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Original Article

Intralesional and peripheral plasma of oral lichenoid reactions exhibit different cytokine profiles: A preliminary study

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Abstract *Background/purpose:* Oral lichenoid reactions (OLRs) are commonly characterized by the infiltration and activation of inflammatory cells at the interface of the oral mucosa. This study aimed to compare the cytokine profiles between intralesional and peripheral plasma from patients with OLRs and elucidate the cytokine profile in the OLR microenvironment.
Materials and methods: A total of 26 paired intralesional and peripheral plasma samples were collected from patients with OLRs. A panel of 15 cytokines was measured using a Luminex assay. The reticular, erythema, and ulcerative score was used to evaluate the degree of OLR severity.
Results: IL-10 was detected in a fewer number of intralesional samples (19/26) compared to peripheral samples (26/26, $p = 0.01$). The intralesional plasma exhibited significantly

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elevated levels of granzyme B (median 108.94 *vs.* 16.00), TGF-β1 (mean 30448.92 *vs.* 10199.04), TGF-β2 (mean 1659.73 *vs.* 1308.49), and TGF-β3 (mean 914.33 *vs.* 573.13) compared to the peripheral plasma ($p = 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). The levels of intralesional IL-2 (median 2.84 *vs.* 3.45, $p = 0.019$) and TNF-α (median 7.66 *vs.* 10.34, $p = 0.048$) were significantly lower in the intralesional plasma compared to the peripheral plasma.

Conclusion: The intralesional concentrations of granzyme B and TGF-β were elevated, whereas IL-2 and TNF-α were decreased in the OLR microenvironment compared to the peripheral plasma. These findings may contribute to establishing a panel of biomarkers that can be used to monitor the disease activity of OLRs in a large cohort study in future.

Introduction

Oral lichenoid reactions (OLRs) represent a spectrum of inflammatory diseases involving the interface of the oral mucosa. OLRs can be classified as oral lichen planus (OLP) and its sister disease, oral lichenoid lesion (OLL), which includes oral lichenoid contact reactions, oral lichenoid drug eruptions, and oral lichenoid reactions associated with graft-versus-host disease.[1] Although the precise etiology of OLRs has not been fully elucidated,[2] most clinicians agree that inflammatory cell infiltration and activation is a common feature.[3]

A large number of cytokines function to recruit inflammatory cells, a hallmark of OLRs. The cytokines produced by immune cells can be divided into T helper (Th)1, Th2, Th17, and other subgroups. Our previous study detected a panel of cytokines, including interleukin (IL) −2, IL-4, IL-5, IL-6, IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-γ, and transforming growth factor (TGF)-β in saliva samples from patients with OLRs.[4] The cytokine profiles associated with inflammatory cells in relation to OLRs have also been widely investigated in peripheral blood samples. However, different types of samples may express altered cytokine levels, making it challenging to draw a definitive conclusion.[5–7] In most oral mucosal diseases, high cytokine concentrations in the local microenvironment play a transient immunomodulatory function in an autocrine or paracrine manner.[8–12] Therefore, intralesional blood samples may be optimal to reflect the actual pattern of inflammation in the OLR microenvironment, and has previously been explored in the field of dermatology.[13] In addition, due to a lack of available objective indicators that can directly reflect the level of local inflammation, cytokine concentrations in the inflammatory region provide a potential approach.

In this study, we hypothesized that the cytokine profile in the intralesional blood would differ from that of the peripheral blood. Thus, to compare the cytokine profiles between these two sample types and elucidate the cytokine features in the OLR microenvironment, paired intralesional and peripheral blood samples were collected from patients with OLRs. These findings may contribute to establishing a panel of biomarkers that can be used to monitor the disease activity of OLRs.

Materials and methods

Study design

This study was independently reviewed and approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (approval ID: SH9H-2020-TK376-1, approved on May 25th, 2020) and conducted according to the Declaration of Helsinki and the additional requirements. This study was undertaken with adequate understanding and written consent of each participant. We recruited patients with OLRs who visited the Department of Oral Medicine, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. Patients aged ≥18 years with a diagnosis of OLRs based on both clinical and histopathological criteria in accordance with the modified WHO criteria were included.[14] K.S. and Y.W. independently performed the clinical diagnosis and two experienced pathologists independently performed the histopathological diagnosis. Patients who used topical or systemic corticosteroids within 3 months were excluded. A total of 26 pairs of intralesional and peripheral blood samples were collected for this study.

Collection of intralesional and peripheral blood samples

The patients received a daily administration of 50 mg fluconazole and a 1% sodium bicarbonate solution for approximately 2 weeks prior to blood collection. Under local anesthesia, a 5-mm incision was made in the lesion at a depth of approximately 3 mm. Intralesional blood from the site of the lesion (buccal mucosa or tongue) was collected and immediately transferred into an EDTA-embedded Eppendorf tube, followed by centrifugation at 6000 rpm for 8 min. The peripheral blood was collected and treated with the same protocol. The supernatant plasma was collected and stored at −80 °C.

Luminex assay of cytokine concentrations

Based on the results of our previous study,[4] the cytokine profile in this study consisted of a selection of a total of 15 cytokines that were divided into four subgroups: 1) Th1

cytokines: IL-2, TNF-α, IFN-α, IFN-β, IFN-γ, and IL-12p70; 2) Th2 cytokines: IL-4, IL-5, IL-6, and IL-10; 3) Th17 cytokines: IL17A; and 4) other cytokines: TGF-β1, TGF-β2, TGF-β3, and granzyme B.

The cytokine profile was detected and measured using a Human XL Cytokine Luminex Performance Panel Premixed Kit (R&D Systems Inc., Minneapolis, MN, USA) and Bio-Plex Pro™ TGF-β 3-plex Assay (Bio-Rad Laboratories Inc., California, CA, USA) in accordance with the manufacturers' protocols. Briefly, 50 μL of diluted resuspended beads were embedded into each well of a 96-well plate for antibody capture. Then, 50 μL of the standard substance and samples were added. Diluted biotin labeled antibody complex, streptavidin labeled PE, and detergent were added accordingly to each well. Fluorescence intensity readings for the standards and samples were detected on a Luminex 200 sorting and detection platform (Luminex Corp, Austin, TX, USA). Fluorescence data were further converted into the corresponding cytokine concentrations using R&D Analyst software.

The reliable working range of each cytokine was determined and the detection rate was calculated from the standard curve. The detection rates could be compared when the detection rate of a given cytokine in any of the intralesional or peripheral plasma groups was above 60%. The concentrations could be numerically compared when the detection rate of a given cytokine in both the intralesional and peripheral plasma groups was above 80%.[15]

Evaluation of severity

The reticular, erythema, and ulcerative (REU) scoring system was used to evaluate OLR severity as previously described.[16] In brief, the oral cavity was divided into 10 sites: labial mucosa; right buccal mucosa; left buccal mucosa; dorsal tongue; ventral tongue; floor of mouth; hard palate mucosa; soft palate/tonsillar pillars; maxillary gingiva; and mandibular gingiva. In this system, R represents reticular/hyperkeratotic lesions and each site scored from 0 to 1; E and U represent erosive/erythematous and ulcerative areas, respectively, and both were scored from 0 to 3 in each site. The REU score was calculated as: REU score $= 1 \times \Sigma R + 1.5 \times \Sigma E + 2 \times \Sigma U$.

Statistical analysis

The results of the continuous variables were presented as the mean $\pm$ standard deviations (SD) or median (interquartile range, IQR). An independent *t*-test and Mann—Whitney *U* test were performed for normally distributed and non-normally distributed data, respectively. A Chi-squared or Fisher's exact probability test were used to calculate the differences in the detection rates. A Spearman's correlation test was used to analyze the correlation between REU scores and cytokine concentrations, and results were presented as the coefficient of correlation (R). The REU score was not applicable for one subject and the missing value was replaced with the series mean. Data analysis was performed using SPSS 24.0 software (IBM Corp., Armonk, NY, USA). Graph Prism 7 (Graph Pad Software Corp., San Diego, CA, USA) was used to create graphic figures. A $p < 0.05$ was considered statistically significant.

Results

Participant characteristics

A total of 26 participants with OLRs were enrolled in our study, of whom 14 (53.85%) were diagnosed as OLP and 12 (46.15%) as OLL. There was a higher number of female participants than male participants (17 females and 9 males), and the average age was 49.27 $\pm$ 13.77 years. Table 1 lists detailed information regarding the lesion site, systemic diseases, drugs used within 3 months, and REU score for each participant.

Cytokine detection rate

The detection rates for granzyme B, IL-2, IL-12p70, TNF-α, TGF-β1, TGF-β2, and TGF-β3 were over 80% for both the intralesional and peripheral plasma. The concentrations of these seven cytokines were further analyzed. The detection rates of IFN-α, IL-6, IL-10, and IL-17A were compared (Table 2). Since the detection rate in both samples was less than 60% for IFN-β, IFN-γ, IL-4 and IL-5, these cytokines could not be analyzed qualitatively or quantitatively.

IL-10 was detected within the reliable working range for 19 intralesional samples (19/26, 73.08%) and all of the 26 peripheral samples (26/26, 100%, $p = 0.010$). IFN-α was detected in 23 intralesional samples (23/26, 88.46%) compared to 20 peripheral samples (20/26, 76.92%, $p = 0.465$). IL-6 was detected in 19 intralesional samples (19/26, 73.08%) compared to 21 peripheral samples (21/26, 80.77%, $p = 0.510$). The detection rate of IL-17A was slightly lower in the intralesional samples (16/26, 61.54%) compared to that of the peripheral samples (19/26, 73.08%, $p = 0.375$). These data indicate that the detection rate of IL-10 was higher in the peripheral plasma compared to that in the intralesional plasma (Fig. 1).

Comparison of cytokine concentrations

The concentrations of granzyme B, TGF-β1, TGF-β2, and TGF-β3 in the intralesional plasma of the participants with OLRs were significantly higher compared to that observed in the peripheral plasma ($p = 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively; Table 3; Fig. 2A – D). The concentrations of IL-2 and TNF-α in the intralesional plasma were significantly lower than those in the peripheral plasma ($p = 0.009$ and $p = 0.048$, respectively; Table 3; Fig. 2E and F). No significant differences were observed for IL-12p70 ($p = 0.697$; Fig. 2G).

Correlation between REU score and cytokine concentrations

The concentration of IL-12p70 in the peripheral plasma was positively correlated with the REU score in the patients with OLRs (coefficient of correlation $= 0.463$, $p = 0.02$;

Table 1 Clinical characteristics of the participants with OLRs.

No.	Gender	Age (years)	Lesion Site	Systemic Disease	Drugs Used within 3 Months	REU score	Diagnosis
1	F	69	dorsum of tongue	osteoporosis, rheumatoid arthritis	vitamin D3, calcium, glucosamine hydrochloride	8	OLP
2	F	56	buccal mucosa	hypertension, hyperlipidemia	telmisartan, amlodipine aspartate, fenofibrate	5	OLL
3	F	45	buccal mucosa	denied	denied	5	OLP
4	M	34	buccal mucosa	denied	denied	2	OLP
5	F	58	buccal mucosa	hypertension	amlodipine, vitamins	2	OLL
6	F	62	buccal mucosa	hypertension, oral leukoplakia	valsartan, concentrated tinidazole gargles	3	OLP
7	M	39	buccal mucosa	denied	denied	4	OLL
8	F	49	buccal mucosa	denied	cefdinir	3	OLL
9	M	32	dorsum of tongue	denied	denied	7.5	OLL
10	F	61	buccal mucosa	carotid plaque, chronic gastritis, facial paralysis	rosuvastatin calcium, omeprazole, artificial bezoar metronidazole, mecobalamin	15	OLP
11	F	64	buccal mucosa	denied	denied	11.5	OLL
12	F	39	buccal mucosa	malignant teratoma	denied	6.5	OLP
13	M	65	buccal mucosa	diabetes mellitus, hypertension, cerebral insufficiency of blood supply	metformin hydrochloride, irbesartan and hydrochlorothiazide, TCM for promoting blood circulation (Yin Dan Xin Nao Tong capsule)	13	OLL
14	F	67	buccal mucosa	denied	denied	5.5	OLP
15	M	30	buccal mucosa	denied	denied	8	OLP
16	F	25	buccal mucosa	NA	NA	NA	OLP
17	F	44	buccal mucosa	denied	denied	3	OLP
18	M	25	ventral tongue	denied	denied	7	OLP
19	M	58	buccal mucosa	diabetes mellitus, lacunar infarction, hypertension	metformin hydrochloride, atorvastatin calcium, valsartan	10	OLL
20	M	46	buccal mucosa	denied	denied	4.5	OLL
21	F	56	buccal mucosa	diabetes mellitus, psoriasis	metformin hydrochloride	10	OLP
22	F	55	buccal mucosa	lumbar disc protrusion	mecobalamin	10	OLP
23	M	55	ventral tongue	denied	TCM for clearing heat and toxic material (Bi Bai Ke caspule)	4.5	OLL
24	F	48	buccal mucosa	chronic gastritis	vitamin U, belladonna and aluminium capsules, cefdinir	6	OLL
25	F	32	buccal mucosa	denied	denied	4	OLP
26	F	67	buccal mucosa	denied	vitamins	7	OLL

OLRs: oral lichenoid reactions; REU: reticular, erythema, and ulcerative; OLL: oral lichenoid lesion; F: female; M: male; TCM: traditional Chinese medicine; NA: not applicable.

Table 4). No significant correlation between the REU score and cytokine concentration was found for IL-12p70 in the intralesional plasma or the other detected cytokines.

Discussion

In this study, patients with OLRs were enrolled instead of those with OLP alone. To date, there is a lack of diagnostic criteria that can be used to precisely distinguish OLP from OLL.[17] Moreover, OLL has frequently been used to indicate an uncertain diagnosis of OLP.[18] However, a diagnosis of OLR is more clinically applicable since it represents the typical spectrum of interface oral mucositis. Moreover, OLRs could also reflect the features of the OLP microenvironment.

There have been few recent studies on the regional immune characteristics of the oral mucosa. OLRs are a form of intractable inflammation mainly caused by an immune deficiency or dysregulation of the oral immune system.[19] In addition, cytokines can be used as reliable biomarkers to monitor the pattern and severity of inflammatory diseases.[20,21] In this study, a panel of four inflammatory cell-related cytokine subgroups were used to profile the

Table 2 Detection rate for each cytokine.

Cytokines	Range (pg/ml)	Number of samples		p
		Intralesional	Peripheral	
Granzyme B*	3.46—29884	25/26	24/26	1.000
IFN-α*	1.54—38240	23/26	20/26	0.465
IFN-β*	1.27—29364	1/26	5/26	0.191
IFN-γ	2.94—42396	0/26	12/26	<0.001
IL-2*	1.22—31590	26/26	26/26	—
IL-4	0.41—9564	4/26	14/26	0.004
IL-5*	2.49—47546	0/26	4/26	0.110
IL-6	3.84—71032	19/26	21/26	0.510
IL-10*	18.42—203564	19/26	26/26	0.010
IL-12p70*	7.57—155,916	23/26	25/26	0.610
IL-17A	2.84—80204	16/26	19/26	0.375
TNF-α*	4.53—106272	23/26	23/26	1.000
TGF-β1	5.50—334,970	26/26	26/26	—
TGF-β2	12.40—388,090	26/26	26/26	—
TGF-β3*	4.05—370,010	25/26	26/26	1.000

Chi-square was used. *: A Fisher's exact probability test was used. The concentrations of granzyme B, IL-2, IL-12p70, TNF-α, TGF-β1, TGF-β2, and TGF-β3 should be further analyzed due to the high detection rate. IFN: interferon; IL: interleukin; TNF: tumor necrosis factor; TGF: transforming growth factor; p: probability.

Table 3 Comparison of the cytokine concentrations from the intralesional and peripheral plasma of OLR samples.

Cytokines	Concentrations (pg/ml)		p
	Intralesional	Peripheral	
Granzyme B	108.94 (57.80,404.94)	26.00 (20.25,58.98)	<0.001
IL-2	2.84 (2.04,3.26)	3.45 (2.49—5.53)	0.019
IL-12p70	10.59 (8.41,12.93)	10.59 (8.41,11.76)	0.697
TNF-α	7.66 (6.82,9.42)	10.34 (7.66,12.24)	0.048
TGF-β1	30448.92 ± 19210.18	10199.04 ± 5303.33	<0.001
TGF-β2	1659.73 ± 319.49	1308.49 ± 166.85	<0.001
TGF-β3	914.33 ± 232.90	573.13 ± 208.32	<0.001

Data are presented as the mean ± standard deviations or median (25% interquartile, 75% interquartile) where appropriate. OLR: oral lichenoid reaction; p: probability; IL: interleukin; TNF: tumor necrosis factor; TGF: transforming growth factor.

intralesional microenvironment of OLRs. Many previous studies of cytokine profiles derived from peripheral blood mononuclear cells (PBMCs) compared between diseased and healthy subjects. In contrast, our study detected cytokine concentrations in the intralesional and peripheral plasma, with the aim of obtaining a more localized inflammatory profile of OLRs and exploring a reliable panel of biomarkers that can be used to monitor the severity of OLRs at a molecular level.

Our results revealed that the granzyme B and TGF-β concentrations were higher in the intralesional plasma of OLRs compared to the peripheral plasma. Increased granzyme B expression has been previously reported in OLP lesions compared to the cutaneous lichen planus, which is consistent with our findings.[22] Thus, intralesional granzyme B may represent a potential biomarker for the differential diagnosis of oral or cutaneous lesions. The concentration of TGF-β in OLRs is controversial. In our previous study, salivary TGF-β did not significantly differ between patients with OLP and healthy controls,[4] while Zenouz et al. found a significant decrease in the levels of serum TGF-β in OLP patients compared to healthy controls.[23] Given these discrepancies, further studies are warranted to evaluate whether intralesional granzyme B or TGF-β can be used to monitor the disease activity and therapeutic effects of OLRs.

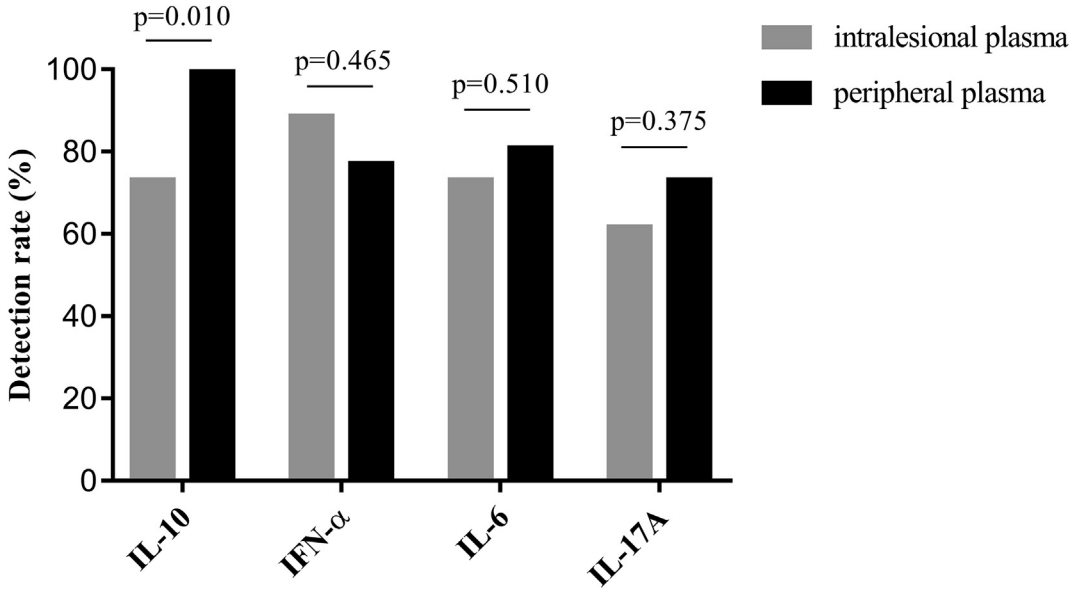

Figure 1 Comparison of the detection rate for IFN-α, IL-6, IL-10, and IL-17A. The detection rate of these cytokines met the criteria for qualitative analysis. IFN, interferon; IL, interleukin.

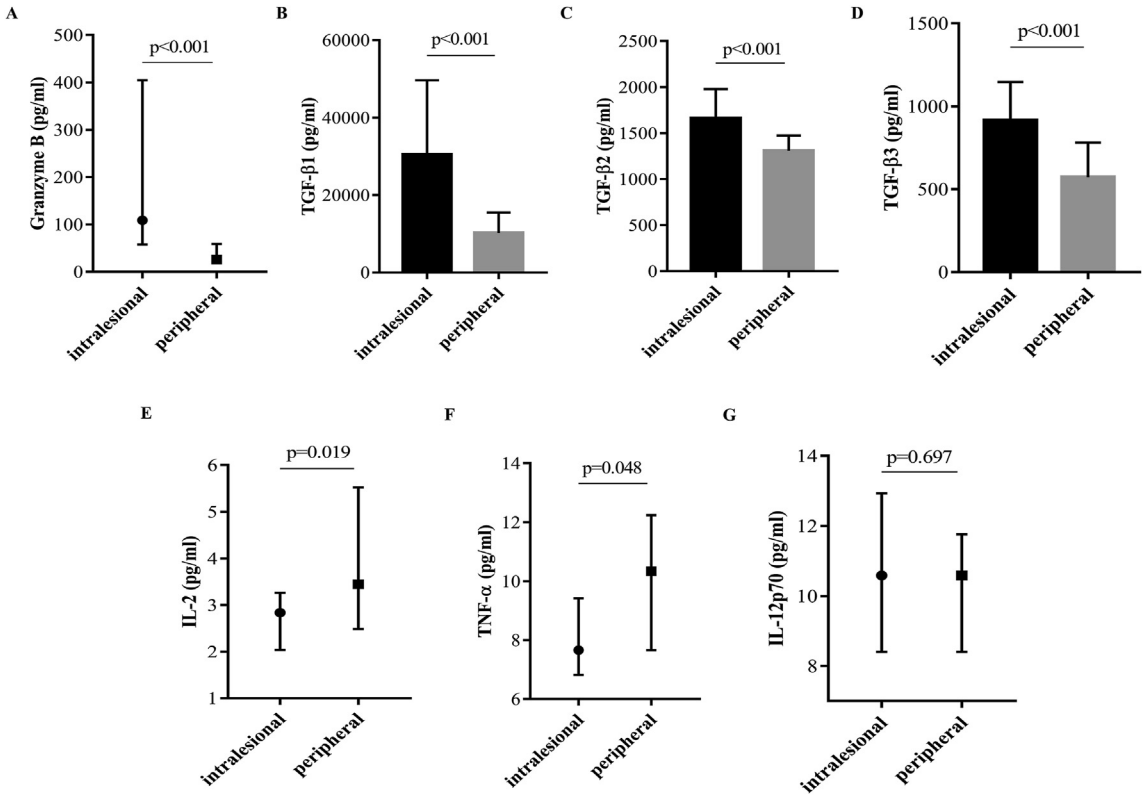

Figure 2 Comparison of the concentrations of granzyme B, IL-2, IL-12p70, TNF-α, TGF-β1, TGF-β2, and TGF-β3. The detection rate was above 80% in both groups. The data were analyzed using a *t*-test or Mann–Whitney test. IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor.

Table 4 Correlations between the REU score and each cytokine.

Cytokines	Intralesional		Peripheral	
	R	p	R	p
Granzyme B	0.017	0.936	−0.102	0.634
IFN-α	0.012	0.955	0.165	0.499
IL-2	0.072	0.725	0.093	0.652
IL-4	0.943	0.057	−0.08	0.785
IL-6	−0.116	0.637	0.015	0.947
IL-10	−0.105	0.669	0.028	0.891
IL-12p70	0.339	0.113	0.463	0.02*
IL-17A	0.041	0.879	0.031	0.899
TNF-α	−0.035	0.876	0.046	0.836
TGF-β1	−0.254	0.21	0.077	0.707
TGF-β2	−0.117	0.57	0.357	0.073
TGF-β3	−0.254	0.22	0.175	0.392

REU: reticular, erythema, and ulcerative; R: coefficient of correlation; p: probability; *: $p < 0.05$. IL: interleukin; TNF: tumor necrosis factor; TGF: transforming growth factor.

Similar to granzyme B, TNF-α is believed to be another important "killer" cytokine. In our study, the concentration of TNF-α was lower in the intralesional plasma compared to peripheral plasma of OLR patients, which may be due to the compensatory effect of granzyme B. It has also been reported that different detection methods may lead to different results. Most studies have observed elevated levels of TNF-α in the serum of patients with OLRs using an ELISA.[24,25] In contrast, no statistical differences in the serum levels of TNF-α were found between patients with OLRs and healthy controls when bead-based flow cytometry was used.[4,26] Thus, further comparisons between different methods of cytokine detection are required.

In this study, the concentration of IL-12p70 in the peripheral plasma was positively correlated with the REU score. Higher levels of IL-12 have been found in systemic lupus erythematosus patients compared to controls, and serum levels of the p40 subunit were found to be positively correlated with disease severity,[27] which is consistent with our results. Moreover, Ohno et al. reported that PBMCs from OLP patients produced higher amounts of IL-12 than those from healthy controls.[28] Similarly, Janardhanam et al. reported that salivary epithelial cells from OLP subjects express increased levels of IL-12 compared to healthy controls.[29] These findings suggest that IL-12 may represent a potential diagnostic and monitory biomarker for OLP. However, in our study, the intralesional concentration of IL-12p70 and other cytokines were not statistically correlated with the REU score. We speculate that this difference may be attributed to the fact that the local site of the biopsy does not reflect the overall profile of the oral lesions. Some other biomarkers of OLRs should be explored in future studies.

There are some limitations associated with this preliminary study. The concentrations of some cytokines are

below the lower limits of detection. Neither the detection rate of IFN-γ or IL-4, which represent Th1 and Th2 cytokines, respectively, met the criteria for data analysis in this study. Since the Th1/Th2 imbalance is controversial,[30] further studies using methods with greater sensitivity are needed in the future. Moreover, during the process of intralesional blood collection, the volume of the collected blood from some of the patients did not meet the requirements for a Luminex assay. Therefore, an optimized protocol for the collection of intralesional blood is essential for future studies. In addition, due to ethical concerns, we could not collect the intralesional plasma from healthy control subjects or participants after treatment, which restricted our ability to compare our data with these control groups.

In conclusion, the intralesional concentrations of granzyme B and TGF-β were found to be higher, whereas the concentrations of IL-2 and TNF-α were decreased in the OLR microenvironment compared to the peripheral plasma. A large cohort study should be considered to further confirm our findings.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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