ORAL DISEASES

Oral Diseases (2010) 16, 76–82. doi:10.1111/j.1601-0825.2009.01608.x

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ORIGINAL ARTICLE

FOXP3$^+$ T regulatory cells in lesions of oral lichen planus correlated with disease activity

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OBJECTIVE: The aim of this study was to determine the correlation between the number of FOXP3$^+$ T cell in lesions and the disease activity of patients with oral lichen planus (OLP).

MATERIALS AND METHODS: The expression of FOXP3 was investigated using immunohistochemical staining and real-time RT-PCR in 23 OLP lesions and 12 controls. Changes of FOXP3$^+$ Treg in peripheral blood from three patients' pre and post-treatment were assessed using flow cytometry.

RESULTS: Few FOXP3$^+$ cells were detected in controls, but an increased number of FOXP3$^+$ cells were observed in lesions (n = 20, 40.99 ± 24.68 cells per high-power field – hpf). Furthermore, the frequency of FOXP3$^+$ Treg in reticular OLP (n = 7, 63.6 ± 23.2 cells per hpf) was significantly higher than that in erythematous/erosive OLP (n = 13, 28.8 ± 16.8 cells per hpf, P = 0.001). In addition, negative correlation was found between the number of FOXP3$^+$ Treg and disease activity (correlation oefficient = −0.557, P = 0.013). The proportion of FOXP3$^+$ Treg showed remarkable increase in peripheral blood from patients after treatment (1.39 ± 0.71% *vs* 4.91 ± 1.59%).

CONCLUSIONS: These data indicated that FOXP3$^+$ Treg were involved in the pathogenesis of OLP and correlated with disease's subtype and activity.
Oral Diseases (2010) **16**, 76–82

Keywords: oral lichen planus; FOXP3; regulatory T cell

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This work was supported by grants from the National Natural Scientific Foundation of China (30672317, 30872874), Specialized Research Fund for the Doctoral Program of Higher Education of China (200805580082), Provincial Natural Scientific Foundation of Guangdong (8151008901000080), and the Priming Scientific Research Foundation for the Junior Teachers of Medicine in Sun Yat-sen University (2008016).
Received 22 December 2008; revised 20 May 2009; accepted 29 June 2009

Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease which affects 0.1–4% of the general adult population (Lodi *et al*, 2005). This disease is characterized histologically by a dense subepithelial lymphocytic infiltrate, degeneration of basal keratinocytes and basement membrane disruption (Sugerman *et al*, 2002). Typical oral lesions are striations (striae of Wickham), papules, plaques, mucosal atrophy, erosions (shallow ulcers) or blisters affecting the buccal mucosa, tongue, and gingiva. Erythematous and erosive lesions are often sensitive or painful and there is ongoing concern that OLP may be premalignant (Eisen *et al*, 2005). A large body of evidence supports a role of immune dysregulation in the pathogenesis of OLP, especially involving the cellular arm of the immune system. Antigen-specific mechanisms in OLP include antigen presentation by basal keratinocytes and antigen-specific keratinocyte killing by CD8$^+$ cytotoxic T-cells. Non-specific mechanisms include mast cell degranulation and matrix metalloproteinase (MMP) activation in OLP lesions. These mechanisms may combine to cause T-cell accumulation in the superficial lamina propria, basement membrane disruption, intra-epithelial T-cell migration, and keratinocyte apoptosis in OLP. Obviously, the abnormality of immunological regulation may contribute to its pathogenesis.

CD4$^+$ CD25$^+$ regulatory T cells (Treg) are a subset of T lymphocytes that play a central role in inducing and maintaining immunologic tolerance to self and non-self (Maggi *et al*, 2005; Sakaguchi, 2005). Most surface proteins that are expressed on CD4$^+$ CD25$^+$ Treg cells such as CD25, CD152 (T-lymphocyte-associated antigen-4, CTLA-4), GITR (Glucocorticoid-induced TNFR-related protein) and PD-L1 (programmed cell death 1 ligands 1) can also be found on activated T responder cells. On the other hand, FOXP3, an X chromosome–encoded forkhead transcription factor family member, is indispensable for the development and function of CD4$^+$ CD25$^+$ Tregs (Zheng *et al*, 2007), which indicate that FOXP3 is a faithful and

specific marker for Tregs. In recent years, abnormalities in number and function of FOXP3-expressing $CD4^+$ $CD25^+$ Treg have been identified in a number of autoimmune and inflammatory diseases, including psoriasis, multiple sclerosis, autoimmune polyglandular syndrome type II, rheumatoid arthritis, myasthenia gravis and type I diabetes (Ehrenstein *et al*, 2004; Kriegel *et al*, 2004; Viglietta *et al*, 2004; Balandina *et al*, 2005; Lindley *et al*, 2005; Sugiyama *et al*, 2005; Rieger *et al*, 2006). Moreover, this evidence indicates that FOXP3-expressing $CD4^+CD25^+$ Treg may be a target for the development of new treatments inflammatory/autoimmune diseases in future.

Recently, our microarray study demonstrated that FOXP3 transcription significantly elevated in OLP lesions comparing with the controls (Tao *et al*, 2009). This result directly suggested that $FOXP3^+$ Treg cells may participate in the pathogenesis of OLP. Considering the pathogenic roles of immune response mediated by T cells in OLP, it was crucial to determine the number and function of $FOXP3^+$ Treg in subjects with OLP. Therefore, we investigated the number of $FOXP3^+$ Treg in both the lesions and peripheral blood of subjects with various subtypes of OLP.

Materials and methods

Study participants

This study was approved by the Institutional Review Boards at the Sun Yat-sen University. The enrolled subjects included 23 patients with OLP (age: 19–72, 46.3 ± 3.39 years), and 12 healthy controls (age: 20–44, 31 ± 1.68 years) who received orthognathic surgery. Before the study, informed consent was obtained from each subject. Given that different OLP lesions display a vast spectrum in aspects of clinical presentation, malignant transformation, immune activity, etc., it should be noticeable that $FOXP3^+$ T regulatory cells may play different role(s) in different clinical forms of OLP lesions. Therefore, all OLP patients were further divided into reticular and erythematous/erosive subgroup according to the previous criteria (Tao *et al*, 2007). Patients and controls with an adequate amount of tissue were utilized for the analysis. Nine patients and nine controls had adequate material for RT-PCR, 20 patients and six controls had adequate tissue for immunohistochemical staining (specimens of six patients and three controls were involved in both assays). To detect the change of $FOXP3^+$ Treg cells in peripheral blood, venous blood was obtained from three selected patients with erythematous/erosive OLP before and after treatment with 0.1% tacrolimus ointment for 4 weeks.

The inclusion criteria of OLP patients have been described in our previous studies (Rhodus *et al*, 2005; Tao *et al*, 2007). Briefly, all subjects neither had detectable gingival and/or periodontal inflammation nor any visible oral lesions under careful examination; moreover, they were not taking drugs inducing hyposalivation, or any other prescription or non-prescription drugs, such as anticholinergics, antihistamines, antihypertensives and beta-adrenergic blockers. Furthermore, none of the subjects received treatment for the OLP within 60 days before specimen collection and had any history, symptoms, and/or signs of systematic infections, allergies, and smoking.

Clinical data, including age, gender, site, disease activity score and history of past illness, were obtained at the time of sample acquisition (Table 1). The scores of disease activity were assessed according to the criteria (REU scoring system) as described in the previous studies (Piboonniyom *et al*, 2005; Xia *et al*, 2006). In brief, the oral cavity of each individual was divided into 10 sites: upper/lower 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 gingival. Surface areas of erythema and ulceration in each site were measured with a sterile flexible periodontal scale probe, and were estimated in squared millimeters. The lesions were scored according to the surface areas: (1) Reticular/hyperkeratotic lesion (R), 0 = no white striations, 1 = presence of white striations or keratotic papules; (2) Erosive/erythematous lesion (E), 0 = no lesion, 1 = less than 100 mm^2, 2 = from 100 to 300 mm^2, 3 = larger than 300 mm^2; (3) Ulcerative lesion (U), 0 = no lesion, 1 = less than 100 mm^2, 2 = from 100 to 300 mm^2, 3 = larger than 300 mm^2. The score of every site was calculated according to the following formula: site score = $R + E \times 1.5 + U \times 2.0$. The total score of each subject was derived by summation of the scores of all 10 sites.

Immunohistochemistry staining

For immunostaining, 4-μm thick serial sections were cut, deparaffinized, and subjected to a heat-induced epitope retrieval step before incubation with antibody as

Table 1 Clinical data of patients participating in this study

Case no.	*Sex*	*Age (years)*	*Affected site*	*Clinic form*	*Disease activity score*
1	F	66	Cheek	Erythematous/erosive	14.5
2	M	30	Cheek	Erythematous/erosive	25.5
3	F	50	Cheek	Erythematous/erosive	7.5
4	F	56	Gingiva	Erythematous/erosive	34.5
5	M	18	Tongue	Erythematous/erosive	9
6	M	63	Cheek	Reticular	5
7	M	34	Cheek	Erythematous/erosive	6
8	F	41	Cheek	Reticular	5
9	F	62	Cheek	Reticular	4
10	F	72	Cheek	Erythematous/erosive	12
11	F	40	Cheek	Reticular	2
12	M	36	Cheek	Erythematous/erosive	12
13	M	32	Tongue	Reticular	2
14	M	46	Tongue	Erythematous/erosive	17.5
15	M	53	Cheek	Reticular	4
16	M	70	Tongue	Reticular	2
17	F	70	Cheek	Erythematous/erosive	9
18	F	18	Tongue	Erythematous/erosive	6.5
19	F	36	Cheek	Erythematous/erosive	8
20	M	32	Cheek	Reticular	8
21	M	38	Tongue	Erythematous/erosive	16
22	F	60	Tongue	Erythematous/erosive	9
23	M	43	Cheek	Erythematous/erosive	11

described in a previous study (de Boer *et al*, 2007). The primary antibodies included polyclonal antibody against FOXP3 (Clone: Poly6238, dilution 1:60, BioLegend Limited, San Diego, CA, USA), monoclonal antibodies against CD4 (Clone: 4B12, dilution 1:20, Lab Vision Corp., Fremont, CA, USA) and CD25 (Clone: IL2R.1, dilution 1:60, Lab Vision Corp.). Briefly, heat-induced antigen retrieval was performed using preheated Tris-EDTA buffer at pH 9.0 in a microwave oven for 20 min. Non-specific binding was blocked by incubating the sections for 10 min in normal goat serum, and subsequently with the primary antibody at room temperature for 1 h. Detection was carried out using the relevant biotin-conjugated secondary antibody, followed by streptavidin-peroxidase complex (Lab Vision Corp.) for 15 min at room temperature. All stages were separated by PBS washes, and final product was visualized by diaminobenzidine (DAB) for 5 min. The sections were then counterstained with hematoxylin. PBS was substituted for the primary antibodies of FOXP3, CD4 and CD25 as a negative control. Deltopectoral lymphoid nodes were taken as positive controls.

To exclude non-specific staining of the FOXP3 antibody, we double labeled FOXP3 and CD25, FOXP3 and CD4 in part of biopsies, and confirmed the co-expression of CD4 and CD25 on all FOXP3-postive cells. For double labeling of FOXP3/CD4 and FOXP3/CD25, the following sequence of steps was applied using a commercial double staining kit (Kit-9999, Maixing Inc., Fuzhou, Fujian, China): endogenous peroxidase activity was blocked using 0.3% peroxide; non-immune serum block; anti-FOXP3 overnight at 4°C; biotinylated goat anti-rabbit immunoglobulin; AP-conjugated streptavidin; AP activity visualization with bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT); non-immune serum; CD4 or CD25 antibody, respectively; EnVision+ anti-mouse/HRP polymer; peroxidase activity visualization with 3-amino-9-ethylcarbazole (AEC).

Two investigators determined the number of FOXP3-positive cells in each slide independently and blinded to subjects' outcomes. FOXP3-positive cells were counted in the 10 consecutive areas at 400× magnification using light microscopy (Rieger *et al*, 2006). The mean of positive cells per 1 high-power field (hpf) was designed as the number of FOXP3-positive cells for each subject.

Real time quantitative RT-PCR

The transcripts of forkhead box protein 3 (FOXP3) were verified by quantitative real-time RT-PCR with the Perkin-Elmer 9600 GeneAmp PCR System (Perkin-Elmer, Boston, MA, USA). PCR primer pairs were designed using Primer express software (Forward Primer: CACCTGGCTGGGAAAATGG, Reverse Primer: GGAGCCCTTGTCGGATGA). Each amplification reaction was carried out using SYBR Green Mastermix (ABI Inc., Foster City, CA, USA) for 5 min at 93°C for the initial denaturing, followed by 40 cycles of 93°C for 1 min, 55°C for 1 min and 72°C for 1 min. FOXP3 mRNA expression in each sample was calculated using the relative quantization method, with human β-actin which was used as an internal control.

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized venous blood by density gradient centrifugation with lymphoprep™ (AXIS-SHIELD Inc., Oslo, Norway). Then, cells were immediately stained for flow cytometry. In current study, human regulatory T cell staining kit no. 2 (eBioscience Inc., San Diego, CA, USA) was used for staining $FOXP3^+$ $CD4^+$ $CD25^+$ Treg cells. The following monoclonal antibodies were included: anti-Foxp3-APC (Clone: PCH101), anti-CD4-FITC (Clone: RPA-T4), anti-CD25-PE (Clone: BC96). In brief, appropriate CD4/CD25 cocktail was added in 100 μl prepared cells and incubated for 30 min in the dark at 4°C. After that, cells were washed and resuspended in freshly prepared eBioscience Fixation/Permeabilization Buffer for intracellular FOXP3 protein staining according to the manufacturer's protocol. Acquisition of data was performed on an EPICS ALTRA flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA). The WINMDI 2.9 software was used to quantify the fluorescence signals and to set the logical electronic-gating parameters. T-lymphocyte populations were gated based on forward and side scatter, and at least 50 000 $CD4^+$ lymphocytes were acquired for analysis of $FOXP3^+$ cells.

Statistical analysis

All calculations were performed using SPSS statistical software package (SPSS 11.0 for Windows, SPSS Inc, Chicago, IL, USA). Results were expressed as mean ± standard deviation (SD). Wilcoxon Signed Ranks Test was used to analyze the flow cytometric result. Other data were analyzed using student T-test. P value less than 0.05 was considered to be statistically significant.

Results

We observed that only a few weakly CD25-stained cells were negative for FOXP3 staining, and most of $FOXP3^+$ cells co-expressed CD4 in this study. These findings are consisted with those of Rieger *et al* and de Boer *et al* (Rieger *et al*, 2006; de Boer *et al*, 2007). All these indicated that FOXP3 demonstrated a preferential staining of the $CD4^+CD25^+$ T cell population (Figure 1a, b). Representative tissue sections, stained for FOXP3, are shown in Figure 1c–e. Immunohistochemistry demonstrated few $FOXP3^+$ cells in tissue sections from normal oral mucosa. However, $FOXP3^+$ T cells were easily detectable in OLP lesions, and mainly dispersed at the lamina propria of lesions (n = 20, 40.99 ± 24.68 per hpf). Moreover, the results from Pearson correlation analysis showed that there was a negative correlation between the density of $FOXP3^+$ T cells and scores of disease activity in OLP subjects (correlation coefficient = −0.557, P = 0.013). Interestingly, we observed that the mean of $FOXP3^+$ T cells in erythematous/erosive OLP lesions (n = 13,

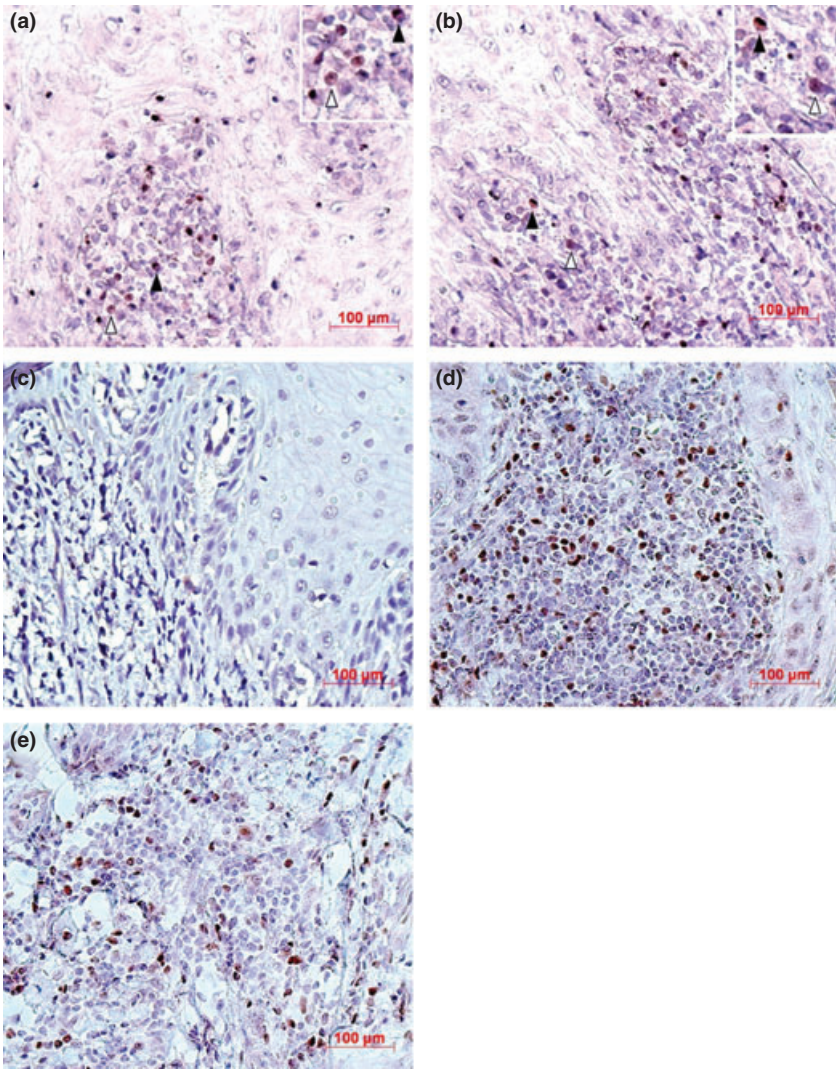

Figure 1 Immunohistochemistry staining for $CD4^+$ $FOXP3^+$, $CD25^+$ $FOXP3^+$, $FOXP3^+$ T cells of representative oral biopsies. (**a**) CD4 (brownish red) and FOXP3 (black) double staining of OLP lesion. CD4 and FOXP3 double-positive cells (▲), CD4 single-positive cell (△). (**b**) CD25 (brownish red) and FOXP3 (black) double staining of OLP lesion. CD25 and FOXP3 double-positive cells (△), CD25 single-positive cell (▲). (**c**) Few FOXP3-positive cells were detected in the epithelium of healthy tissues. The number of $FOXP3^+$ Treg cells in atrophy OLP lesions (**d**) was significantly higher than that in erythematous/erosive OLP lesions (**e**). (Original magnification: 400×)

28.8 ± 16.8 per hpf) was significantly lower than that in reticular OLP lesions ($n = 7$, 63.6 ± 23.2 per hpf, $P = 0.001$).

In this study, real-time quantitative RT-PCR for OLP lesions demonstrated 23.16 times higher numbers of FOXP3 mRNA copies than normal control specimens ($P = 0.0001$). These findings correlated with the observations of IHC showing a marked increase of $FOXP3^+$ T cells were detected in OLP lesions compared with the controls. In addition, results of flow cytometric analysis indicated that the frequency of peripheral $FOXP3^+$ Tregs (Tregs per $CD4^+$ T cells) demonstrated marked increase in OLP subjects after treatment (1.39 ± 0.71% *vs* 4.91 ± 1.59%), although the sample size was too small to provide significant results ($P = 0.109$, Figure 2).

Discussion

As T cell-mediated autoimmunity is considered to be involved in the pathogenesis of OLP (Sugerman *et al*, 2002), the immune regulatory dysfunction may contribute to development and refractoriness of OLP. Several previous investigations indicated that OLP chronicity may be due, in part, to a defect in the tumor growth factor-ß (TGF-ß)/interleukin-4 (IL-4) immunosuppressive pathway involving insufficient numbers of IL-4-secreting Th2 and TGF-ß-secreting Th3 regulatory T-cells (Sugerman *et al*, 2002; Eisen *et al*, 2005). But our recent observations showed that the expression of IL-4 was upregulated in local lesions of OLP and its protective role seems not to attenuate effectively the excessive immune response mediated by IFN-gamma in OLP (Tao *et al*, 2008). Therefore, there may be other more important mechanisms of immunological regulations contributing to OLP pathogenesis. In recent years, the crucial roles of $CD4^+CD25^+FOXP3^+$ Treg cell have been identified in a series of autoimmune and/or inflammatory diseases (Maggi *et al*, 2005; Sakaguchi, 2005; Zheng and Rudensky, 2007), its role in pathol-genesis of OLP should not be overlooked. Sparse data are available on $FOX3^+$ T regulatory cells in OLP. In this small pilot study, the increased numbers of $FOXP3^+$ Treg cells were detected in OLP lesions, and the transcription of FOXP3 was found to be significantly increased in OLP lesions. To our knowledge, this

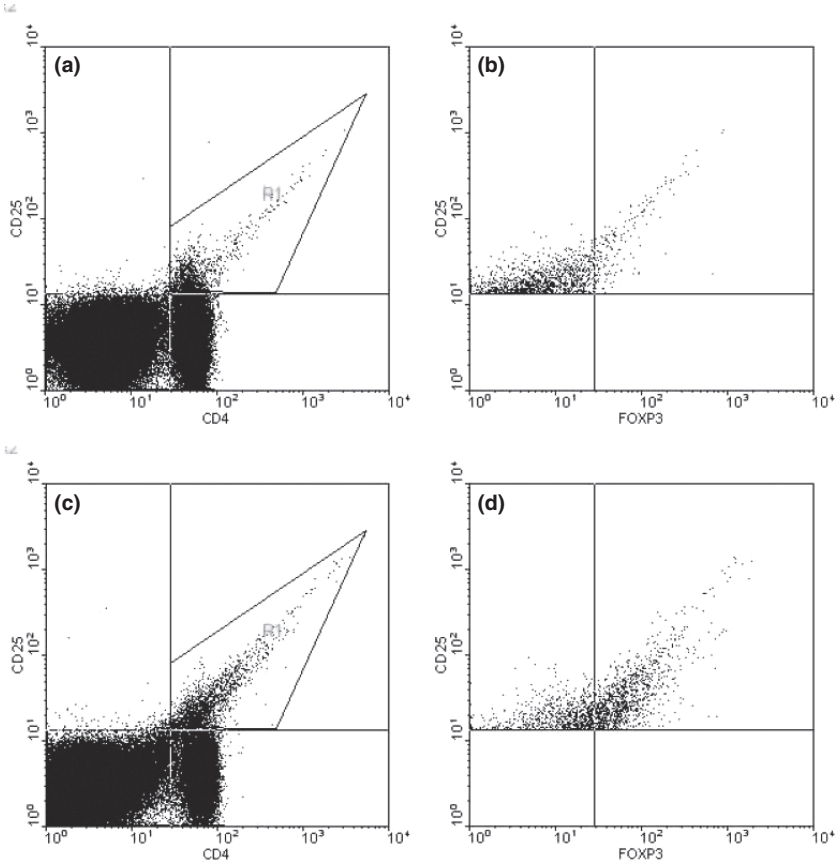

Figure 2 The representative sample of $CD4^+$ $CD25^+FOXP3^+$ Treg cells staining in peripheral blood from patients with oral lichen planus. The frequencies of $CD4^+$ $CD25^+FOXP3^+$ Treg cells (per $CD4^+$ T cells) in patients before (**a** and **b**) and after (**c** and **d**) treatment were quantified using WINMDI 2.9 software. Although the frequency of peripheral $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs demonstrated marked increase in OLP patients after treatment ($1.39 \pm 0.71\%$ *vs* $4.91 \pm 1.59\%$), the difference was not significant due to the small sample size

is the first time that $FOXP3^+$ Treg cells have been shown to be involved in the pathogenesis of OLP.

Noteworthy, we observed that the mean of $FOXP3^+$ Treg cells in erythematous/erosive lesions was significantly lower than that in reticular lesions of OLP. Furthermore, the density of $FOXP3^+$ T cells in lesions was negatively correlated with the score of disease activity. Similar findings have been reported in other inflammatory or autoimmune disorders, including systemic lupus erythematosus, mycobacterium tuberculosis, experimental myasthenia gravis and Guillain-Barré syndrome (Baráth *et al*, 2007; Chen *et al*, 2007; Chi *et al*, 2007; Valencia *et al*, 2007; Azab *et al*, 2008; Sheng *et al*, 2008). Therefore, these results indicated that $FOXP3^+$ Treg cells were significantly correlated with the clinic forms and activity of OLP. Because all biopsy samples from subjects with OLP were obtained during the initial presentation and prior to the start of topical and/or systemic treatment, the difference in the number of $FOXP3^+$ cells is not attributable to the duration of any individual mucosal lesions. However, more work needs to be performed with regard to the recruited mechanism of $FOXP3^+$ Treg cells in OLP lesions, which may provide great help for us to understand the difference in the number of $FOXP3^+$ Treg cells between erythematous/erosive and reticular OLP lesions.

Given the fact that $FOXP3^+$ Treg cells have an important role in the mechanisms of peripheral immune tolerance and the prevention of pathogenic autoimmunity, investigators have endeavored to explore the potentials of $FOXP3^+$ Treg cells in the treatment of inflammatory and autoimmune disorders. Subsequently, many studies have shown that selective expansion or/and targeting immigration of $FOXP3^+$ Treg cells allowed effective therapy or retroconversion of several inflammatory/autoimmue-related disorders, including type 1 autoimmune diabetes, experimental colitis and experimental autoimmune encephalomyelitis (Beyersdorf *et al*, 2005; Chai *et al*, 2005; Loser *et al*, 2005; Piccirillo *et al*, 2005; Fantini *et al*, 2006; Ephrem *et al*, 2008; Radulovic *et al*, 2008). Not surprisingly, we found the number of $FOXP3^+$ Treg cells in lesions had a negative correlation with the score of disease activity and an increasing trend of $CD4^+CD25^+FOXP3^+$ Treg cells in peripheral blood in OLP patients after effective treatment with tacrolimus. It is reasonable to believe that $FOXP3^+$ T regulatory cells could be a new target of OLP treatment. On the other hand, although the effectiveness of tacrolimus in OLP treatment had been presented by several studies, the exact mechanism of tacrolimus to OLP management remains unclear. Recently, Caproni *et al* observed significant expression of TGF-ß in lesions of atopic dermatitis after the patients were treated with tacrolimus ointment (Caproni *et al*, 2006). Our observation raised a possibility that the effectiveness of tacrolimus in OLP treatment may result from the expansion of $FOXP3^+$ T regulatory cells induced by TGF-ß, which need to be elucidated further.

In summary, our data indicated that FOXP3$^+$ Treg cells may play an important role in the pathogenesis of OLP and significantly correlated with its subtype and activity. Selective expansion of FOXP3$^+$ Treg cells may be the new strategy of OLP management.

Acknowledgement

We thank Prof. Xiao-hua Chen for her great help in histopathological analysis of our study.

Author contributions

Bin Cheng contributed to the research design. Xiao-an Tao, Juan Xia and Xiao-bin Cheng contributed to the acquisition and analysis of data. Xiao-an Tao and Nelson L. Rhodus contributed to the interpretation of data and drafted paper. Hong Wang and Yao-hui Dai contributed to the collection of the human samples.

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