Foxp3 gene expression in oral lichen planus: A clinicopathological study

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Abstract. $CD4^+CD25^+$ Forkhead-box protein 3 ($Foxp3^+$) regulatory T cells are important in oral lichen planus (OLP). The present study aimed to investigate Foxp3 expression in $CD4^+CD25^+$ T cells of peripheral blood mononuclear cells and oral lesions in patients diagnosed with OLP, who were grouped as OLP subtype, duration and relapse. Using quantitative polymerase chain reaction (qPCR), western blotting and immunohistochemistry, Foxp3 expression levels in explants of oral lesions and $CD4^+CD25^+$ T cells from 32 patients with OLP were measured and compared, with 10 healthy subjects as the control group. Foxp3 mRNA expression levels in the explants of oral lesions and circulating $CD4^+CD25^+$ T cells in patients with OLP were significantly higher than those in the control group ($P<0.05$). In patients with clinically erosive lesions, Foxp3 mRNA expression was significantly lower in circulating $CD4^+CD25^+$ T cells and tissue explants compared to patients with reticular lesions ($P<0.01$ and $P<0.05$, respectively), and lowest in patients with a history of OLP of >1 year or with a history of relapse ($P<0.05$ and $P<0.01$, respectively). Foxp3 protein levels in reticular OLP were significantly higher than those in erosive OLP and the control group. The incidence of Foxp3 protein expression in OLP tissues was 36.24±18.92 and 10.44±6.51% in normal oral mucosa ($P=0.019$). Atrophic/erosive OLP lesions showed a higher proportion of Foxp3-expressing cells than that of reticular OLP lesions ($P<0.05$). This study indicated that Foxp3 expression in patients with OLP is associated with the severity and duration of the disorder, suggesting altered immune suppression in the development, clinical course and responsiveness to treatment.

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Introduction

Oral lichen planus (OLP) is a chronic inflammatory disorder that frequently presents as a white, lacy and reticular pattern on the mucosa, gingiva or lateral border of the tongue (1). The major subtypes of OLP include reticular, erosive, atrophic and popular lesions. Histological examination has shown that lesions are characterized by infiltrating bands of lymphocytes at the epidermal-dermal junction with damage to the basal cells of the epidermis (2). Although the etiology of OLP remains unclear, it appears to be a T cell-mediated autoimmune disorder in which $CD8^+$ T cells induce apoptosis of epithelial cells within the lesion (3). Clones of lymphocytes (the majority of which are $CD8^+$) isolated from oral lesions in the lichen planus have been found to have suppressive effects on keratinocytes (4,5). Elevations in proinflammatory cytokine synthesis and membrane receptor expression have been identified in local inflammatory cells and keratinocytes, which then activate multiple signaling networks (6). Previous studies suggested there is a T-helper cell (Th)1/Th2 imbalance in the cell-mediated immune response of OLP (7,8), which is consistent with the chronic and vacillating inflammatory nature of OLP. Atrophic or erosive lesions may reflect an exacerbated condition and these forms are associated with malignant development (9). Regardless of the central role of $CD4^+CD25^+$ regulatory T cells (Tregs) in the immunological tolerance and suppression of immune activation, limited information on the role of Tregs in OLP is available.

Tregs are $CD4^+CD25^+$ T cells that have a significant role in immune homeostasis and protect against autoimmunity (10). Tregs are anergic via stimulation through the T-cell receptor. Tregs are capable of suppressing the activation of $CD4^+CD25^-$ and $CD8^+$ T cells through cell contact-dependent mechanisms and cytokine-independent pathways. The aforementioned pathways may involve transforming growth factor-β (TGF-β) or interleukin (IL)-10, which is consistent with the immunosuppressive function (11,12). Tregs are also involved in the immune response in a wide spectrum of pathology, such as autoimmune diseases, infectious diseases, allergies, cancer, and organ transplantation (13). The associated molecular markers of Tregs include CD25, Forkhead-box protein 3 (Foxp3), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and

glucocorticoid-induced tumor necrosis factor receptor (GITR). Foxp3 is a member of the highly conserved forkhead/winged helix transcription protein family that controls the development, differentiation, maturation and function maintenance of $CD4^+CD25^+$ Tregs (14,15).

Considering the critical role of Foxp3 in the development and function of Tregs, Foxp3 is considered a specific molecular marker of the Treg functional activity (16,17). The aim of the present study was to characterize Foxp3 expression in $CD4^+CD25^+$ T cells from peripheral blood lymphocytes and oral lesions in patients wih OLP (including evaluation of the OLP subtype, duration and relapse).

Materials and methods

Study subjects. Thirty-two patients with OLP and 10 healthy volunteers were recruited from The First Affiliated Hospital of Jinan University (Guangzhou, China) from 2007 to 2010. Experimental procedures were conducted in accordance with the guidelines of the Medical Ethics committee of the Health Bureau of Guangdong Province of China. Informed consent was obtained from each participant and the study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Jinan University.

Thirty-two patients (females, 23 and males, 9) with an average age of 42.8±15.4 years (range, 26-61) were diagnosed with OLP. Patients who had been treated with corticosteroid or immunosuppressive medications in the previous 6 months, and patients with diabetes mellitus and autoimmune diseases (e.g., systemic lupus erythematosus and rheumatoid arthritis) were excluded from this study. Ten gender- and age-matched volunteers served as the healthy controls.

Incisional biopsies were obtained from representative oral lesions. Each biopsy specimen was bisected for histological and molecular assessment. Half of the specimens obtained were fixed in 4%-buffered formaldehyde and embedded in paraffin for histopathological verification of OLP. The clinical diagnosis of OLP was verified, based on their histopathological features. The histopathological features included lesions characterized by infiltrating bands of lymphocytes at the epidermal-dermal junction with damage to the basal cells of the epidermis. The remaining specimens were minced with surgical blades and immediately immersed in liquid nitrogen, and then stored at -80˚C until isolation of the total RNA. Gingival biopsies were obtained from 10 gender- and age-matched subjects immediately after the third molar extraction.

All oral lesions were localized without contact with dental restorations. In terms of OLP subtype, 18 of the 32 patients with OLP presented with reticular lesions, while the remaining patients exhibited erosive or atrophic lesions. According to the duration of disease, based on the self-report of the subjects, patients were divided into three groups: <6 months (14 cases); 6-12 months (10 cases); and >12 months (8 cases). Of the 32 patients with OLP, 25 presented with a first diagnosis of OLP and without prior treatment of corticosteroid or immunosuppressive therapy. Seven patients with OLP had previously received corticosteroid or immunosuppressive therapy on ≥2 occasions with clinical relapse. Grouping of the study subjects is shown in Table I.

Preparation of peripheral blood mononuclear cells (PBMCs). Blood samples were collected from all subjects and stored in heparin-containing tubes. PBMCs were isolated immediately using the Ficoll-Hypaque gradient centrifugation technique to further separate $CD4^+CD25^+$ T cells.

$CD4^+CD25^+$ T-cell separation by magnetic beads magnetic cell sorting (MACS). Purified human $CD4^+CD25^+$ T cells were isolated according to the manufacturer's instructions in a two-step procedure. $CD4^+$ T cells were isolated from PBMCs using positive selection with antibody-coated paramagnetic MultiSort MicroBeads [magnetic-activated cell sorting (MACS); Miltenyi Biotec GmbH, Bergisch Gladbach, Germany]. $CD4^+CD25^+$ T cells were obtained from the $CD4^+$ T cells with CD25 MicroBeads (Miltenyi Biotec) by positive selection. The purity of the $CD4^+$ and $CD4^+CD25^+$ T cells was established using flow cytometry of cells stained with anti-human CD4-FITC and anti-human $CD25^-$PE (purity of >95% $CD4^+$ T cells and >90% $CD4^+CD25^+$ T cells).

Total RNA isolation from $CD4^+CD25^+$ T cells and OLP tissues followed by cDNA synthesis. Total RNA was extracted from $CD4^+CD25^+$ T cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. Specimens were homogenized in liquid nitrogen and subjected to total RNA extraction using the Tissue RNA Extraction kit (Takara Biotechnology, Dalian, China) and treated with RNase-free DNase (Ambion®; Invitrogen Life Technologies) to remove contaminating genomic DNA. The RNA yield and purity were determined by a spectrophotometer at 260/280 nm. RNA samples were treated with DNase in strict accordance with the manufacturers' instructions (Invitrogen Life Technologies). The RNA quality was monitored using spectrophotometry and electrophoresis. The cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Corp., Indianapolis, IN, USA) following the manufacturer's instructions.

qPCR. For qPCR, primers for the Foxp3 gene and the reference gene (i.e., β-actin) were: Foxp3; forward, 5'-ACC CCCTTTCACCTACGC-3' and reverse, 5'-CCTTCTC GCTCTCCACTC-3'; and β-actin; forward, 5'-CACCAA CTGGGACGACAT-3' and reverse, 5'-ACAGCCTGGA TAGCAACG-3'. The primers were synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Shanghai, China).

Expression levels of Foxp3 and the reference gene (β-actin) were determined by SYBR-Green I real-time PCR. PCR was performed as previously described by Chen *et al* (18). Correction for inefficiencies in RNA input or reverse transcriptase were made by normalization of the housekeeping gene, β-actin. The 2 ($^{-\Delta}$CT) method was used to compare expression levels of Foxp3 relative to β-actin (19). Briefly, PCR of 25 μl total volume was performed with ~1 μl cDNA, 0.5 μM of each primer and 2.5X RealMasterMix 11.25 μl (Tiangen Biotech Co., Ltd, Beijing, China). After the initial denaturation at 95˚C for 2 min, 45 cycles at 95˚C (15 sec), 60˚C (60 sec) and 82˚C (1 sec) were performed for plate reading using MJ Research DNA Engine Opticon 2 PCR cycler (Bio-Rad, Hercules, CA, USA). Homogeneity of products from each reaction was confirmed by a melt-curve analysis. The size and quantity of amplified

Table I. Grouping of participants in relation to the subtype, duration and relapse of OLP.

	OLP subtype		OLP duration			OLP relapse	
Groups	Erosive OLP	Reticular OLP	>12 months	6-12 months	<6 months	Relapse	First diagnosis
OLP	14	18	8	10	14	7	25
Control	10		10			10	

OLP, oral lichen planus.

products were confirmed by 2% agarose gels and visualized by staining gels with ethidium bromide. The Foxp3 mRNA expression levels were calculated as previously described (20).

Western blot analysis of Foxp3 expression. The samples were solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride for 30 min. Lysates were centrifuged (15,000 x g) at 4˚C for 15 min. Equal concentrations of the soluble proteins were denatured in sodium dodecyl sulfate (SDS), electrophoresed on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and probed with antibodies, including rabbit anti-human polyclonal antibodies against human Foxp3, goat anti-rabbit antibodies and mouse anti-human GAPDH antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). GAPDH was used as an internal control to monitor equal protein loading. Western blot bands were observed using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). The results represent three independent experiments.

Immunohistochemical evaluation of Foxp3 expression. For the immunohistochemical procedure, sections were pre-incubated overnight in Tris-EGTA buffer (0.05 M Tris and 2.5 mM EGTA) at 60˚C followed by 1.5% H_2O_2 in Tris-buffered saline (TBS)/Nonidet (TBS; 0.05 M Tris, pH 7.4, 0.15 M NaCl; with 0.01% Nonidet P-40) (Sigma-Aldrich, Missouri, MO, USA) for 15 min at room temperature (20˚C) to extinguish endogenous peroxidase. Subsequently, sections were incubated in 10% goat serum (*In Vitro* A/S, Fredensborg, Denmark; code 04009-1B) for 30 min at room temperature in order to block non-specific binding. Sections were then incubated with antibodies, including rabbit anti-human polyclonal antibodies against human Foxp3. Sections were always processed and stained simultaneously and under the same laboratory conditions. Foxp3 was mainly expressed in the nucleus. The percentage of staining was estimated by two independent observers, three representative areas were selected and evaluated on high-power fields (magnification, x200). The mean percentage of positive cells was represented by the mean of each slide.

Statistical analysis. Data are expressed as the means ± standard deviation and subject to nonparametric analysis using the Mann-Whitney and Kruskal Wallis tests (IBM SPSS Statistics; IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of Foxp3 mRNA expression between OLP subtypes. Foxp3 mRNA expression was analyzed using qPCR. Correction for inefficiencies in RNA input or reverse transcriptase was made by normalization of the housekeeping gene (β-actin). A melting curve analysis was performed to ensure the specificity of the primers. In circulating $CD4^+CD25^+$ T cells, Foxp3 mRNA expression in reticular lesions of OLP was significantly higher than that in the erosive lesions and the healthy control group (1.59±0.64 versus 0.76±0.21 and 0.58±0.41, respectively) (P<0.01). Compared with that of the control group, Foxp3 mRNA expression in erosive OLP lesions was significantly higher (0.76±0.21 versus 0.58±0.41, respectively; P<0.01) (Fig. 1A).

The *in situ* Foxp3 mRNA expression in reticular OLP lesions was significantly higher than that in erosive OLP lesions and the healthy control group (18.27±7.83 versus 11.41±5.86 and 9.63±3.54, respectively; P<0.05). Compared with that of the healthy control group, Foxp3 mRNA expression in erosive OLP lesions was significantly higher (11.41±5.86 versus 9.63±3.54, respectively; P<0.05) (Fig. 1B).

Comparison of Foxp3 mRNA expression levels between groups of OLP duration. Foxp3 mRNA expression levels in circulating $CD4^+CD25^+$ T cells from patients with OLP duration of >6 months (the >12 months group and the 6-12 months group, 0.79±0.53 and 1.07±0.78, respectively) did not differ significantly from that of the control group (0.58±0.41). Foxp3 mRNA expression levels in patients with OLP duration of <6 months (1.46±0.96) was significantly higher than that in patients with a duration of >12 months and the control group (P<0.01) (Fig. 2A).

In patients with OLP for <6 months, the *in situ* Foxp3 mRNA expression level (21.76±6.38) was significantly higher than that of the patients with OLP for >12 months (11.96±7.13) and the healthy controls (9.63± 3.54) (P<0.05). The *in situ* Foxp3 mRNA expression levels in patients with OLP for 6-12 months (18.13±8.57) failed to present a significant difference as compared to the other groups (P>0.05) (Fig. 2B).

Comparison of Foxp3 mRNA expression between OLP relapse groups. In circulating $CD4^+CD25^+$ T cells, Foxp3 mRNA expression of the first diagnosis group (1.57±0.64) was significantly higher compared with that of the control

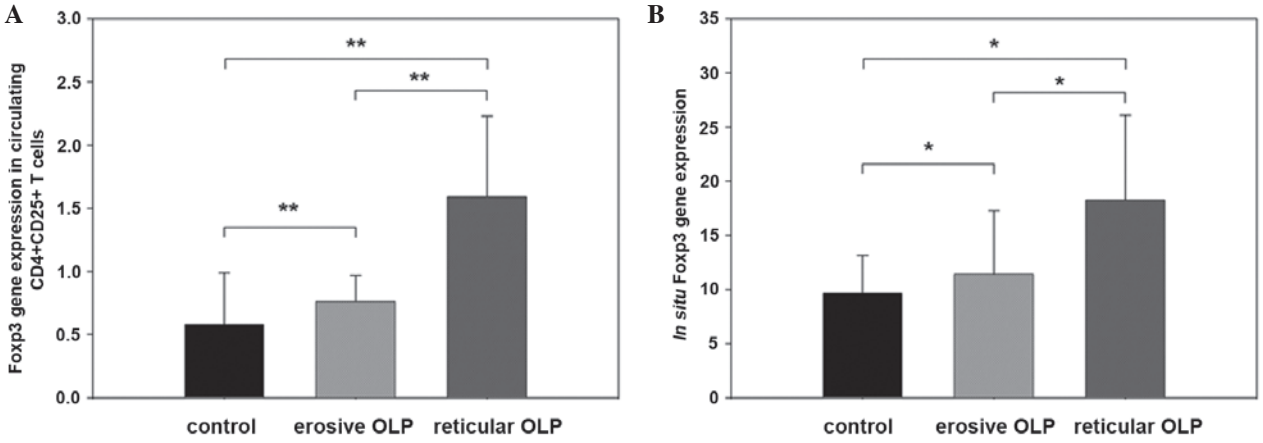

Figure 1. Comparison of Foxp3 gene expression in (A) circulating $CD4^+CD25^+$ T cells and (B) *in situ*, between OLP subtypes ($^{**}P<0.01$, $^{*}P<0.05$). OLP, oral lichen planus.

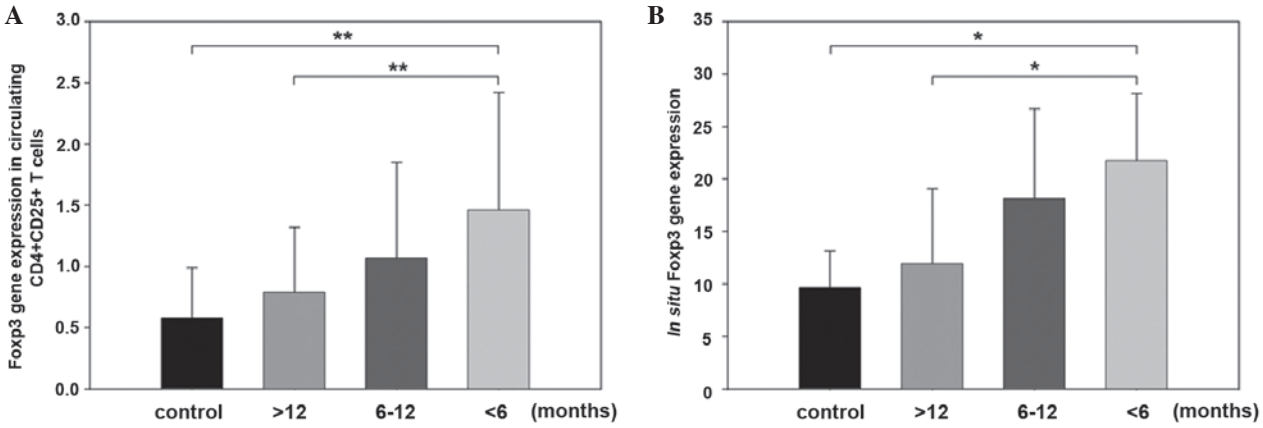

Figure 2. Comparison of Foxp3 gene expression in circulating (A) $CD4^+CD25^+$ T cells and (B) *in situ*, between OLP duration ($^{**}P<0.01$, $^{*}P<0.05$). OLP, oral lichen planus.

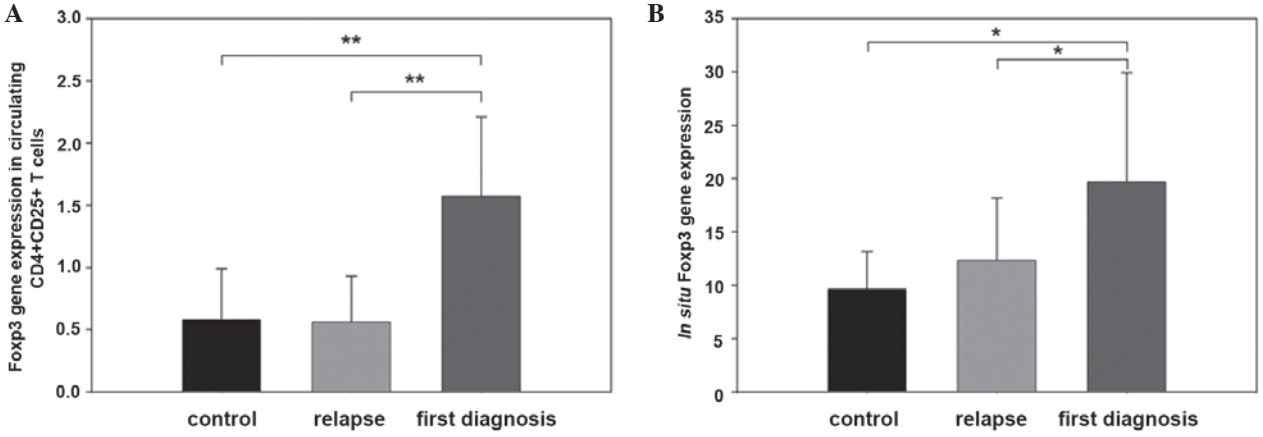

Figure 3. Comparison of Foxp3 gene expression in circulating (A) $CD4^+CD25^+$ T cells and (B) *in situ*, between OLP relapses ($^{**}P<0.01$, $^{*}P<0.05$). OLP, oral lichen planus.

and the relapse groups (0.58±0.41 and 0.56±0.37, respectively) (P<0.01). No differences of Foxp3 mRNA expression were found between the control and relapse groups (Fig. 3A).

In situ mRNA expression of Foxp3 was also higher in tissues from patients in the first diagnosis group (19.68±10.24), compared with that of the relapse and control groups (12.31±5.85 and 9.63±3.54, respectively; P<0.05). No differences of Foxp3 mRNA expression were found between the control group and the relapse group (Fig. 3B).

Foxp3 protein expression was analyzed using western blotting and immunohistochemistry. Foxp3 protein expression

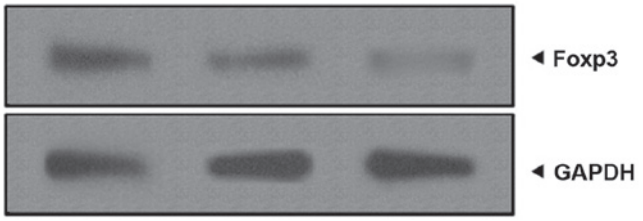

Figure 4. Western blot analysis of Foxp3 expression. Reticular and erosive OLP lesions and the control are shown in lanes from left to right. OLP, oral lichen planus.

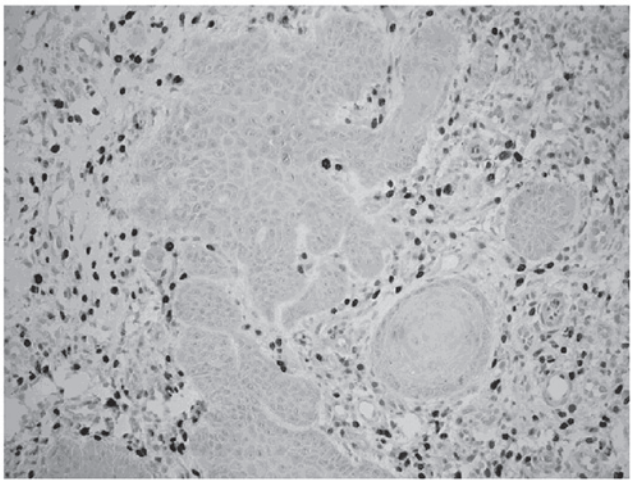

Figure 5. Immunohistochemical staining of Foxp3. Obvious nuclear expression in OLP with streptavidin-peroxidase (magnification, x200).

was analyzed by western blotting. As shown in Fig. 4, Foxp3 protein expression in the reticular OLP lesions was significantly higher than that in the erosive OLP lesions and the healthy control group. In addition, compared with that of the healthy control group, Foxp3 protein expression in the erosive OLP lesions was also high.

Furthermore, as shown in Fig. 5, under a light microscope, the distribution of Foxp3 was mainly observed in the nuclei of lymphocytes, which were sporadically scattered in the lamina propria of local lesions. The incidence of Foxp3 expression in OLP tissues was 36.24±18.92% and 10.44±6.51% in normal oral mucosa, and there was a significant difference (P=0.019). Atrophic/erosive OLP lesions showed a higher proportion of Foxp3-expressing cells in the lamina propria of local lesions than that of the reticular OLP (P<0.05).

Discussion

OLP is typically characterized by a dense, well-defined infiltration of T-lymphocytes in the lamina propria. $CD4^+$ and $CD8^+$ T cells appear to be predominant in local lesions. These cells have been shown to be cytotoxic T cells that migrate through the basement membrane in OLP (21). These cells may trigger keratinocyte apoptosis (22) promoted by Th1 cytokines. Previous studies (23,24) have shown that T cells in OLP were resistant to apoptosis resulting in inappropriate immune homeostasis and accumulation of T cells. A number of mechanisms may contribute to the biological basis of OLP (which largely remains unclear), including activation of the inflammatory mediator, nuclear factor-κB (25), dysfunction of the TGF-β signal transduction on $CD8^+$ T cells (26), and $CD8^+$ and $CD4^+$ T-cell resistance to activation-induced cell death (24), suggesting a dysregulation of local inflammation.

The suppressive function of Tregs is consistent with the hypothesis that the change of Foxp3 expression in T cells may contribute to the pathogenesis of OLP. In the present study, higher levels of Foxp3 mRNA and protein expression in both circulating $CD4^+CD25^+$ T cells and local tissues were found in patients with OLP compared with that of the healthy controls. However, differences in Foxp3 expression were also associated with the clinical form, including reticular versus erosive lesions, and duration and relapse status. Our data suggest that OLP may be associated with the change of Foxp3 expression in $CD4^+CD25^+$ cells.

$Foxp3^+CD25^+CD4^+$ Tregs are produced in the thymus and are also induced in the periphery from naive T cells as a functionally mature T-cell subpopulation forming a functionally distinct T-cell subpopulation in the periphery. Tregs are dedicated to the control of immune responses and mediate the tolerance against harmless non-self or self-antigens (27). The differentiation and function of Treg cells require Foxp3 (11,12). A number of autoimmune diseases, such as arthritis, diabetes mellitus, immunodysregulation polyendocrinopathy, enteropathy and X-linked syndrome, may develop spontaneously following elimination of $CD4^+CD25^+$ Tregs (28,29).

In the present study, Foxp3 mRNA and protein expression levels in peripheral blood $CD4^+CD25^+$ T cells were higher in patients with OLP than that of the healthy controls. Similar elevations in the expression pattern were observed in local lesions of reticular and erosive forms of OLP, although the expression in the erosive form was lower than that in the reticular form. Foxp3 localization observed in this study supported the findings of Tao *et al* (30), who identified a higher Foxp3 expression and frequency of $Foxp3^+$ Tregs in the reticular OLP lesions than that in the erosive OLP lesions. A potential interpretation for the enrichment of $Foxp3^+$ Tregs in patients with OLP was that Tregs differentially traffic to the oral mucosa in response to chemokines, which is possibly elaborated by keratinocytes. Recent studies (31,32) examining $Foxp3^+$ T-cell function in chronic inflammatory conditions, including systemic lupus erythematosus and rheumatoid arthritis, have shown that the $Foxp3^+$ T cells may not have the ability to exert a suppressive function due to the resistance from effector cells. Therefore, such inflammatory diseases may become chronic due to the inability of Tregs to control cytokine-activated T-cell function (33). Our findings also supported the possibility that $Foxp3^+$ Tregs may be dysfunctional in patients with OLP.

Reduced Foxp3 mRNA expression levels were noted in the erosive OLP lesions than those of the reticular OLP lesions, which were similar to the expression levels in the control group, both in the circulating $CD4^+CD25^+$ T cells and *in situ*. Immunoregulatory and histological differences have been documented between T cells in reticular and

atrophic/erosive OLP (8,34). The epithelial cells in the erosive form of OLP showed increased apoptotic cells as compared to the reticular form (35), which is consistent with the more aggressive form of erosive lichen planus. A previous study hypothesized that the erosive form corresponded to a more active and symptomatic stage of the condition, whereas the reticular form corresponded to a quiescent and asymptomatic phase (36). In the present study, the severity of OLP was significantly associated with Foxp3 expression levels; the greater the severity, the lower the Foxp3 expression levels. The latter observation was consistent with the hypothesis that lower Foxp3 expression levels resulted from upregulation of Foxp3-related suppressive mechanisms in the development of the disorder. With increasing disease severity, characterized by the formation and exacerbation of erosive lesions, the abnormally high ratio of activated T cells to Treg cells may have hampered the functional ability of Tregs. In this situation, T cells incur resistance to apoptosis resulting in inappropriate immune homeostasis and accumulation of T cells in the tissues. High proinflammatory cytokine levels may negatively affect the activation of $Foxp3^+$ Tregs cells in local lesions (37,38). In the active inflammatory compartment, overproduction of Th1 proinflammatory cytokines and deficiencies in TGF-β1 (7,39,40) signaling cascades may abrogate Treg-mediated suppression (37,41-44). Moreover, the relative activity of these cytokines may determine the level of immunological activity in OLP lesions and the clinical behavior of the disease (7).

The present study also compared Foxp3 expression and duration of disease, providing an insight into the regulation of the inflammatory response. The results suggest that the protracted disease was associated with reduced Foxp3 expression, which may be partially responsible for the maintenance of the inflammatory infiltrate. Therefore, it was possible that the function and number of Tregs may become increasingly impaired with advancing duration of disease, resulting in continued amplification and extension of local inflammation.

Current therapeutic agents for OLP are largely immunosuppressive and improve the management of symptoms, with clinical relapse commonly occurring after discontinuation of treatment. Pathological alterations in the proportion of immune cells, including regulatory cell subsets, may account for phenotypic differences in the observed inflammatory condition. A diminution of Foxp3 Tregs would represent one possible scenario. In the latter, the failure of Tregs to control autoimmune dysregulation may provide important therapeutic targets for the management of OLP. Several studies have shown that a reduction of Foxp3 levels in natural $CD4^+CD25^+$ Tregs blocked their suppressive activities (45,46), and were associated with relapse of the disease (47). The balance between Tregs and T effector cells may determine corresponding levels of inflammation and therefore, may be linked to periods of relapse or remission.

In conclusion, the present study examined elevated Foxp3 expression levels in circulating $CD4^+CD25^+$ T cells and oral lesions in patients with OLP, suggesting altered immune suppression in the development, clinical course and responsiveness to treatment. The observation of lower Foxp3 expression levels in erosive lesions compared with that of the reticular lesions of lichen planus, further suggested that a dysregulation of suppressor mechanisms contributed to the evolution and chronicity of inflammation.

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