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ORIGINAL ARTICLE

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The preliminary exploration of immune microenvironment in oral leukoplakia concomitant with oral submucosal fibrosis: A comparative immunohistochemical study

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Abstract

Background: Oral leukoplakia concomitant with oral submucous fibrosis is a high-risk oral potentially malignant disorder, but little is known about its immune microenvironment.

Methods: Thirty samples of oral leukoplakia concomitant with oral submucous fibrosis, 30 oral leukoplakia samples, and 30 oral submucous fibrosis samples were collected from two hospitals. Immunohistochemistry was performed to analyze expression of T cell biomarkers [CD3, CD4, CD8, and Forkhead box P3 (Foxp3)], a B cell biomarker (CD20), macrophage biomarkers (CD68 and CD163), an immune inhibitory receptor ligand (PD-L1), and Ki-67.

Results: The numbers of $CD3^+$ ($p < 0.001$), $CD4^+$ ($p = 0.018$), and $CD8^+$ ($p = 0.031$) cells in oral leukoplakia concomitant with oral submucous fibrosis were less than those in oral leukoplakia. The number of $CD4^+$ cells ($p = 0.035$) in oral leukoplakia concomitant with oral leukoplakia was higher than that in oral submucous fibrosis. More $CD3^+$($p < 0.001$), $CD4^+$($p < 0.001$), $Foxp3^+$($p = 0.019$), and $CD163^+$($p = 0.029$) cells were found in oral leukoplakia than in oral submucous fibrosis.

Conclusion: Various levels of immune infiltration were observed among oral leukoplakia concomitant with oral submucous fibrosis, oral leukoplakia, and oral submucous fibrosis. Characterization of the immune microenvironment may contribute to personalized immunotherapy.

KEYWORDS
immune cell biomarkers, immune microenvironment, oral leukoplakia, oral submucous fibrosis

1 | INTRODUCTION

As one of the most common oral potentially malignant disorders (OPMD), oral leukoplakia (OLK) is a white plaque-like lesion that is mainly caused by tobacco smoking, which significantly increases the risk of oral squamous cell carcinoma (OSCC).[1] The malignant transformation rate of OLK is approximately 10% and is associated with the development and severity of epithelial dysplasia.[2,3] Oral submucosal fibrosis (OSF) is another common precursor lesion of OSCC with a malignant rate of approximately 6%.[4] Stimulated by areca nut chewing, subepithelial collagen fibers in OSF patients gradually accumulate, resulting in restriction of mouth opening.[5] Because of the synergistic effects of tobacco smoking and areca nut chewing, OLK and OSF can occur simultaneously on the oral mucosa,[5] and the malignant risk of OLK concomitant with OSF was different from that of OLK or OSF, respectively.[6]

The immune microenvironment is complex, in which various immune cells interact with other components, and is closely related to the development and progression of OPMD.[7] Immune cells, such as T cells and macrophages, play major roles in OLK. Alterations of immune cells are associated with epithelial dysplasia and might participate in malignant transformation.[8] There are few studies on OSF, although some alterations of immune cells have been demonstrated.[9,10] Furthermore, there is a lack of studies on the immune microenvironment of OLK concomitant with OSF and the differences between OLK and OSF. Investigation of the immune microenvironment and immune cells among OLK concomitant with OSF, OLK, and OSF may facilitate understanding the synergistic effects of these diseases and the risk of malignant transformation.

This study explored the expression of T cell biomarkers [CD3, CD4, CD8, and Forkhead box P3 (Foxp3)], a B cell biomarker (CD20), macrophage biomarkers (CD68 and CD163), an immune inhibitory receptor ligand (PD-L1), and Ki-67 in OLK concomitant with OSF (OLKwOSF), OLK, and OSF to deepen our understanding of the immune microenvironment of OPMD and contribute to the development of immunotherapies.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

This study recruited 30 male patients with OLKwOSF from the Xiangya Stomatological Hospital of Central South University, no female patients were found. Thirty male OLK patients and 30 male OSF patients were recruited from Peking University Stomatological Hospital. The histopathological grades of epithelial dysplasia of OLKwOSF and OLK were strictly matched. There were 19 cases of mild dysplasia and 11 cases of moderate/severe dysplasia in OLKwOSF and OLK groups, respectively. And no dysplasia was observed in OSF group. Histopathological grading and diagnosis were performed by two experienced pathologists in accordance with the World Health Organization classification.[1,11] Clinicopathological parameters are shown in Table 1. This study was approved by the hospital institutional review board.

2.2 | Tissue processing and immunohistochemistry

Formalin-fixed, paraffin-embedded samples were cut into tissue sections (3 μm thick) and mounted on adhesive slides. Immunohistochemical staining was conducted in a fully automated immunohistochemistry instrument (BOND III; Leica Biosystems Melbourne Pty Ltd, Australia, 2017) by deparaffinization, rehydration, antigen retrieval, endogenous peroxidase inactivation, incubation with primary and secondary antibodies (BOND Polymer Refine Detection), and counterstaining with

TABLE 1 Clinicopathological parameters of patients with oral leukoplakia (OLK), oral leukoplakia concomitant with oral submucous fibrosis (OLKwOSF), and oral submucous fibrosis (OSF).

Variables	OLK	OLKwOSF	OSF	*p* value
Age	46.7 ± 9.3	46.1 ± 8.6	28.9 ± 6.6	<0.001
Site				<0.001
Buccal	9 (30.0%)	20 (66.7%)	27 (90.0%)	
Tongue	17 (56.7%)	8 (26.7%)	2 (6.7%)	
Gingiva	4 (13.3%)	1 (3.3%)	0 (0.0%)	
Lip	0 (0.0%)	1 (3.3%)	1 (3.3%)	
Tobacco smoking				0.008
No	16 (53.3%)	8 (26.7%)	5 (16.7%)	
Yes	14 (46.7%)	22 (73.3%)	25 (83.3%)	
Alcohol drinking				0.523
No	18 (60.0%)	14 (46.7%)	14 (46.7%)	
Yes	12 (40.0%)	16 (53.3%)	16 (53.3%)	
Areca nut chewing				<0.001
No	26 (86.7%)	0 (0.0%)	1 (3.3%)	
Yes	4 (13.3%)	30 (100.0%)	29 (96.7%)	

hematoxylin. The primary antibodies included a Mouse anti-CD3 monoclonal antibody(Maxim Biomedical) at a 1:200dilution, Rabbit anti-CD4 monoclonal antibody(Maxim Biomedical) at a 1:100 dilution, Rabbit anti-CD8 monoclonal antibody(ZSGB-BIO) at a 1: 200 dilution, Mouse anti-Foxp3 monoclonal antibody(Maxim Biomedical) at a 1: 200 dilution, Mouse anti-CD20 monoclonal antibody(ZSGB-BIO) at a 1:200 dilution, Mouse anti-CD68 monoclonal antibody(Gene Tech) at 1:200 dilution, Mouse anti-CD163 monoclonal antibody(Gene Tech) at a 1: 200 dilution, Rabbit anti-PD-L1 monoclonal antibody(Maxim Biomedical) at a 1:200 dilution, and Mouse anti-Ki-67 monoclonal antibody, (Maxim Biomedical) at a 1: 200 dilution.

Immunohistochemical assessment of CD3, CD4, CD8, Foxp3, CD20, CD68, and CD163 was performed as follows. Five high-density positive staining areas were imaged at high magnification (×400). Positive cells in each image were counted by ImageJ version 1.53, and the average number of cells in the five images was considered to be the final staining evaluation. PD-L1 and Ki-67 expression was assessed by scoring the staining intensity at high magnification (×400) with a score of 0 indicating negative expression, one indicating weak expression, two indicating moderate expression, and three indicating strong expression. Immunohistochemistry was performed by two experienced pathologists.

2.3 | Statistical analysis

Data analysis and visualization were performed using IBM SPSS Statistics 26.0 and GraphPad Prism 8.3.0. One-way ANOVA was used to compare the immune cell number and ratio, Tukey's multiple comparisons test was used to compare between two groups in multiple group analyses and Fisher's exact test was used to compare PD-L1

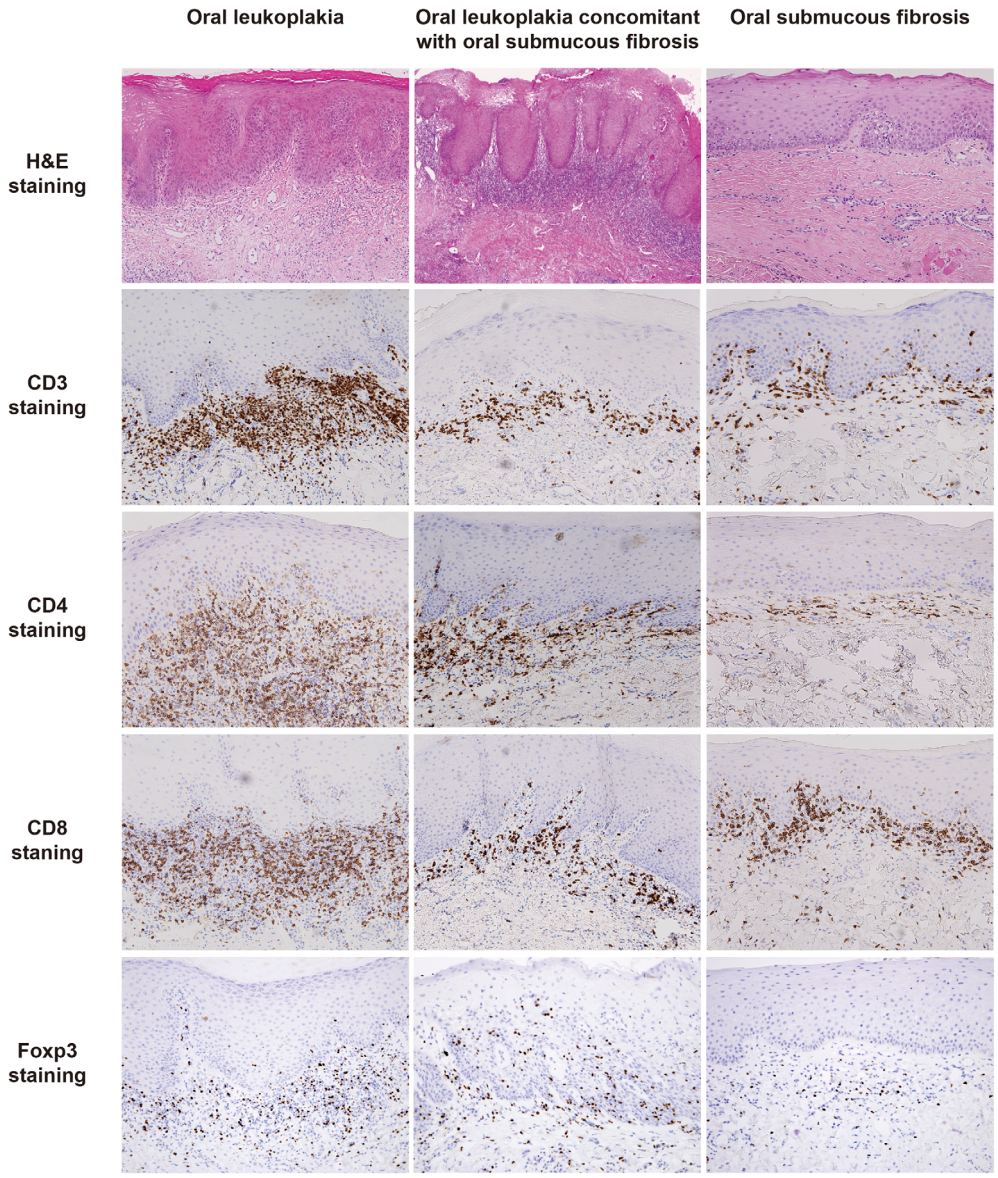

FIGURE 1 H&E staining, CD3, CD4, CD8, and Foxp3 staining in oral leukoplakia, oral leukoplakia concomitant with oral submucous fibrosis, and oral submucous fibrosis specimens.

and Ki-67 expression between groups. $p < 0.05$ was considered to indicate a statistical difference.

3 | RESULTS

3.1 | Clinicopathological features of OLK, OLKwOSF, and OSF groups

As shown in Table 1, the age of OLK group and OLKwOSF group were both higher than that of OSF group ($p < 0.001$). OLK was more common in the tongue, while OLKwOSF and OSF were more common in the buccal ($p < 0.001$). There was a higher rate of tobacco smoking in OLK group ($p = 0.008$), while OLKwOSF group and OSF group were found with higher rates of areca nut chewing ($p < 0.001$). There was no significant difference in the rate of alcohol drinking between three groups ($p = 0.523$).H&E staining of OLK, OLKwOSF, and OSF specimens is shown in Figure 1.

3.2 | T cell infiltration

As shown in Figures 1 and 2, more $CD3^+$ cells were found in OLK than in OLKwOSF ($p < 0.001$) and OSF ($p < 0.001$), but there was no significant difference in $CD3^+$ cells in OLKwOSF and OSF ($p = 0.310$). More $CD4^+$ cells appeared in OLK than in OLKwOSF ($p = 0.018$) and OSF ($p < 0.001$), and more $CD4^+$ cells appeared in OLKwOSF than in OSF ($p = 0.035$). More $CD8^+$ cells were found in OLK than in OLKwOSF ($p = 0.031$), but there was no difference in $CD8^+$ cells between OLK and OSF ($p = 0.126$) or OLKwOSF and OSF ($p = 0.818$). The ratio of $CD4^+/CD8^+$ cells in OLKwOSF was significantly higher than that in OSF ($p = 0.007$), but there was no difference in the ratio of $CD4^+/CD8^+$ cells between OLK and OLKwOSF ($p = 0.703$) or OLK and OSF ($p = 0.059$). The number of $Foxp3^+$ cells in OLK was more than that in OSF ($p = 0.019$), but there was no significant difference in $Foxp3^+$ cells between OLK and OLKwOSF ($p = 0.830$) or OLKwOSF and OSF ($p = 0.079$).

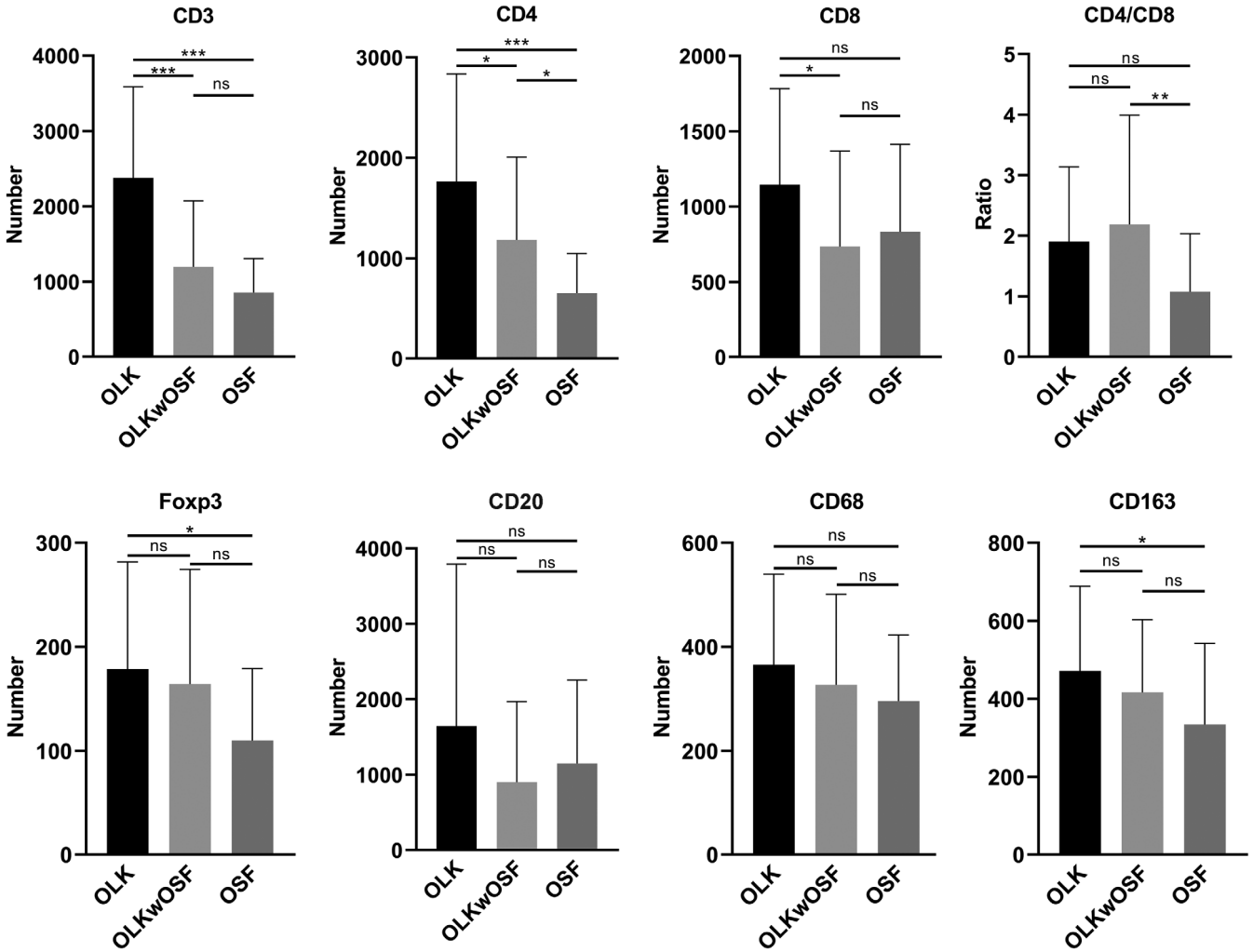

FIGURE 2 Differences in the numbers (mean ± SD) of $CD3^+$, $CD4^+$, $CD8^+$, $Foxp3^+$, $CD20^+$, $CD68^+$, and $CD163^+$ cells, and the $CD4^+/CD8^+$ cell ratio among oral leukoplakia (OLK), oral leukoplakia concomitant with oral submucous fibrosis (OLKwOSF), and oral submucous fibrosis (OSF) specimens.

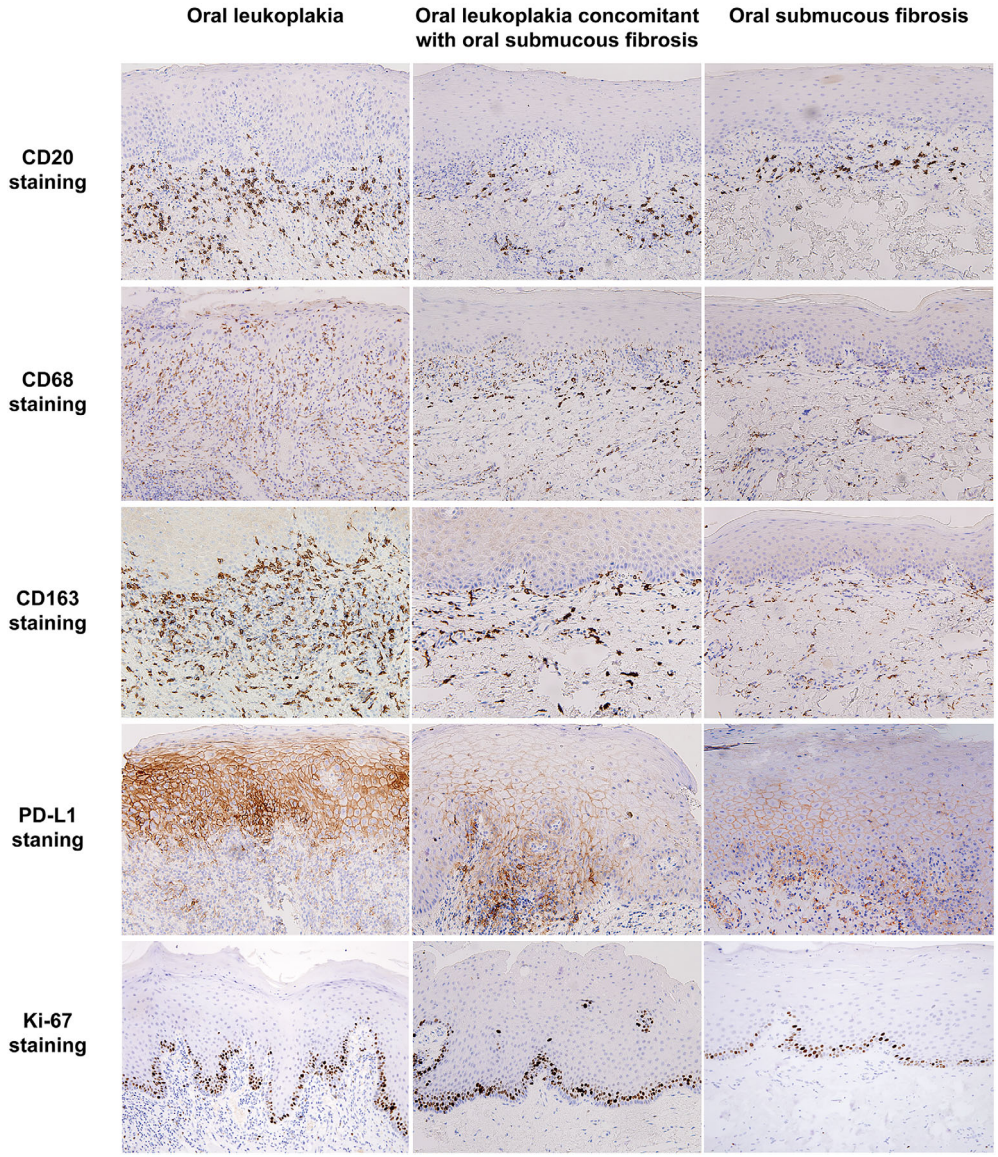

FIGURE 3 CD20, CD68, CD163, PD-L1, and Ki-67 staining in oral leukoplakia, oral leukoplakia concomitant with oral submucous fibrosis, and oral submucous fibrosis specimens.

Markers	Assessment	OLK	OLKwOSF	OSF	*p* value
PD-L1	Negative	15 (50.0%)	20 (66.7%)	27 (90.0%)	0.015
	Weak	12 (40.0%)	7 (23.3%)	3 (10.0%)	
	Moderate	2 (6.7%)	1 (3.3%)	0 (0.0%)	
	Strong	1 (3.3%)	2 (6.7%)	0 (0.0%)	
Ki-67	Weak	0 (0.0%)	9 (30.0%)	4 (13.3%)	<0.001
	Moderate	26 (86.7%)	15 (50.0%)	26 (86.7%)	
	Strong	4 (13.3%)	6 (20.0%)	0 (0.0%)	

TABLE 2 Expression of Ki-67 and PD-L1 in oral leukoplakia (OLK), oral leukoplakia concomitant with oral submucous fibrosis (OLKwOSF), and oral submucous fibrosis (OSF) specimens.

3.3 | B cell and macrophage infiltration

There were no significant differences in the numbers of $CD20^+$and$CD68^+$ cells between OLK and OLKwOSF ($p = 0.123$, $p = 0.621$, Figures 2 and 3), OLK and OSF ($p = 0.388$, $p = 0.219$), or OLKwOSF and OSF ($p = 0.787$, $p = 0.737$). The number of $CD163^+$ cells in OLK was higher than that in OSF ($p = 0.029$), but there was no difference in $CD163^+$ cells between OLK and OLKwOSF ($p = 0.557$) or OLKwOSF and OSF ($p = 0.267$).

3.4 | PD-L1 and Ki-67 expression

PD-L1 expression was higher in OLK than in OSF ($p = 0.003$, Table 2 and Figure 3), but there were no differences between OLK and OLKwOSF ($p = 0.537$) or OLKwOSF and OSF ($p = 0.100$). Ki-67 expression was higher in OLK than in OLKwOSF ($p = 0.002$) and OSF ($p = 0.013$), and different between OSF and OLKwOSF ($p = 0.003$).

4 | DISCUSSION

OLK and OSF are two common OPMDs with malignant potentials.[1,11] Yang et al. reported that the synergistic effect of OLK and OSF lead to a higher risk of malignant transformation in patients with OLK concomitant with OSF.[6] However, there is a lack of studies on the immune landscape of OLK concomitant with OSF and the difference in microenvironments between OLK and OSF.

This study performed a comparative analysis between different types of oral potentially malignant disorders. More patients of OLK smoked tobacco, while patients of OLKwOSF and OSF always chewed areca nut. The tobacco smoking and areca nut chewing have been considered as significant risk factors for OLK and OSF, respectively,[5,12,13] and patients with together risk factors increased the risk for concomitant diseases. We found that OLK and OLKwOSF occurred more common in the tongue with higher age than OSF. This might be associated with increased transformation rates of OLK and OLKwOSF, because tongue and increasing age were identified as risk factors for malignant transformation of OPMD.[2,14]

This study revealed the different inflammatory features and immune microenvironments among OLK, OSF, and OLK concomitant with OSF. It has been shown that the number of T cells in OLK and OSF is higher than that in normal oral mucosa.[9,10,15] In this study, we found more $CD3^+$, $CD4^+$, and $Foxp3^+$ T cells in OLK than in OSF. T cell infiltration in OLK was stronger than that in OSF, and $CD163^+$ macrophages and PD-L1 expression showed the same trend. However, $CD68^+$ macrophage and $CD20^+$ B cell infiltration was not different among the three lesions. This might be related to the biological behavior of OSF. Fibrous degeneration of connective tissue leads to gradual accumulation of subepithelial collagen fibrous tissue and vascular stenosis or occlusion.[5] Because of the dense fibrous tissue obstruction, immune cells cannot migrate to the subepithelial stroma. Normal connective tissue in OLK does not interfere with cell migration or aggregation. Therefore, immune cells infiltrate and aggregate in the subepithelial layer.

Epithelial dysplasia in OLK and OLK concomitant with OSF causes Ki-67 overexpression. However, through interference of collagen fibrous tissue accumulation, Ki-67 expression was lower in OLK concomitant with OSF than in OLK.

Studies have shown upregulation of immune reactivity in epithelial dysplastic lesions with increased infiltration of $CD4^+$ T cells, $CD8^+$ T cells, and $CD163^+$ macrophages, and increased expression of PD-L1.[15–18] Epithelial dysplasia is a common risk factor for malignant transformation of OLK.[2] To resist this cancer-promoting effect, the immune microenvironmental with immune surveillance features has been elucidated in OLK with epithelial dysplasia.[8,19] In this study, epithelial dysplasia occurred in all samples of OLK and OLK concomitant with OSF, but not observed in all OSF samples. Thus, immune cell infiltration in both OLK and OLK concomitant with OSF was higher than that in OSF. Furthermore, a reduction of $CD8^+$ T cell infiltration is associated with malignant transformation of OPMD.[20] The lower number of $CD8^+$ T cells in OLK concomitant with OSF than in OLK might be associated with an increased risk of OSCC development reported by Yang et al.[6] In summary, the epithelial dysplasia in OLK concomitant with OSF lesions might contribute to a proinflammatory state, but the accumulation of collagen fibers may affect the migration and aggregation of immune cells. The unique biological behaviors of OLK and OSF lead to the complex immune microenvironment in OLK concomitant with OSF.

5 | CONCLUSION

The study found differences in the immune microenvironment among OLK, OSF, and OLK concomitant with OSF. The higher immune cell infiltration was identified in OLK and OLK concomitant with OSF. The delineation of the immune landscape in various OPMDs may contribute to the development of personalized immunotherapy.

AUTHOR CONTRIBUTIONS

Tiejun Li, Heyu Zhang and Long Li designed the study, collected and analyzed the data, obtained copies of studies and revised the writing. Xinjia Cai, Jianyun Zhang, Yinghong Peng, Zhigang Yao, Junhui Huang, Jiaying Bai, and Jing Yan conducted experiments, collected and analyzed the data, and wrote the draft of the manuscript. All authors read and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

All authors declared that there were no conflicts of interest with the contents of this article.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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