Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

The expression of Th17/Treg in oral submucosal fibrosis carcinogenesis and the significance in the development of mucosal lesions

Dan Wu, Xun Liu, Tingting Zhou, Jin Tan*

Department of Stomatology, First Affiliated Hospital of Hunan University of Chinese Medicine, Changsha, Hunan, 410021, China

ARTICLE INFO

Original paper

Article history:
Received: August 10, 2023
Accepted: September 17, 2023
Published: September 30, 2023

Keywords:

Lesions, oral submucosal fibrosis and carcinogenesis, Th17 cells, treg cells

ABSTRACT

It targets to explore the expression of Th17/Treg in oral submucous fibrosis (OSF) carcinogenesis and its significance in the development of mucosal lesions. In this research, 100 patients with OSF who visited our hospital for surgical treatment from March 2020 to April 2022 were selected. Based on pathological examination results, the patients were divided into 27 patients with oral leukoplakia (OK) group, 14 patients with oral lichen planus (OLP) group, 9 patients with oral squamous cell carcinoma (OSCC) group, and 50 patients with OSF group. It adopted flow cytometry (FC) to calculate the ratio of peripheral blood Th17 cells and Treg cells in four groups, and the Th17/Treg ratio was calculated; The area of oral mucosal lesions (OML) from patients was collected. It needs to compare the differences in Th17/Treg ratio and OML area among four groups and determine the correlation between indicators. ROC curve was used to analyze the diagnostic threshold of the Th17/Treg ratio for carcinogenesis. Except for the OK and OLP, it had statistical significance differences in Th17, Treg cells, and Th17/Treg ratio (P<0.001); The area of OML in the OK, OLP, and OSCC was higher than that in the simple OSF, with statistical significance (P<0.001); Th17 (%), Treg (%), and Th17/Treg all had direct ratio with the area of OML; The area of OML has directed ratio with the development of mucosal lesions (r>0, P<0.05); The areas under the ROC curve for patients with OSF combined with OK, OLP, or OSCC with Th17 (%), Treg (%), Th17/Treg, and OML area were 0.560, 0.986, 0.936, and 0.466, respectively. The expression of Th17/Treg is elevated in oral submucosal fibrosis and carcinogenesis. When mucosal lesions progress or become cancerous, the Th17/Treg ratio increases accordingly, and it has more clinical value than the increase in the OML area.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.9.12

Introduction

Oral submucous fibrosis (OSF) is a chronic disease, which mainly affects the fibrous tissue under the oral mucosa, leading to the stiffening and thickening of the oral mucosa, with various degrees of mouth cracks, mouth opening restrictions and other symptoms. In serious cases, it can develop into oral cancer. A survey shows that 13.7% of OSF patients are early-stage oral squamous cell carcinoma (OSCC) patients (1). OSF is prone to progression into OSCC patients. At present, the pathogenesis of OSF is not fully understood, but some studies have explored it. OSF is a potentially malignant oral disease with a high malignant transformation rate, but its pathogenesis is still unclear (2). Inflammatory reactions, oxidative stress, and fibroblast proliferation under the oral mucosa are closely related to the occurrence of OSF and the development of mucosal lesions. From the distribution of diseases, OSF is mainly distributed in Asia, especially in India, Bangladesh, Pakistan and other regions, where there is a phenomenon of long-term consumption of betel nut with high caffeine content. This lifestyle habit is one of the main causes of OSF. The long-term effects of stimulating substances such as tobacco, alcohol, and betel nut can lead to inflammation and oxidative stress in the oral submucosa, thereby promoting the proliferation and fibrosis of fibroblasts. In addition, some genetic factors may also be related to the occurrence of OSF and the development of mucosal lesions (3,4). Inflammatory reactions and fibroblasts are key factors in the conversion of OSF to OSCC. Chronic inflammation is the infiltration of various immune cells, including T cells, B cells, macrophages, and dendritic cells, into affected tissues. The balance between pro-inflammatory Th17 cells and anti-inflammatory Treg cells during inflammation has been proven to play a crucial role in the pathogenesis of chronic inflammation-related diseases such as autoimmune diseases, inflammatory bowel disease and cancer. Fibroblasts are the main cells in which OSF occurs. Arecoline can affect inflammatory cytokines produced by fibroblasts, while fibroblasts act on immune cells Th17 cells and Treg cells to change them (5). At the same time, OSF is often accompanied by Leukoplakia (OK), OLP, and other fibrous changes, and it has developed into OSCC many years later. Scholars have found that Th17/Treg imbalance can have an impact on the occurrence and deterioration of OSF (6). However, the role of Th17/Treg cells in the progression of OSF to OSCC is not fully understood. Therefore, further observation and research are needed on the Th17/Treg balance status in patients with OSF accompanied by OK and OLP. At the same time, it is still necessary to further determine the evaluation value of Th17/Treg balance status for the development of mucosal lesions. The purpose of this study is to investigate the expression of Th17/Treg in OSF and its significance for the

* Corresponding author. Email: chunjiangsnyxz@163.com

development of mucosal lesions. The results are reported as follows.

Materials and Methods

Baseline materials

In this study,100 patients with OSF who visited our hospital for surgical treatment from March 2020 to April 2022 were selected. Inclusion criteria: ① Meeting the relevant diagnostic criteria for OSF in the Journal of Oral Mucosal Diseases and confirmed by postoperative pathological tissue examination; ② No history of tumor indicators such as chemotherapy or radiotherapy before surgery; ③ Important organs such as liver and kidney function well. Exclusion criteria: ① Those with abnormal immune or blood system functions; ② Patients with concurrent mucosal diseases or periodontitis at the lesion tissue; ③ Missing medical record data or merging with other tumor patients; ④ During the onset of infectious diseases. According to the pathological examination results, patients are divided into a simple OSF group, OK group, OLP group, and OSCC group. Among them, there are 50 cases in the simple OSF, with 28 males and 22 females; The age is 34-48, with an average age of 40.18 ± 3.63. The chewing time of betel nut for 1-13 years, of which 9 cases were less than 5 years old; There were 27 cases in the OK, including 15 males and 12 females; The age is 34-48, with an average age of 40.48 ± 3.43. The chewing time of betel nut is 1-14 years, of which 5 cases are less than 5 years old; There were 14 cases in the OLP, containing 8 males and 6 females; The age is 34-48, with an average age 40.36 ± 4.16. The chewing time of betel nut is 1-15 years, with 2 cases less than 5 years old; There were 9 cases in the OSCC, including 5 males and 4 females; The age is 34-48 with an average age of 35.89 ± 5.90. The chewing time of betel nut is 3-17 years, with 1 case being less than 5 years old; There has been no statistically significant difference in gender, age, and betel nut chewing time among the four groups of patients (P>0.05).

Research methods

Th17/Treg ratio

It needs to take 5ml of fasting elbow venous blood on the day the patient plans to operate and add Foxp3 fluorescent antibody 20 μ L. PE-IL17 fluorescent antibody 10 μ L. The fluorescent antibodies are all provided by ThermoFisher in the United States. They are kept away from light and allowed to stand at 4℃ for 30 minutes. 2ml of membrane breaker is added and centrifuged using a fully automatic centrifuge. The centrifugation radius is 7.5cm, and the supernatant is discarded after centrifugation at 1200r/min. After adding 2ml of Congo red staining solution and mixing, the percentage of Th17 cells and Treg cells are evaluated using an FC. The FC is produced by ThermoFisher in the United States, and the Congo red staining solution is provided by Nanjing Shenghang Biotechnology Co., Ltd. The Th17/Treg ratio is calculated.

Area and development of OML

OML area: A highly experienced professional doctor measures the patient's OML area, taking an average of three measurements. The patient is advised to relax as much as possible during the measurement. First, it needs to place sulfuric acid paper on the surface of the oral mucosa and record the patient's lesion status. Finally, the recording is conducted after the sulfuric acid paper is taken out and lay it flat on a grid paper which is in 1mm standard units. *Development of mucosal lesions:* Patients with OK, OLP, and OSCC are assigned values of 1, 2, and 3 respectively, while those with simple OSF are assigned values of 0.

Statistical methods

SPSS 26.0 statistical software package is adopted to statistically deal with the data, and the measurement data conforming to the normal distribution is described as x±s. Independent sample t-test or analysis of variance is applied to group comparison, and GraphPad software is used to draw a histogram; The counting data is represented as an example (%), and the comparison between groups is conducted using the four grid table method χ^2 -test. Pearson correlation analysis is applied to identify the correlation between data. ROC curve analysis is used to evaluate the diagnostic efficacy of the Th17/Treg ratio in the growth of OML and the correct level α= 0.05.

Results

Comparison of peripheral blood Th17 and Treg cells among four groups of patients

After analysis of variance, it has a statistical significance difference in Th17, Treg cells, and Th17/Treg proportion in peripheral blood in the four groups (P<0.001) (Table 1); From Figure 1, it has a statistical significance difference (P<0.001) in the comparison among the groups except for the OK group and the OLP group.

Table 1. Comparison of peripheral blood Th17 and Treg cells among four groups of patients.

Group (n)	Th17 (%)	Treg (%)	Th17/Treg
Simple OSF group (50)	1.07±0.23	2.65±0.35	0.41±0.10
OK group (27)	1.92±0.36	4.19±1.18	0.50±0.22
OLP group (14)	3.03±0.69	5.51±0.83	0.56±0.15
OSCC group (9)	3.25±0.42	4.50±0.89	0.74±0.11
F	159.033	62.657	14.117
P	<0.001	<0.001	<0.001

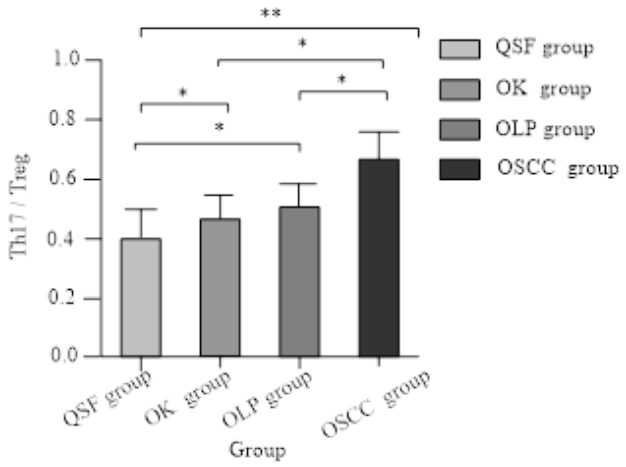

Figure 1. Comparison of peripheral blood Th17/Treg cell ratios between different groups.

Comparison of OML area among four groups of patients

After analysis of variance, it has s statistical significance difference in the area of OML in the four groups (P<0.001) (Table 2); From Figure 2, the OML area of the OK, OLP, and OSCC is higher than the simple OSF, and it has statistical significance difference (P<0.001)

Correlation between Th17/Treg and the area and development of OML

Pearson correlation analysis found that Th17 (%), Treg (%), and Th17/Treg had a direct ratio with the area of OML which was also positively correlated with the development of OML (r>0, P<0.001) (Table 3).

ROC curve analysis results

The areas under the ROC curve for patients with OSF combined with OK, OLP, or OSCC with Th17 (%), Treg (%), Th17/Treg, and OML areas were 0.560, 0.986, 0.936, and 0.466, respectively (Figure 3).

Discussion

OSF is a progressive disease caused by oral mucositis, and its pathogenesis is complex. Long-term non-healing of oral mucosal tissue damage can lead to debilitating diseases, affect the quality of life, and has a higher likelihood of developing into malignancy. Once it develops into precancerous lesions or cancerous transformation, it can even lead to hearing loss (7,8). From the pathological mechanism, OSF is featured by extreme fibrosis of the submucosa, and angiogenesis contributes to oral mucosal fibrosis (9). OSF is considered to have a potential premalignant disease, accompanied by trace element disorders and genetic susceptibility (10). Clinicians must make judgments based on the evaluation of each case. The most common OPMD is vitiligo, while others include lichen planus, oral submucosal fibrosis, and erythema. Factors of increased risk of malignant transformation include gender, segment and lesion type, smoking and drinking habits, as well as the presence of epithelial dysplasia on histological examination (11). OSF belongs to non-reversible injury, and tissue biopsy is mainly chosen as the gold standard. However, because of the invasive nature of tissue biopsy, it needs to explore more non-invasive and convenient diagnostic methods. Changes in some biochemical indicators during the OSF process have been discovered (12). However, the biochemical indicators for the progression and deterioration of DSF are always in the embryonic stage, and no typical end-of-life indicators have been found.

Fibrosis is the widespread deposition of fibrous connective tissue, featured by the accumulation of collagen and other extracellular matrix (ECM) components. As a disease mainly occurring in the oral cavity, OSF has similar pathological mechanisms to diseases such as renal interstitial fibrosis, myocardial infarction and systemic sclerosis (13). Both innate and adaptive immunity are related to the occurrence of various fibrotic diseases. A study on chronic heart failure found that a rise in Th17 cells and a reduction in Tregs exacerbate myocardial fibrosis and heart failure by inducing LOX expression (14). The expression of Th17 and Treg in the blood is at high levels in patients with OSF and OSCC, and the Th17 and Treg ratios will form an upward direction during the progression of OSF. They share a common pre-somatic cell (naive CD4 T cells) and need a common tumor growth factor (TGF)- β signal to begin differentiation (15). Th17-derived pro-inflammatory factors, containing IL-17A, IL-17F, IL-21, IL-22, and IL-26, are important in the pathogenesis of these diseases (16). Th17 cells have a unique ability to adapt to local

Table 2. Comparison of preoperative OML area among four groups of patients.

Group (n)	Area of OML (mm2)
Simple OSF group (50)	38.42±6.23
OK group (27)	46.25±6.20
OLP group (14)	45.07±6.15
OSCC group (9)	42.56±5.57
F	11.635
P	<0.001

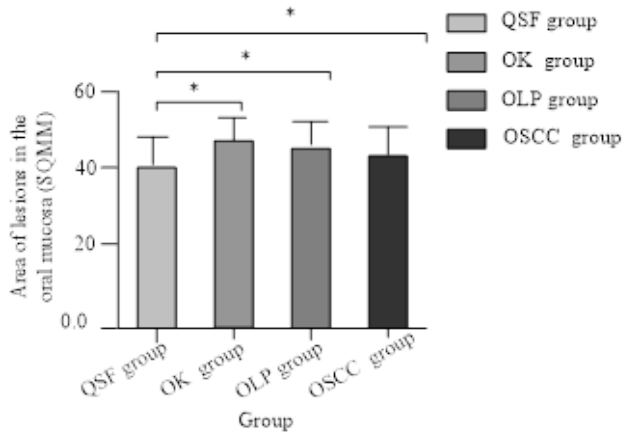

Figure 2. Comparison of OML area between different groups.

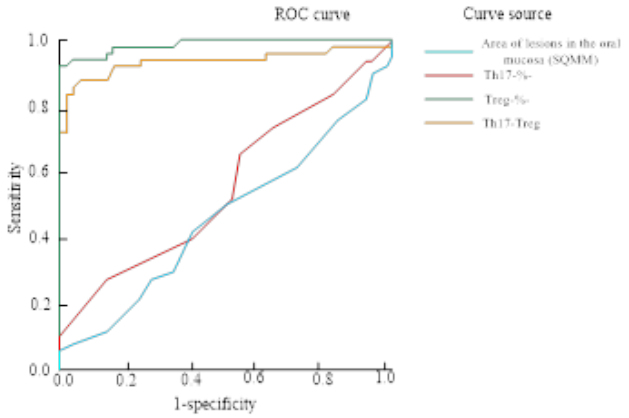

Figure 3. ROC curves of different indicators for cancer transformation.

Table 3. Correlation between Th17/Treg and the area and development of OML.

Index	Area of OML		Development of mucosal lesions	
	r	*P*	*r*	*P*
Th17 (%)	0.346	<0.001	0.860	<0.001
Treg (%)	0.359	<0.001	0.631	<0.001
Th17/Treg	0.207	0.039	0.552	<0.001
Area of OML	/	/	0.294	<0.001

molecular signals to promote or inhibit inflammation (17). There is a clear correlation between Th17, Th17/Treg, and OSF.

The results of this study indicated that it has statistical significance differences in Th17, Treg cells, and Th17/Treg ratio among the groups except for the OK and OLP (P<0.001); They were equivalent between the OK and the OLP but showed higher levels in the OSCC. The reason for this is that the oral mucosa belongs to the barrier tissue of the oral cavity, which is an important portal tissue that connects the body with microorganisms, food, and air particles, and is connected to the esophagus, trachea, and gastrointestinal tract, with mucosal immune characteristics. IL-17/Th17 is crucial for the protective immune and inflammatory responses of the oral mucosa (18). An animal experiment on periodontitis found that the local proliferation characteristics of Th17 cells in periodontitis mice were different from those of steady-state mouse oral Th17 cell proliferation in vivo. The dilation of Th17 cells in periodontitis mice more relied on the local flora disorder, and interleukin-6 and interleukin-23 were also involved in this process. This experiment also found a relationship between genetic defects in Th17 cell differentiation and periodontitis. Meanwhile, Th17 cell defects were associated with periodontitis and bone loss. Th17 cells had unique functions in the occurrence and development of oral immunity and inflammation, and have become a new target for the therapy of periodontitis (19). Periodontitis and oral mucosal fibrosis are both related to the health of the oral mucosa, and periodontitis is an early stage of oral mucosal fibrosis. With the local inflammation of periodontitis and the emergence of pathogens, there will be an imbalance between the ecology and immunity in the oral cavity, resulting in a rise in Th17 production (20).

At the same time, the OML area of the OK, OLP, and OSCC was bigger than that of the simple OSF, with statistical significance (P<0.001). Compared with simple OSF, patients with OK, OLP, and OSCC had a larger area of OML. When OK occurred in the oral cavity, *Candida albicans*, a pathogen, could invade the oral epithelium, resulting in tissue lesions, and releasing inflammatory mediators. These cytokines produce Th17 cell differentiation and IL-17 and/or IL-22 mediated production of antifungal protective immune inflammatory responses in infected mucosa (21). The oral immune system includes various antigen-presenting cells, containing Langerhans cells, myeloid and plasma cell-like dendritic cells, which are located in the mucosa along the lamina propria and the upper and subcutaneous tissues of the mucosa respectively. Without danger signals, all these dendritic cell subsets are tolerant. Oral tissue contains a limited number of mast cells and eosinophils, most of which are located in the submucosal area. Th1, Th2, and Th17 cells living in the oral cavity are distributed along the lamina propria, which is the body's defense mechanism against infectious pathogens (22). OLP belongs to an immune disease, where the T cell subpopulations Th17 and Tregs cells are known to participate in immune regulation, accompanied by abnormal Th17/Treg cell balance, and local immune regulatory dysfunction (23). Oral mucosal fibrosis's lesion size is directly involved in the seriousness of the disease. The evaluation results of this study, it was not entirely based on the area of OML to determine whether a patient had cancer or progression of mucosal lesions. However, Th17 (%), Treg (%), and Th17/Treg were not only related to the area of OML, but also increased with the development of mucosal lesions combined with OK, OLP, and OSCC. At the same time, Th17 (%), Treg (%), Th17/Treg, and the area of OML used for the ROC curve of patients with OSF combined with OK, OLP, or OSCC were 0.560, 0.986, 0.936, and 0.466, respectively. Th17, Treg, and Th17/Treg all had a higher area under the ROC curve compared to the area of OML. Based on the above reasons, Th17/Treg can be used as a non-invasive and easily accessible effective identification method for mucosal lesions and OSF disease progression. However, the clinical data obtained in this study is relatively small, which inevitably leads to data bias. In the future, a prospective multicenter cohort study will be considered to remedy the shortcomings of this study.

In summary, there is an increase in the expression of Th17/Treg in OSF and carcinogenesis. When mucosal lesions progress or carcinogenesis occurs, the Th17/Treg ratio increases accordingly, and it is more clinically valuable than the increase in OML area.

References

1. Saalim M, Sansare K, Karjodkar FR, Johaley S, Ali IK, Sharma SR, Mehra A, Rahman B. The prevalence of oral squamous cell carcinoma with oral submucous fibrosis. J Cancer Res Ther 2021; 17(6): 1510-1514. https://doi.org/10.4103/jcrt.JCRT_719_19
2. Ray JG, Chatterjee R, Chaudhuri K. Oral submucous fibrosis: A global challenge. Rising incidence, risk factors, management, and research priorities. Periodontol 2000 2019; 80(1): 200-212. https://doi.org/10.1111/prd.12277
3. Tejasvi MA, Anulekha CK, Afroze MM, Shenai KP, Chatra L, Bhayya H. A correlation between oral mucosal lesions and various quid-chewing habit patterns: A cross-sectional study. J Cancer Res Ther 2019; 15(3): 620-624. https://doi.org/10.4103/jcrt.JCRT_620_14
4. Yongxiu D, Dongye S, Xinchun J, Qiuhua M, Yanan C, Pu X. [Epidemiological investigation of chewing fresh or dried betel nut and oral mucosal disease]. Hua Xi Kou Qiang Yi Xue Za Zhi 2016; 34(4): 391-394. Chinese. https://doi.org/10.7518/hxkq.2016.04.014
5. Wang L, Gu L, Tang Z. Cytokines secreted by arecoline activate fibroblasts that affect the balance of TH17 and Treg. J Oral Pathol Med 2020; 49(2): 156-163. https://doi.org/10.1111/jop.12965
6. Liu S, Liu Z, Shan Z, Liu Y, Chen T, Fang L, Quan H. Skewed Th17/Treg balance during progression and malignant transformation of oral submucous fibrosis. Oral Dis 2022; 28(8): 2119-2130. https://doi.org/10.1111/odi.13853
7. Gayathri K, Malathi N, Gayathri V, Adtani PN, Ranganathan K. Molecular pathways of oral submucous fibrosis and its progression to malignancy. Arch Oral Biol 2023; 4(148): 105644. https://doi.org/10.1016/j.archoralbio.2023.105644
8. Singh R, Rana AK, Johri N. Oral Submucous Fibrosis: Association of Clinical and Histological Severity with Hearing Loss and Middle Ear Function. Indian J Otolaryngol Head Neck Surg 2022; 74(Suppl 3): 4441-4449. https://doi.org/10.1007/s12070-022-03077-2
9. Choudhari SS, Kulkarni DG, Patankar S, Kheur SM, Sarode SC, Sarode GS, Patil S. Angiogenesis and Fibrogenesis in Oral Submucous Fibrosis: A Viewpoint. J Contemp Dent Pract 2018; 19(2): 242-245. https://doi.org/10.5005/jp-journals-10024-2244
10. Xu H, Lyu FY, Song JY, Xu YM, Jiang EH, Shang ZJ, Chen LL, Xu Z. Research Achievements of Oral Submucous Fibrosis: Progress and Prospect. Biomed Res Int 2021; 2021: 6631856. https://

doi.org/10.1155/2021/6631856

11. Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: risk of progression to malignancy. Oral Surg Oral Med Oral Pathol Oral Radiol 2018; 125(6): 612-627. https://doi.org/10.1016/j.oooo.2017.12.011
12. Qin X, Ning Y, Zhou L, Zhu Y. Oral Submucous Fibrosis: Etiological Mechanism, Malignant Transformation, Therapeutic Approaches and Targets. Int J Mol Sci 2023; 24(5): 4992. https://doi.org/10.3390/ijms24054992
13. Zhang M, Zhang S. T Cells in Fibrosis and Fibrotic Diseases. Front Immunol 2020; 11: 1142. https://doi.org/10.3389/fimmu.2020.01142
14. Lu M, Qin X, Yao J, Yang Y, Zhao M, Sun L. Th17/Treg imbalance modulates rat myocardial fibrosis and heart failure by regulating LOX expression. Acta Physiol (Oxf) 2020; 230(3): e13537. https://doi.org/10.1111/apha.13537
15. Lee GR. The Balance of Th17 versus Treg Cells in Autoimmunity. Int J Mol Sci 2018; 19(3): 730. https://doi.org/10.3390/ijms19030730
16. Li B, Huang L, Lv P, Li X, Liu G, Chen Y, Wang Z, Qian X, Shen Y, Li Y, Fang W. The role of Th17 cells in psoriasis. Immunol Res 2020; 68(5): 296-309. https://doi.org/10.1007/s12026-020-09149-1
17. Crouser ED. Role of imbalance between Th17 and regulatory T-cells in sarcoidosis. Curr Opin Pulm Med 2018; 24(5): 521-526. https://doi.org/10.1097/MCP.0000000000000498
18. Gaffen SL, Moutsopoulos NM. Regulation of host-microbe interactions at oral mucosal barriers by type 17 immunity. Sci Immunol 2020; 5(43): eaau4594. https://doi.org/10.1126/sciimmunol.aau4594
19. Dutzan N, Kajikawa T, Abusleme L, Greenwell-Wild T, Zuazo CE, Ikeuchi T, Brenchley L, Abe T, Hurabielle C, Martin D, Morell RJ, Freeman AF, Lazarevic V, Trinchieri G, Diaz PI, Holland SM, Belkaid Y, Hajishengallis G, Moutsopoulos NM. A dysbiotic microbiome triggers T_H17 cells to mediate oral mucosal immunopathology in mice and humans. Sci Transl Med 2018; 10(463): eaat0797. https://doi.org/10.1126/scitranslmed.aat0797
20. Meghil MM, Cutler CW. Oral Microbes and Mucosal Dendritic Cells, "Spark and Flame" of Local and Distant Inflammatory Diseases. Int J Mol Sci 2020; 21(5): 1643. https://doi.org/10.3390/ijms21051643
21. Feller L, Khammissa RA, Chandran R, Altini M, Lemmer J. Oral candidosis in relation to oral immunity. J Oral Pathol Med 2014; 43(8): 563-569. https://doi.org/10.1111/jop.12120
22. Moingeon P, Mascarell L. Induction of tolerance via the sublingual route: mechanisms and applications. Clin Dev Immunol 2012; 2012: 623474. https://doi.org/10.1155/2012/623474
23. Firth FA, Friedlander LT, Parachuru VP, Kardos TB, Seymour GJ, Rich AM. Regulation of immune cells in oral lichen planus. Arch Dermatol Res 2015; 307(4): 333-339. https://doi.org/10.1007/s00403-015-1540-8