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Original Article

Direct immunofluorescence and immune function in patients with oral lichen planus

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REU score;
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Background/Purpose: Direct immunofluorescence and immune function and patients with oral lichen planusThe etiology of oral lichen planus (OLP) is unknown, our purpose was to evaluate the diagnostic value of direct immunofluorescence (DIF) and to investigate the immune functions in OLP.

Materials and methods: We enrolled 65 patients with suspected lesions of OLP and 47 controls. In all participants, clinical and serologic testing were conducted. The histopathologic and DIF tests were conducted in 65 patients. The severity of OLP was evaluated by reticular/hyperkeratotic, erosive/erythematous, ulcerative (REU) scoring system.

Results: By hematoxylin and eosin (H&E) staining and DIF examination, 71.2% (42/59) were diagnosed as OLP, 28.8% (17/59) were diagnosed as non-OLP. DIF demonstrated 64.3% positive reactivity with 2 distinct distribution patterns and 8 staining patterns. Compared to the controls, serum IgA in OLP was higher ($P < 0.01$), and serum CD3+ cells, IgM, IgE, C3 and C4 were lower ($P < 0.05$). Pearson correlation analysis in OLP revealed correlations between REU score and IgM, IgA of DIF ($r = 0.54$, $P = 0.026$; and $r = 0.56$, $P = 0.020$, respectively), between serum IgG and IgG of DIF ($r = 0.51$, $P = 0.038$), between serum CD4+ and the ratio of CD4+/CD8+, IgM in DIF ($r = -0.50$, $P = 0.048$; and $r = -0.54$, $P = 0.031$, respectively), between serum CD8+ and IgM, IgA in DIF ($r = 0.52$, $P = 0.038$; and $r = -0.50$, $P = 0.047$, respectively).

Conclusion: A combination of H&E test and DIF is useful for the diagnosis of OLP. Compared to controls, immune changes happen to patients with OLP. There are significant associations

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between the OLP lesions and general cellular and humoral immune status, localized humoral immune response.
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Introduction

Oral lichen planus (OLP) is an immune-mediated condition in the oral cavity and affects 1—2% of the population. OLP has a greater impact on women than men at a ratio of 1.4:1, mainly in adults over 40 years old.[1] Any site of oral mucosa can be involved, such as the buccal, tongue, palate and gingival mucosa. Different clinical forms of OLP including reticular, plaque, atrophic, erosive, ulcerative, and bullous presentations, are described in the literature. Among them, the reticular form is the most manifestations. OLP is a premalignant condition whose malignant transformation rate is 0—2%.[2]

Under the microscope, there is dense infiltration of subepithelial lymphocytes and degeneration of basal keratinocytes. Shaggy deposition of fibrinogen along the basement membrane zone (BMZ) is the unique pattern of direct immunofluorescence (DIF).[3] The previous studies almost concentrated on linear or shaggy fibrinogen and colloid body at the BMZ of OLP, only a few studies described the antibodies and complement in DIF examinations of OLP.[4—7] In this study, we overlooked the fibrinogen and colloid body and focused on the antibodies and complement of DIF results.

The previous studies suggested that OLP was a chronic inflammation mediated by T lymphocytes.[8—10] Only a few studies described the general immune status and localized humoral immune response of OLP. Langerhans cells present antigens to CD4+ helper (Th) cells and CD8+ cytotoxic T cells (CTL). The activated Th cells can promote the proliferation of CTL by secreting interferon-gamma and interleukin-2. In final, CTL can cause apoptosis and degeneration of basal cells.[11—15]

To explore the definite role of immune factors in OLP, we examined cellular immunity and humoral immunity to determine the factors related to OLP. We measured the levels of CD3+ cells (lymphocytes), CD4+ cells (Th cells), CD8+ cells (CTL), and CD4+/CD8+ cell ratio (an immunomodulatory index) as indicators of cellular immunity, immunoglobulin (including IgG, IgA, IgM, IgE) and complement C3 and C4 as measures of the humoral immunity. We further evaluated the correlations between DIF results and serologic testing, the severity of OLP to find the relationship between OLP lesions and localized and systemic immune function.

Materials and methods

Participants

We enrolled 65 patients with suspected oral mucosal lesions of OLP. The inclusion criteria was a suspected clinical diagnosis of OLP, these patients had no systemic immune diseases, had not received immunotherapy, systemic medication, concomitant chemotherapy, and/or radiotherapy in the past 3 months, and there was no amalgam in their mouths.

A total of 47 age- and gender-matched controls were involved. These normal subjects reported no systemic diseases or problems associated with OLP and no soft tissue lesions in the oral cavity in the past. Clinical, and serologic testing were conducted in all participants. The histopathologic, DIF studies were conducted for patients with suspected oral mucosal lesions of OLP.

In this study, all the patients and controls were from the Department of Oral Medicine, West China College of Stomatology, Sichuan University. All the studies were carried out by the guidelines of the Medical Ethics Committee of West China Hospital of Stomatology. All patients provided informed consent before their participation. The study was carried out following the principles of the Declaration of Helsinki.

Hematoxylin and eosin (H&E) staining

Six patients lost to follow-up were excluded, and 59 patients were biopsied in the Oral Medicine Department of West China Hospital of Stomatology of Sichuan University. Specimens were cut to two parts equally for H&E testing and direct immunofluorescence respectively. The samples for H&E examinations were stored in 10% formalin, then sent to the Oral Pathology Department of West China Hospital of Stomatology. The tissues were processed overnight, then we used alcohol to dehydrate, xylene to clear, paraffin to infuse the tissues, then they were cut to 4-mm-thick sections, stained with H & E, dried, and observed under a light microscope at 500 magnification.

Direct immunofluorescence

The specimens submitted for DIF were received in normal saline, then sent to the Pathology Department of West China Hospital of Sichuan University. The biopsies were frozen on dry ice and cut to 4-mm-thick sections, stained for IgM, IgA, IgG, and C3 using fluorescein-labeled goat anti-human conjugates. The interpretation was carried out by a fluorescence microscope at 200 magnification.

Serologic testing

All the blood samples were collected at the same time after the patient fasted overnight. The expression of CD3+, CD4+, CD8+, and CD4+/CD8+ cells were detected by flow

cytometry, and the expression of IgG, IgA, IgM, IgE, C3, C4 were detected by immunoturbidimetry. All serological tests were carried out in the cooperating Laboratory of Clinical Immunology, West China Hospital, Sichuan University, and reagents were obtained commercially.

Reticular/hyperkeratotic, erosive/erythematous, ulcerative scoring system

Reticular/hyperkeratotic, erosive/erythematous, ulcerative (REU) scoring system was used chairside to evaluate the severity of OLP and completed after the initial interview.[16] The oral mucosa was divided into 10 sites (labial, right buccal, left buccal, dorsal tongue, ventral tongue, floor of mouth, hard palate, soft palate/tonsillar pillars, maxillary gingiva, mandibular gingiva). Area of each part was measured with a flexible periodontal scale probe.[17] The severity was measured as follows: reticular (R) (0 = no white striations, 1 = presence of white striations or papules); erosive/erythematous (E) and ulcerative (U) (0 = no lesion, 1 = lesions less than 1 cm^2, 2 = lesions from 1 to 3 cm^2, 3 = lesions greater than 3 cm^2). REU score was summation of the scores: reticular score = R, erythema score = E, and ulcerative score = U with a total weighted score of $R + E \times 1.5 + U \times 2.0$.[18]

Diagnosis

The diagnosis for each patient was assessed by clinical appearance and histopathological test rendered by pathologists. When some non-OLP patients had a vague HE diagnosis, DIF testing characterized the lesions further and determined the underlying pathologic features. Since the diagnosis of H&E and DIF results in some non-OLP patients was not specific, an oral medicine expert with more than 10 years of clinical experience could give the final diagnosis through combined clinical appearance and pathology. Diagnostic algorithm and testing process for all the participants are shown in Fig. 1.

Statistical analysis

We used a built-in function in the R language (version 4.0.3) to analyze the data. Correlation analysis of data was performed using the "corrplot" R package. Descriptive statistical methods (mean, standard deviation, frequency) were used to evaluate the data, student's t-test to analyze quantitative data, Chi-square test to analyze qualitative data. Pearson correlation coefficients were used to evaluate correlations among the identified variables. P values of <0.05 were significant, and the level in confidence intervals was 95%.

Results

Demographic characteristics

We excluded patients who were pathologically diagnosed as erythema multiforme, benign mucous membrane pemphigoid, lichen planus pemphigoid, discoid lupus erythematosus,

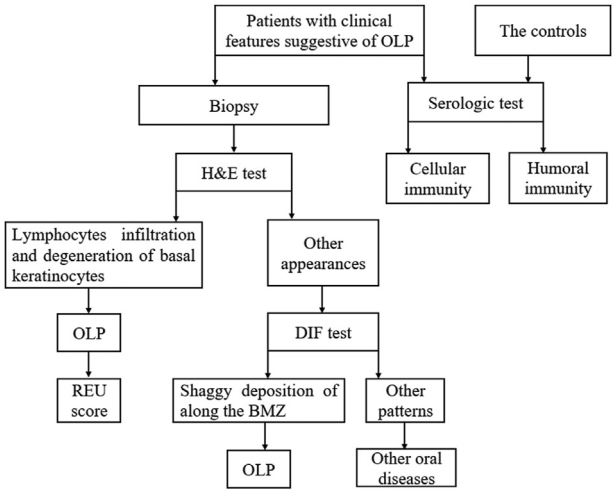

Figure 1 Diagnostic algorithm for oral lichen planus and testing process for all the participants. OLP, oral lichen planus; H&E, hematoxylin and eosin staining; DIF; direct immunofluorescence; REU score (REU: reticular/hyperkeratotic, erosive/erythematous, ulcerative); BMZ, basement membrane zone. IgG, Immunoglobulin G; IgA, Immunoglobulin A; IgM, Immunoglobulin M; IgE, Immunoglobulin E; C3, Complement C3; C4, Complement C4.

oral leukoplakia, white sponge nevus, lichenoid reaction. 17 patients were diagnosed as other oral diseases, and 6 patients were lost to follow-up, so 42 patients with OLP and 47 controls were included. The average age of OLP was 39.6 ± 13.7 years old, while that of the control group was 48.1 ± 12.0 years old. Of the 47 patients with OLP, 26 were female (61.9%), 16 were male (38.1%), and of the 47 controls, 35 were female (74.5%) and 12 were male (25.5%). There was no significant difference in age and sex between OLP and the controls (P = 0.296).

Clinical features

Lesions of 42 OLP patients only involve the oral mucosa. No skin involvement or nail involvement were seen. Aeras of involving included buccal (n = 39.92.9%), tongue (n = 23,54.8%), gingival (n = 14,33.3%), lip (n = 13,31.0%), vestibular groove (n = 17,40.5%) lesions, 31 patients (73.8%) showed more than 2 involvement sites. Clinical presentation showed reticular (n = 17,40.5%), atrophic/erythematous (n = 6,14.3%), erosive (n = 9,21.4%), ulcerative (n = 7,16.7%), bullous (n = 3,7.1%) lesions. Referring to the clinical presentation of lesions, we divided 42 OLP patients into 2 clinical subtypes: reticular (n = 17) and ulcer subtype (including atrophic/erythematous, erosion, ulcer, bullous type, n = 25). The clinical appearances of representative cases (case 23, case 34) are shown in Fig. 2.

Hematoxylin and eosin staining

Microscopic features of 42 OLP patients included hyperparakeratosis, hyperorthokeratosis, basal cell liquefaction, and a band-like dense infiltration of subepithelial

Table 1 The staining patterns of DIF detection in OLP.

IgM	IgA	IgG	C3	layer	n (%)
−	−	−	−	/	15 (35.7%)
−	−	−	+	B	10 (23.8%)
+	−	−	−	L	7 (16.7%)
+	+	−	−	L	4 (9.5%)
+	+	+	+	L	2 (4.8%)
+	−	−	+	L	2 (4.8%)
+	−	+	+	L	1 (2.4%)
+	−	−	+	B	1 (2.4%)

DIF, direct immunofluorescence; OLP indicates oral lichen planus; IgM, Immunoglobulin M; IgA, Immunoglobulin A; IgG, Immunoglobulin G; C3, Complement C3; B, basement membrane zone; L, lamina propria;/, not available.

lymphocytes. The microscopic features of representative cases (case 23, case 34) are shown in Fig. 2.

Direct immunofluorescence

DIF demonstrated positive reactivity was 64.3% [27 of 42], with 2 distinct distribution patterns (Fig. 2). By the distribution of antibodies or complement, these included positive reactivity with dermal side (35.7% [15 of 42]), with basement membrane zone (BMZ) (28.6% [12 of 42]). By the subtypes of antibodies or complement, suspicious results were regarded as positive results, the IGM reactivity (17,40.5%) was the most common, followed by C3 reactivity (16,38.1%), IGA (6,14.3%) and IGG (4,9.5%). The negative reactivities were IGM (25,59.5%), IGA (36,85.7%), IGG (38,90.5%), C3 (26,61.9%).

DIF demonstrated reactivity with 8 distinct staining patterns (Table 1). The top 3 staining patterns were negative staining pattern (35.7% [15 of 42]), positive C3 reactivity with BMZ (23.8% [10 of 42]) and positive IgM reactivity with lamina propria (16.7% [7 of 42]), only 2 (4.8%) patients showed consistent positive reactivity of all the antibodies and complement.

As for DIF results, the 2 clinical subtypes did not differ from each other ($P > 0.05$).

Cellular immunity

Table 2 summarizes the level of serum cellular immunity in patients with OLP and control groups. The ratio of serum CD3+ cells in OLP group was lower than controls ($P < 0.01$). There were no differences in the ratio of CD4+, CD8+ CD4+/CD8+ cells between OLP and the controls ($P > 0.05$). No significant difference was found between the ulcerative OLP and reticular OLP groups ($P > 0.05$).

Humoral immunity

Table 2 summarizes the level of serum humoral immunity in patients with OLP and control groups. The concentration of serum IgA in OLP was higher than the controls significantly ($P < 0.01$), and the concentrations of serum IgM, IgE, complement C3 and C4 were significantly lower than the controls ($P < 0.05$). The concentration of IgG in serum in patients with OLP did not differ from those in the healthy controls ($P > 0.05$). No significant difference was found between the ulcerative OLP and reticular OLP groups ($P > 0.05$).

The relationship between the variables of serological results, DIF results and REU scores

When the patients were analyzed together, Pearson correlation analysis revealed a strong correlation between REU score and IgM, IgA of DIF results in patients with OLP ($r = 0.54$, $P = 0.026$; and $r = 0.56$, $P = 0.020$, respectively; Fig. 3). Moreover, a positive relationship was found between IgG in serum and IgG of DIF results ($r = 0.51$, $P = 0.038$; Fig. 3). There were relations between cellular immunity and DIF results, CD4+ and the ratio of CD4+/CD8+ in patients with OLP were negatively correlated with the concentration of IgM in DIF results ($r = -0.50$, $P = 0.048$; and $r = -0.54$,

Table 2 Serologic detection evaluation of serum lymphocyte levels and immunoglobulin levels in OLP and control groups.

	OLP/Control			U/R		
	Control	OLP	P	R	U	P
CD3	72.0 ± 5.7	67.8 ± 8.5	0.003**	67.3 ± 7.3	68.5 ± 9.5	0.451
CD4	38.2 ± 4.4	36.5 ± 8.5	0.212	35.1 ± 6.9	38.3 ± 10.4	0.114
CD8	25.1 ± 4.7	24.4 ± 8.9	0.632	25.6 ± 8.3	23.7 ± 9.4	0.698
CD4/CD8	1.7 ± 0.2	1.7 ± 0.9	0.995	1.6 ± 0.8	1.9 ± 1.0	0.244
IGG	11.9 ± 1.6	12.3 ± 2.2	0.276	12.0 ± 2.2	12.5 ± 2.2	0.419
IGA	1903.4 ± 672.3	2356.6 ± 933.6	0.003**	2220.6 ± 898.9	2449.0 ± 963.5	0.412
IGM	1520.6 ± 710.3	1317.5 ± 574.8	0.028*	1197.5 ± 618.8	1405.8 ± 537.7	0.117
IGE	80.5 ± 54.2	48.3 ± 44.6	<0.001***	37.4 ± 29.4	55.5 ± 51.7	0.419
C3	1.2 ± 0.1	0.9 ± 0.2	<0.001***	0.9 ± 0.1	0.9 ± 0.2	0.444
C4	0.26 ± 0.6	0.2 ± 0.06	<0.001***	0.2 ± 0.04	0.2 ± 0.07	0.221

OLP indicates oral lichen planus.
R, reticular subtype of OLP; U, ulcer subtype of OLP (including atrophic/erythematous, erosion, ulcer, bullous).
IgG, Immunoglobulin G; IgA, Immunoglobulin A; IgM, Immunoglobulin M; IgE, Immunoglobulin E;
C3, Complement C3; C4, Complement C4.
Data are mean ± SD, unless otherwise indicated.
Student's t-test *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

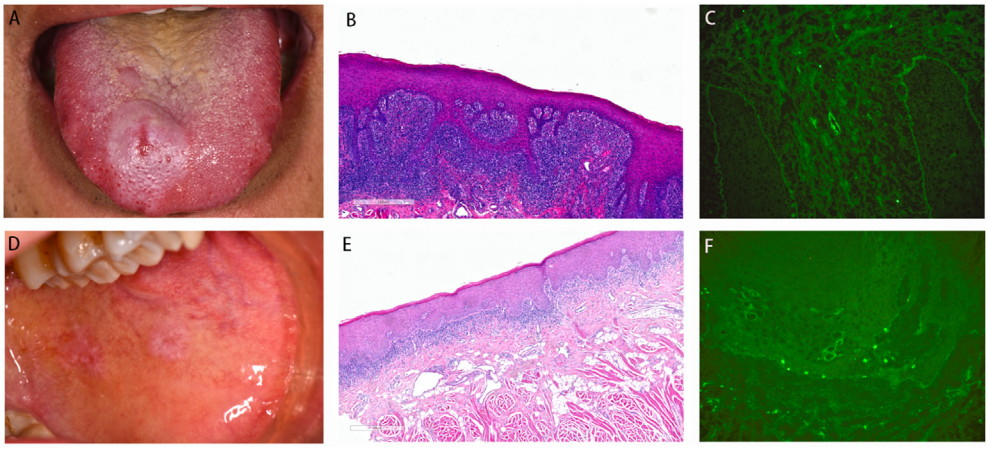

Figure 2 Clinical presentations, histologic presentations and direct immunofluorescence findings of oral lichen planus. A.D. Clinical presentations of oral lichen planus. A. erosive lesions, case 23; D. reticular lesions, case 34; B.E. Histologic presentations in hematoxylin and eosin staining (original magnification ×500) showed basal cell liquefaction and a band-like dense infiltration of subepithelial lymphocytes. C.F. Direct immunofluorescence findings (original magnification ×200). C. positive complement C3 reactivity with basement membrane zone, case 23; F. positive immunoglobulin M reactivity with lamina propria, case 34.

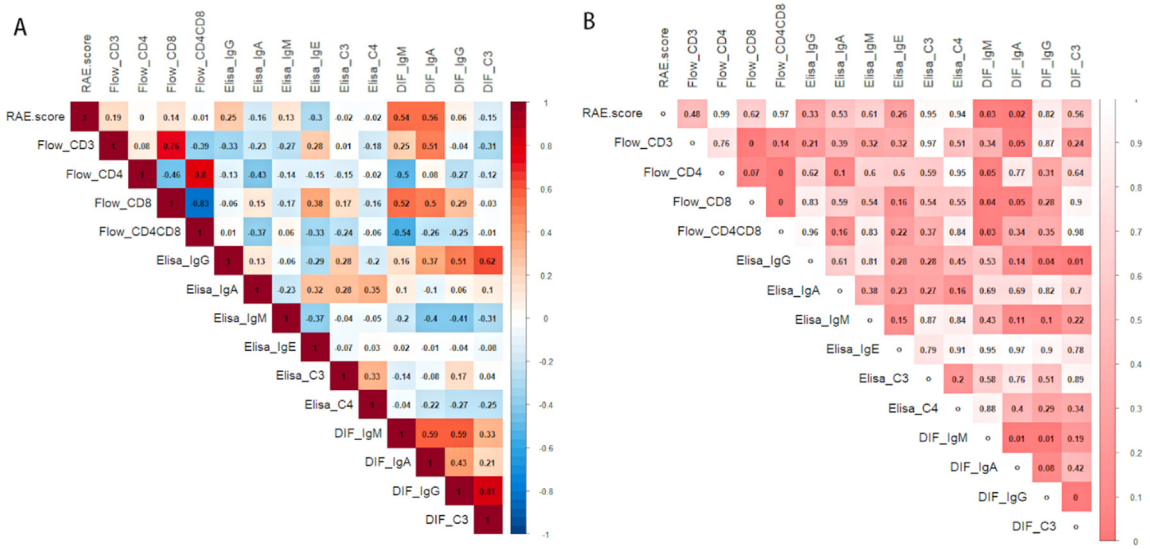

Figure 3 The correlation analysis between the variables of serological results, DIF results and REU scores. A. correlation coefficients of correlation analysis between these variables. B. p-value of correlation analysis between these variables. DIF; direct immunofluorescence; REU scores (REU: reticular/hyperkeratotic, erosive/erythematous, ulcerative); "Flow_" means the variables of cellular immunity; "Elisa_" means the variables of humoral immunity; "DIF_" means the variables of DIF; correlation analysis method : Pearson correlation.

P = 0.031, respectively; Fig. 3), CD8+ was positively correlated with the concentration of IgM and IgA in DIF results (r = 0.52, P = 0.038; and r = −0.5, P = 0.047, respectively; Fig. 3). By the way, Pearson correlation analysis revealed correlations among the IgG, IgM, and C3 of DIF results, among CD3+, CD4+, CD8+ in cellular immunity, but these correlations made no sense.

Discussion

Although there were 59 patients with suspected oral mucosal lesions of OLP due to their white lines or striae, by H&E and DIF examination, 71.2% (42/59) patients were diagnosed as OLP, 28.8% (17/59) were diagnosed as non-OLP. It suggests that we cannot rashly diagnose oral striae diseases as OLP only based on clinical manifestations, all patients considered for a diagnosis of OLP are required to have an oral mucosal biopsy. DIF is usually a necessary method to distinguish OLP from autoimmune blistering diseases, which are usually characterized by desquamative gingivitis.[19] In this way, clinicopathologic correlation is proved, the possibility of accurate diagnosis is increased.

Previous DIF studies showed that the positive rate was 37–97%, whereas positive rate in our study was 64.3% [27 of 42].[5,20,21] As same as Masquijo-Bisio described, the most

antibody deposition was IgM and C3, suggesting that IgM and C3 may play a key role in the localized humoral immunity of OLP.[22]

As same as Al-Fouzan et al., the ratio of CD3+ T cells was significantly lower in OLP than the controls.[23] Previous studies have reported a reduced CD8 lymphocyte,[24] reduced expression of CD4 lymphocytes was underlined by Porter et al.[25] However, our results did not follow these findings.

The results of different researches about immunoglobulins in OLP were very contradictory.[26,27] Dolores Biocina-Lukenda found that elevated serum IgA, which corresponded to our study, supporting that systemic immune changes happen to OLP.[28]

The previous studies suggested that OLP was mediated by a localized cellular immune response.[8–10] In our study, however, Pearson correlation analysis demonstrated that IgM and IgA of DIF results in patients with OLP were positively correlated with their REU scores, in other words, IgM and IgA were positively correlated to the severity of OLP lesions, which suggested that IgM- and IgA-dominated localized humoral immune response may be important in the immunopathogenesis of OLP. We consume that IgM and IgA in lesions could be potential indicators of OLP, this needs further validation.

As for systematic immune response, relationships were observed between IgG in serum and IgG of DIF results, between CD4+ and the ratio of CD4+/CD8+ in serum and IgM in DIF results, between CD8+ in serum and IgM, IgA in DIF results, it showed that significant associations between the OLP lesions and general cellular and humoral immune status. This study had not evaluated localized cellular immunity of lesions, then we could not determine the relationship between localized cellular immunity and OLP lesions.

The production mechanisms of various antibodies in the tissue and serum are still unknown. The nuclear antigens of the damaged areas may be modified by unknown factors and presented to Th cells by the Langerhans cells and macrophages. Under the effect of Th cells, the activated B cells can produce antibodies in the oral lesions and blood. In conclusion, the changes found in humoral immunity may be the result of injury of basal keratinocytes.

Our study evidences significant associations between the OLP lesions and general cellular and humoral immune status, localized humoral immune response. The immunopathogenesis mechanism of OLP needed to be further investigated.

Our findings revealed that a combination of the H&E test and DIF test improved the diagnosis of OLP. There are significant associations between the OLP lesions and general immune status, localized humoral immune response. Especially IgM- and IgA-dominated localized humoral immune response may play a key role in the immunopathogenesis of OLP.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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