36.57 ± 17.45	–
Age (years)	<39	4	45.40 ± 11.27	*P* = 0.5473	60.80 ± 24.23		31.50 ± 7.78		28.65 ± 4.48	
	40–49	4	38.05 ± 38.40		54.40 ± 54.43		34.80 ± 25.90		28.25 ± 15.97	
	50–59	11	38.93 ± 25.13		68.03 ± 81.79	*P* = 0.5477	34.58 ± 19.47	*P* = 0.0254*	28.13 ± 17.73	*P* = 0.0645
	60–69	25	55.88 ± 33.01		78.74 ± 50.30		47.10 ± 17.62		43.83 ± 19.01	
	70–79	22	47.79 ± 20.19		78.24 ± 62.45		51.13 ± 18.96		50.12 ± 15.29	
	≥80	16	54.39 ± 31.31		95.10 ± 80.12		35.74 ± 16.04		30.20 ± 15.37	
Sex	Male	36	49.23 ± 30.01	*P* = 0.1755	70.76 ± 60.56	*P* = 0.1156	39.77 ± 18.54	*P* = 0.0548	32.24 ± 15.35	*P* = 0.0095**
	Female	46	50.44 ± 25.77		87.93 ± 66.54		46.96 ± 19.00		42.09 ± 18.58	
Site	Tongue	46	47.79 ± 29.08	*P* = 0.3691	73.45 ± 66.81	*P* = 0.2243	40.27 ± 19.40	*P* = 0.4503	33.92 ± 17.28	*P* = 0.4479
	Upper gingiva	8	44.48 ± 17.79		71.83 ± 68.59		47.73 ± 17.64		45.13 ± 12.67	
	Lower gingiva	10	58.92 ± 32.88		96.96 ± 73.03		49.46 ± 19.02		44.24 ± 20.14	
	Buccal mucosa	10	50.38 ± 13.56		95.60 ± 47.51		48.72 ± 17.84		38.34 ± 18.20	
	Floor of mouth	5	35.96 ± 23.14		51.08 ± 40.00		37.04 ± 16.24		30.40 ± 15.64	
	Lower lip	3	84.53 ± 45.82		95.33 ± 49.98		39.47 ± 26.20		33.20 ± 17.28	
T classification	1	29	43.16 ± 24.51		69.65 ± 62.90		39.74 ± 21.37		33.59 ± 20.32	
	2	27	44.77 ± 22.89	*P* = 0.0368*	64.07 ± 37.60	*P* = 0.2975	42.19 ± 17.32	*P* = 0.3052	34.02 ± 13.60	*P* = 0.0585
	3	7	74.60 ± 33.11		90.34 ± 79.50		49.97 ± 8.65		53.03 ± 13.69	
	4	19	57.79 ± 32.75		107.27 ± 80.24		46.22 ± 20.07		38.67 ± 16.23	
N classification	0	65	48.82 ± 28.63	*P* = 0.4228	74.63 ± 61.70	*P* = 0.3114	42.07 ± 19.51	*P* = 0.0147*	35.80 ± 18.35	*P* = 0.1222
	1	10	44.34 ± 15.04		77.10 ± 65.40		36.54 ± 11.71		33.52 ± 9.58	
	≥2	7	66.23 ± 34.33		114.03 ± 75.32		59.91 ± 13.33		48.06 ± 14.19	
M classification	0	78	50.13 ± 28.68	*P* = 0.7144	78.59 ± 63.51	*P* = 0.6668	43.35 ± 18.78	*P* = 0.3014	37.10 ± 17.39	*P* = 0.2238
	1	4	42.60 ± 9.62		72.55 ± 71.5		34.55 ± 23.68		26.15 ± 17.32	
TNM stage	I	27	40.23 ± 20.16		65.96 ± 62.52		38.38 ± 21.21		31.81 ± 19.26	
	II	21	48.17 ± 24.72	*P* = 0.2064	69.29 ± 40.42	*P* = 0.3103	44.04 ± 17.93	*P* = 0.3574	36.00 ± 12.98	*P* = 0.1794
	III	10	54.16 ± 31.00		78.36 ± 68.61		45.14 ± 10.71		45.50 ± 16.84	
	IV	24	60.05 ± 34.35		100.04 ± 75.79		46.13 ± 19.90		38.70 ± 18.13	
Recurrence	Negative	64	49.47 ± 28.89	*P* = 0.4597	79.48 ± 67.95	*P* = 0.7033	42.72 ± 19.19	*P* = 0.6540	35.61 ± 17.52	*P* = 0.2872
	Positive	18	50.81 ± 25.70		74.08 ± 45.18		43.64 ± 18.68		39.99 ± 17.24	
Postoperative metastasis	Negative	72	48.05 ± 27.15	*P* = 0.1760	77.05 ± 65.06	*P* = 0.3570	42.46 ± 19.11	*P* = 0.3952	36.13 ± 17.38	*P* = 0.4274
	Positive	10	62.08 ± 32.92		87.26 ± 52.21		46.22 ± 18.55		39.74 ± 18.54	
Survival	Alive	62	54.39 ± 34.46	*P* = 0.4441	73.27 ± 60.10	*P* = 0.5671	45.35 ± 19.06	*P* = 0.4346	43.45 ± 16.17	*P* = 0.0836
	Dead	15	49.11 ± 26.81		95.04 ± 79.06		41.81 ± 19.55		34.26 ± 14.99	

OSCC, oral squamous cell carcinoma; Tregs, regulatory T cells; TAMs, tumour-associated macrophages. Cell count: mean ± standard deviation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3. Associations between pathological variables and immunoreactivity of CD25, FoxP3, CD163, and CD204 in 82 OSCC cases.

		Number of cases	Tregs				TAMs			
			$CD25^+$ T cell count	*P*-value	$FoxP3^+$ T cell count	*P*-value	$CD163^+$ macrophage count	*P*-value	$CD204^+$ macrophage count	*P*-value
All cases		82	49.76 ± 28.07	–	78.29 ± 63.43	–	42.92 ± 18.97	–	36.57 ± 17.45	–
Degree of differentiation	Well	60	47.49 ± 27.46		73.55 ± 65.35		40.31 ± 19.46		33.33 ± 16.91	
	Moderate	20	56.71 ± 30.79	*P* = 0.4268	92.40 ± 59.30	*P* = 0.2210	49.10 ± 16.33	*P* = 0.0371*	45.40 ± 16.60	*P* = 0.0180*
	Poor	2	48.60 ± 4.52		79.70 ± 42.85		59.60 ± 2.83		45.40 ± 14.14	
Stromal lymphocytic reaction	Slight	11	31.91 ± 15.17		41.56 ± 30.15		30.64 ± 14.68		25.25 ± 13.54	
	Moderate	69	52.88 ± 28.98	*P* = 0. 0263*	85.24 ± 65.95	*P* = 0.0227*	45.09 ± 18.76	*P* = 0.0635	38.89 ± 17.09	*P* = 0.0157*
	Severe	2	40.30 ± 7.21		40.9 ± 30.15		35.60 ± 31.11		18.80 ± 23.48	
Mode of invasion	2C	12	32.81 ± 20.20		41.32 ± 29.61		34.78 ± 23.36		32.78 ± 23.19	
	3C	47	48.84 ± 27.80	*P* = 0.0288*	75.87 ± 65.29	*P* = 0.0101*	40.93 ± 18.45	*P* = 0.0181*	34.10 ± 16.05	*P* = 0.1378
	4C	13	61.65 ± 27.11		87.12 ± 49.67		50.85 ± 16.40		42.88 ± 17.55	
	4D	10	59.00 ± 31.26		122.6 ± 76.94		51.76 ± 16.39		44.52 ± 13.25	
Invasion depth	Micro-invasion	6	20.23 ± 8.13		26.77 ± 18.65		20.63 ± 6.59		14.80 ± 9.06	
	Mucosal invasion	34	47.49 ± 29.49	*P* = 0.0016**	76.86 ± 72.61	*P* = 0.0061**	45.86 ± 22.15	*P* = 0.0063**	37.36 ± 18.72	*P* = 0.0033**
	Submucosal tissue	42	55.82 ± 26.94		86.82 ± 56.56		43.72 ± 15.17		39.04 ± 15.27	

OSCC, oral squamous cell carcinoma; Tregs, regulatory T cells; TAMs, tumour-associated macrophages. Cell count: mean ± standard deviation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

study, it was found that the expression of CD25, FoxP3, and CD204 in OSCC differed significantly depending on the stromal reaction; the highest expression was detected in cases with moderate stromal lymphocytic reactions. The mode of invasion in OSCC is typically assessed based on the tumour–host boundary and is considered closely related to postoperative recurrence and metastasis rates[31]. Geng et al.[11] reported a significant correlation between FoxP3 expression and invasion depth in gastric cancer, whereas Kinoshita et al.[12] showed the association between FoxP3 and vascular or pleural invasion in lung adenocarcinoma. Consistent with these results, it was found that the numbers of Tregs and M2 macrophages were higher in patients with advanced mode and depth of invasion in the present study. Overall, these data suggest that Tregs and M2 TAMs may contribute to the malignant potential of OSCC, thereby promoting an aggressive invasive phenotype in cancer cells.

Although there are individual differences in the local immune status of tumours, the tumour microenvironment is, in general, closely involved in cancerization and cancer progression by inhibiting autoimmunity through different pathways[3,9,19]. Thus, it is important to comprehensively analyze the profiles of immunomodulatory cells and molecules in the tumour milieu to determine the mechanisms underlying cancer immune evasion. In addition to Tregs and M2 macrophages, regulatory dendritic cells, myeloid-derived suppressor cells, regulatory B cells, gamma/delta T cells, and various immunosuppressive agents, such as interleukin (IL)-10, IL-6, prostaglandin E2, transforming growth factor beta, and vascular endothelial growth factor, should also be evaluated. Future studies are needed to develop personalized multifactor immunotherapies aimed at providing effective results with minimal side effects according to the analysis of individual immunological reactions in the tumour parenchyma.

In conclusion, this study showed that Treg and M2 TAM infiltration in OSCC and OEPL was associated with oncogenesis, tumour progression, and a poor prognosis, indicating an important role for these immune cells in shaping the tumour microenvironment. Immunotherapeutic approaches based on controlling Treg and M2 TAM accumulation in the tumour vicinity could be effective for the treatment of advanced OSCC and for improving the patient's prognosis and quality of life.

Table 4. Associations between clinical variables and immunoreactivities of CD25, FoxP3, CD163, and CD204 in 45 OEPL cases.

		Number of cases	Tregs			TAMs			
			$CD25^+$ T cell count	*P*-value	$FoxP3^+$ T cell count	*P*-value	$CD163^+$ macrophage count	*P*-value	$CD204^+$ macrophage count	*P*-value
All cases		45	20.69 ± 17.46	–	21.56 ± 20.96	–	24.38 ± 22.08	–	20.84 ± 28.94	–
Age	Mean (years)	67.31 ± 12.60	$r = 0.2689$	$P = 0.0741$	$r = 0.1271$	$P = 0.4053$	$r = 0.0832$	$P = 0.5865$	$r = 0.1492$	$P = 0.3279$
	30–39	2	11.70 ± 12.02		21.60 ± 17.82		39.70 ± 3.25		38.10 ± 43.98	
	40–49	2	10.60 ± 13.01		13.00 ± 16.69		20.50 ± 19.09		16.40 ± 11.03	
	50–59	5	20.56 ± 19.88	$P = 0.5340$	22.44 ± 11.41	$P = 0.6974$	23.80 ± 23.11	$P = 0.6711$	14.00 ± 14.13	$P = 0.6363$
	60–69	18	18.08 ± 13.35		19.66 ± 23.81		19.28 ± 17.45		13.52 ± 16.04	
	70–79	10	19.60 ± 17.47		18.62 ± 18.92		20.36 ± 14.14		17.74 ± 3.09	
	80–89	8	32.78 ± 24.13		28.83 ± 25.74		38.40 ± 36.37		42.25 ± 52.43	
Sex	Male	21	14.38 ± 15.30	$P = 0.0229$*	12.45 ± 10.45	$P = 0.0159$*	18.32 ± 15.81	$P = 0.1686$	12.30 ± 12.50	$P = 0.1794$
	Female	24	26.20 ± 17.65		28.78 ± 24.82		29.68 ± 25.54		28.31 ± 36.64	
Site	Tongue	19	26.89 ± 18.45		27.14 ± 20.24		26.19 ± 22.73		27.22 ± 38.14	
	Upper gingiva	3	14.67 ± 23.16		9.27 ± 13.64		13.87 ± 12.21		9.40 ± 20.86	
	Lower gingiva	15	15.45 ± 15.92	$P = 0.2482$	17.19 ± 15.96	$P = 0.1691$	24.88 ± 25.89	$P = 0.9326$	17.75 ± 20.86	$P = 0.5076$
	Hard palate	2	10.00 ± 11.60		7.80 ± 11.03		20.10 ± 0.99		3.90 ± 5.52	
	Buccal mucosa	6	20.70 ± 14.53		22.53 ± 35.20		24.10 ± 20.18		19.73 ± 21.32	
Size	Mean (mm)	19.07 ± 13.58	$r = 0.1471$	$P = 0.3350$	$r = 0.0454$	$P = 0.7671$	$r = 0.0038$	$P = 0.9803$	$r = 0.1910$	$P = 0.2089$
	<10	8	13.30 ± 16.44		13.58 ± 13.35		21.78 ± 18.38		13.88 ± 14.61	
	10–19	19	24.20 ± 17.21	$P = 0.2606$	26.54 ± 24.22	$P = 0.3778$	25.69 ± 23.10	$P = 0.9922$	22.09 ± 26.66	$P = 0.7657$
	20–29	8	16.70 ± 17.56		14.85 ± 20.72		23.03 ± 20.91		10.82 ± 10.80	
	≥30	10	23.12 ± 18.78		22.04 ± 18.82		25.06 ± 26.49		32.04 ± 46.29	
Recurrence	Negative	43	18.80 ± 15.29	$P = 0.0178$*	19.10 ± 19.04	$P = 0.0239$*	22.60 ± 20.08	$P = 0.0877$	16.24 ± 18.48	$P = 0.0178$*
	Positive	2	61.40 ± 11.60		65.30 ± 4.38		62.70 ± 38.04		119.37 ± 45.40	
Cancerization	Negative	35	14.92 ± 13.58	$P < 0.001$***	13.55 ± 13.99	$P < 0.001$***	18.23 ± 15.34	$P = 0.0179$*	11.17 ± 10.95	$P = 0.0035$**
	Positive	10	34.89 ± 18.29		39.88 ± 23.90		39.51 ± 28.83		44.65 ± 43.57	

OEPL, oral epithelial precancerous lesion; Tregs, regulatory T cells; TAMs, tumour-associated macrophages. Cell count: mean ± standard deviation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry (2016-3-013).

Patient consent

Not required.

Competing interests

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