


Herpes simplex virus type I–infected disorders alter the balance between Treg and Th17 cells in recurrent herpes labialis patients

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Abstract

Recurrent herpes labialis (RHL) is a common skin disease that is often caused by herpes simplex virus type I (HSV-I), but its immunology and pathogenesis remain unclear. The balance of Th17/Treg cells is crucial for maintaining immune homeostasis. This study aimed to investigate whether the balance of Th17/Treg cells and related cytokines may be a determinant occurrence in patients with RHL. This is a clinical experimental research based on clinical observation and analysis. We collected RHL patients from the outpatient clinic of the Department of Dermatology of Zhejiang Chinese Medical University (Hangzhou, China) in 2017, conducted questionnaire survey and signed informed consent. Peripheral blood was collected from 30 patients with RHL and 30 healthy volunteers. Flow cytometry was used to detect the percentages of Treg cells and Th17 cells. Protein microarrays coated with 20 cytokines related to T-cell subsets were performed. Enzyme-linked immunosorbent assay (ELISA) assay was conducted to further verify the expression levels of the cytokines that were screened by protein microarrays. Percentages of Th17/Treg cells in peripheral blood of RHL patients were significantly increased compared to those in healthy volunteers. The fold changes of GM-CSF, IL-4, TGF- β , IL-12, IL-10, IL-17F, and TNF- α were significantly increased compared with healthy volunteers. In addition, the expression of IL-4, IL-10, and TGF- β in the serum of RHL patients increased significantly. Our results indicated an imbalance of Th17/Treg cells in RHL, and this imbalance is probably an important factor in the occurrence, development, and recovery of RHL.

Keywords

cytokine, herpes simplex virus, recurrent herpes labialis, Th17/Treg

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Introduction

Recurrent herpes labialis (RHL) normally occurs in the lip and skin around the mouth. It is usually caused by herpes simplex virus type I (HSV-1) infection and is a common dermatosis, which may particularly occur among adult females.^{1,2} The clinical manifestations of RHL are blisters, pruritus, skin lesions, pain, and flushing, which are self-healing, but prone to repeated attacks. While the effective treatment to RHL is currently lacking, it is pointed out that HSV-1 is a highly adaptive pathogen, which

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Table 1. The basic information of subjects.

Group	Index				
	Cases (male/female)	Age	Weight	Height	BMI
Control group	30 (8/22)	24.58 ± 2.98	54.47 ± 7.61	1.65 ± 0.08	19.94 ± 7.61
RHL group	30 (7/23)	39.50 ± 17.71*	52.17 ± 4.25	1.55 ± 0.04	21.69 ± 0.83

BMI: body mass index; RHL: recurrent herpes labialis.

Uninfected represented as healthy control and infected represented as patients with RHL. Comparing with uninfected group, * $P < 0.05$.

shows potential patterns in its natural host.^{3,4} T-cell-mediated immune response plays an important role in the latency, pathogenicity, and recurrence of HSV-1,⁵ but it remains unclear which changes in the specific immune microenvironment determine the incidence of RHL. To better understand the involvement of T cells in the pathogenesis of RHL, we must first investigate the mechanisms underlying immune regulation at the site of disease.

Treg and Th17 cells are important CD4⁺ T-cell subsets,^{6,7} which have their own sources of differentiation but share mutual restraint. Previous studies indicate that Treg cells, Th17 cells, and their associated cytokines play an important role in immune diseases,⁸ infectious diseases,^{9–11} and others. Treg cells are immunosuppressive cells in the body, accounting for 5% to 10% of CD4⁺ T cells.^{12,13} Treg cells can be divided into intrinsic Treg (nTreg) cells and inducible Treg (iTreg) cells, based on the source of differentiation. Treg cells inhibit T-cell proliferation and maintain autoimmune tolerance in chronic diseases of viral infection and reduce humoral immunity and cellular immune function by inhibiting CD4⁺ T cells, CD8⁺ T cells, immune memory cells, and antigen-presenting cells.^{14,15} The content of CD4⁺-CD25⁺ regulatory T cells notably changes during the stage of attack and stationary phase in mice after HSV-1 infection.^{16,17} Th17 cells secrete various cytokines, including IL-17A, IL-17F, IL-6, IL-22, and TNF- α , which play crucial roles in promoting immune inflammation. Studies have shown that Th17 levels significantly increase in patients infected with HIV¹⁸ or HBV.¹⁹

Thus, we considered that dysregulation of Treg and Th17 cells might play a major role in the pathogenesis of RHL. To better understand the underlying mechanisms, we detected the contents of Treg and Th17 cells in peripheral blood of RHL patients by flow cytometry (FCM). In addition, the levels of cytokines correlated with Treg and Th17 cells

were measured by protein microarrays and confirmed by enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Patients

This is a clinical experimental research based on clinical observation and analysis. The minimum sample size each group is 30 calculated from $n = Z^2 \times (P \times (1 - p)) / E^2$. This study involved 30 RHL adult outpatients (8 males and 22 females). The inclusion criteria of infected group were that age is between 18 and 65 years; the onset of clinical symptoms of RHL such as obvious herpes of lip with blisters, pain, fever, and pruritus was happened in the past 48 h; the previous clinical history of RHL was at least four outbreaks 1 year; the anti-HSV-1 IgG was all positive; and the participant was willing to provide informed consent and adhere to study protocol. In addition, 30 control adults (7 males and 23 females) with no symptom above and no anti-HSV-1 IgG were included for comparison. Moreover, the exclusion criteria of both groups were that participant with history of immunodeficiency, immunosuppression, or autoimmune disease and pregnancy in this period. The basic information of all subjects is shown in Table 1. All patients and control participants were recruited from the outpatient clinic of the Department of Dermatology of Zhejiang Chinese Medical University (Hangzhou, China). The experimental protocol was approved by the Zhejiang Chinese Medical University (2014zjtc-001), and informed consent was obtained from all study participants of the study. A maximum amount of 8 mL of blood per subject was collected into heparinized tubes.

To further verify the occurrence, blood samples were collected in RHL patients when they had an outbreak, serum was extracted, and the presence of

anti-HSV-1 IgG was detected by ELISA to differentiate infected condition. Serum samples were also collected from control individuals. The assay was performed according to the manufacturer's instructions (CUSABIO). Positive judgment rule: if sample OD value \geq judgment value; otherwise, the result was deemed negative. Judgment value = $0.1 + \text{average OD value of the negative hole}$. The positivity rate of HSV-1 IgG in herpes labialis patients was 100%. The OD values are shown in Supplemental Table S1.

Reagents

Monoclonal antibodies and irritant used for FCM analysis were purchased from the following sources: phycoerythrin (PE)-conjugated anti-IL-17A (cat. 560486), PE-conjugated anti-CD25 (cat. 555432), FITC-conjugated anti-CD4 (cat. 555346), and Alexa Fluor[®] 647-Foxp3 (cat. 560045), Leukocyte Activation Cocktail, and BD GolgiPlug[™] (cat. 550583) from BD Biosciences (Becton, Dickinson and Company, San Jose, CA, USA). The Protein Microarray of Human Th1/Th2/Th17 Array Kit (QAH-TH17-1-1) was purchased from RayBiotech. The FIX&PERM Kit was used for intracellular cytokines purchased from Multi Sciences (GAS006, Hangzhou, China), as were the ELISA kits for IL-17 (211770513), TNF- α (218270315), IL-2 (210270513), IL-4 (210470455), IL-10 (211070452), GM-CSF (216370232), and TGF- β (218170454).

Protein microarray

Overall, 20 cytokines were detected with the Human Th1/Th2/Th17 Array Kit and measured according to the kit's instructions. In brief, the processed plasma samples were combined with the antibody array and incubated for 1–2 h. The newly biotinylated samples were placed on the array membrane and incubated for 1–2 h, followed by incubation with Cy3-conjugated streptavidin for 1 h. The signals were visualized, and the relative protein expression levels were quantified by densitometry.

Cell culture and FCM analysis of IL-17A

A total of 250 μ L of heparinized venous blood was added to 250 μ L of RPMI-1640 in a 24-well plate and then incubated with 1.5 μ L of Leukocyte

Activation Cocktail from the BD GolgiPlug kit, including phorbol ester, phorbol 12-myristate 13-acetate, a calcium ionophore (ionomycin), and the protein transport inhibitor BD GolgiPlug (Brefeldin A) for 4 h, at 37°C and 7% CO₂. Subsequently, 200 μ L of the sample was obtained and centrifuged at 400g for 5 min. Blood precipitate was stained with FITC-conjugated anti-CD4 for 30 min, at 37°C. The blood sample was fixed with 100 μ L of fix solution for 15 min at room temperature (RT) and centrifuged at 400g for 5 min. The precipitate was resuspended in 100 μ L of permeabilizing solution, and then stained with PE-conjugated anti-IL-17A, at 37°C, for 30 min. After permeabilization, the cells were washed twice with stain buffer and resuspended in fetal bovine serum (FBS). The percentage changes of Th17 cells were detected by FCM.

Staining and FCM analyses of CD4⁺-CD25⁺-Foxp3⁺-Treg cells

The staining process of CD4⁺-CD25⁺-Foxp3⁺-Treg cells was conducted according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). In brief, 100 μ L of peripheral blood samples was added to a 1.5-mL tube with 5 μ L of FIT-CD4 and 10 μ L of PE-CD25. The mixture was then incubated for 30 min at 37°C and centrifuged at 400g for 5 min. The supernatant was removed, and 100 μ L of reagent A (fixation and permeation) was added, left to stand for 15 min, and centrifuged at 400g for 5 min. The cells in each group were resuspended with 100 μ L of reagent B (permeation) and 15 μ L of Alexa Fluor 647-Foxp3 for 30 min at 37°C. After centrifugation at 400g for 5 min, the cells in each group were resuspended with 500 μ L of FBS. The percentage changes in CD4⁺-CD25⁺-Treg cells were detected by FCM.

Verification of the protein microarray results via ELISA

ELISA was performed according to the instructions of the manufacturer. In brief, 50 μ L of appropriately diluted sample was added to each well and incubated for 120 min at RT. Subsequently, the plate was washed twice with phosphate-buffered saline (PBS). About 100 μ L of horseradish peroxidase-labeled streptavidin was added to each well, and the plate was incubated for 45 min at RT. The

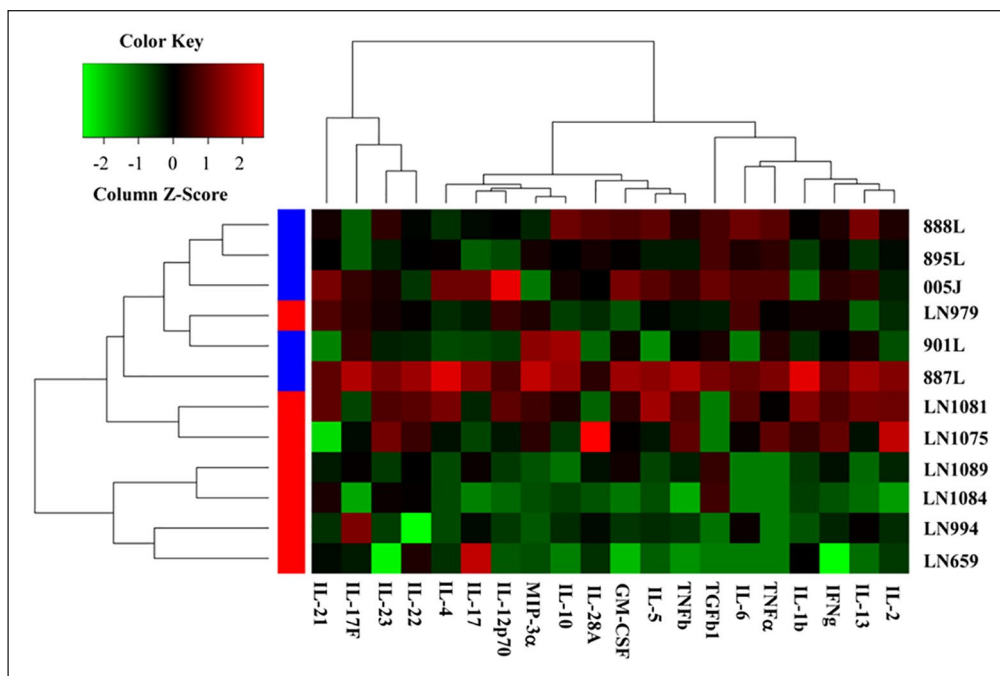


Figure 1. Cluster analysis of 20 proteins in serum samples. Heat map obtained from protein microarray data reflecting protein expression values in all enrolled participants. The right y-axis represents sample name and x-axis represents protein name; the left y-axis illustrates that red color samples represent uninfected group, and blue color samples represent infected group. The antibody clustering tree is shown at the top. Red color represents a high relative expression level, while green represents low level ($n=5$, $n=7$).

plate was washed four times with PBS, and 100 μ L of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate solution was added to the plate. After incubation for 30 min at RT, 100 μ L of stop solution was added to the plate. The OD values were measured by a microplate reader (Thermo Multiskan MK3) at 450 and 570 nm.

Statistical analysis

All values were expressed as the mean \pm standard deviation. The data were subjected to independent sample t tests. Differences were considered statistically significant if the P value was less than 0.05. All analyses were performed using SPSS version 15.0 software.

Results

Hierarchical clustering analysis of cytokines related to Treg/Th17 cell differentiation

To understand the different environments of Foxp3⁺ Treg and Th17 cells in patients with RHL, we chose 20 cytokines related to their differentiation. These cytokines were analyzed using protein microarrays.

Initially, hierarchical clustering analysis identified two major clusters based on 20 kinds of serum proteins in the serum of five healthy patients (888L, 895L, 005L, 901L, and 887L) and seven healthy volunteers (LN979, LN659, LN1081, LN1075, LN1089, and LN1084, LN994). The RayBio L-series Human Antibody Array chip figure was extracted by scanning the chip data, and the differential expression levels of 20 types of proteins were analyzed between the two groups (Figure 1).

Foxp3⁺ Treg and Th17 cell differentiation levels among patients infected with HSV-1

After normalization and analysis of the expression levels of 20 types of proteins, seven proteins were screened due to the differential expression. Compared with the uninfected group, the levels of GM-CSF, IL-4, TGF- β , IL-12, IL-10, IL-17F, and TNF α increased by at least 1.85-fold, and TGF β 1 increased the most among these seven proteins. As shown in Figure 2, four proteins were significantly upregulated. The expression of GM-CSF, TGF β 1, IL-10, and TNF- α significantly increased compared with that in the uninfected group ($P < 0.01$, 0.05).

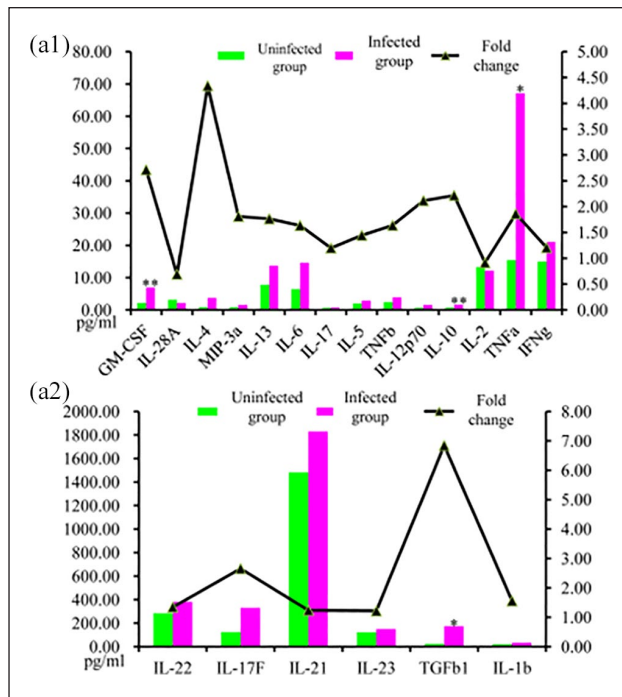


Figure 2. Changes of 20 cytokines in serum samples. The left y-axis represents contents of cytokines and the right y-axis represents fold change, while the x-axis represents names of cytokines. Uninfected represented as healthy control and infected represented as patients with RHL. Compared with uninfected group, * $P < 0.05$; ** $P < 0.01$; $n = 5$, $n = 7$, $n = 10$, $n = 9$.

Percentage changes in Th17 and Treg in peripheral blood lymphocytes

To understand the changes in Th17 and Treg cells among patients with RHL, we evaluated the levels of Foxp3⁺ Treg and Th17 cells in peripheral blood. The results are shown in Figure 3. Peripheral blood levels of IL-17A were consistent with Th17 cell frequency, whereas those of CD4⁺-CD25⁺-Foxp3⁺ were consistent with Treg cell frequency. Foxp3⁺ Treg cells and Th17 were all derived from the same naive T-cell pool. The frequency of Foxp3⁺ Treg and Th17 cells simultaneously increased after HSV-1 infection compared with that in the healthy controls. These indicate that pro-inflammatory and anti-inflammatory are both activated.

Differential expression of GM-CSF, IL-4, IL-10, and TGF- β via ELISA

The protein microarray experiment revealed that GM-CSF, IL-4, IL-10, and TGF- β were significantly increased in patients with RHL compared to healthy controls. To confirm these findings, serum

samples were further analyzed by ELISA. Compared with those in healthy individuals, the serum levels of IL-4, IL-10, and TGF- β were significantly increased in the RHL group ($P < 0.05$), whereas no significant change was observed in the level of GM-CSF (Figure 4). This indicates that RHL has a self-healing development mechanism.

Discussion

RHL is a very common infection affecting about one-third of the general population,²⁰ which was infected mostly by HSV-1 with the incubation period of days to years. Recurrences of HSV-1 reactivation are triggered by menstruation, fever, sunlight, and trauma.^{21,22} Serum was separated from blood samples collected above and HSV-1 IgG was first detected by ELISA to judge positive and native, and the result indicated that all RHL were HSV-1 seropositivity in our study. It is proved that the occurrence of RHL is mainly caused by the infection of HSV-1.

The number and function of Th17 cells and CD4⁺-CD25⁺ Treg cells are closely related to the occurrence and development of viral infectious diseases.^{11,19} Th17/Treg levels were found to be significantly increased in patients infected with varicella-zoster virus (VZV).²³ Th17 cells can express the transcription factor ROR γ t, chemokine receptor 6 (CCR6), CD161, and so on, which is a kind of common pro-inflammatory cells, and play a pro-inflammatory role by secreting IL-17. When the organism is invaded by pathogens, endogenous IL-6, TGF- β will promote the differentiation of Th17, producing abundant IL-17, and then stimulate the secretion of GM-CSF, TNF, IL-6, TGF- β , and other inflammatory factors. And IL-17 can reduce the response of effector CD8⁺ T cells by regulating the negative transcription factors to inhibit the elimination of pathogens. The changes of IL-17 and GM-CSF levels in this study indicate that the inflammatory system in the RHL patients is activated due to the differentiation of Th17, which may be one of the important mechanisms of HSV-1 leading to herpes attack in RHL patients. In addition, the removal of CD4⁺-CD25⁺-Treg cells from an animal model of herpes simplex infection significantly enhanced the proliferation of effector T cells, as well as the cytotoxicity of T cells. Similar to other viral infections, the repeated attack of latent HSV-1 infection may be strongly related

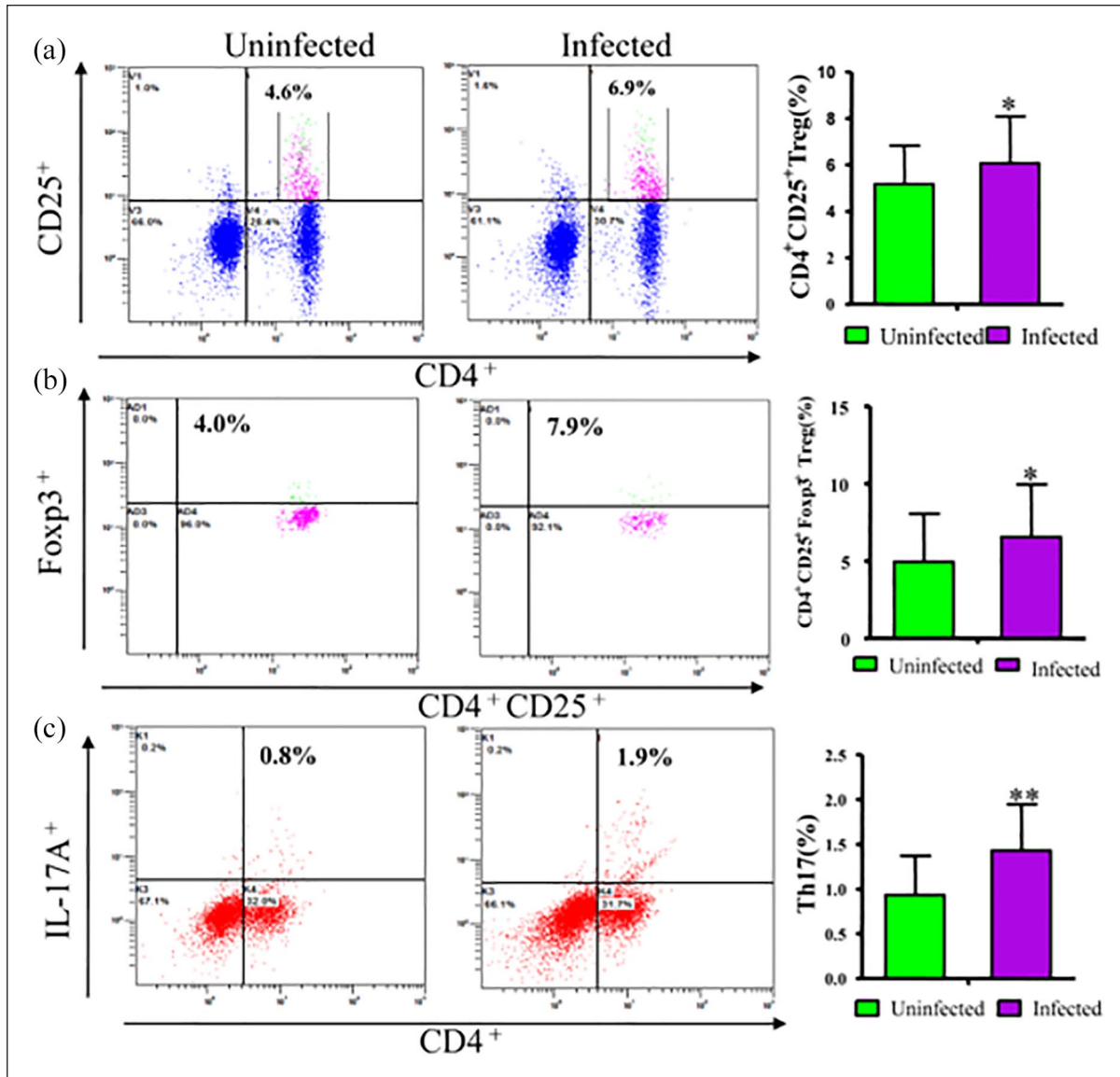


Figure 3. Percentage changes of Th17 and Treg in peripheral blood lymphocytes: (a) FCM of CD4⁺-CD25⁺-Treg cells in peripheral blood lymphocytes; (b) percentage of CD4⁺-CD25⁺-Foxp3⁺-Treg cells in the peripheral blood lymphocytes; (c) percentage of Th17 cells in the peripheral blood lymphocytes. Uninfected represented as healthy control and infected represented as patients with RHL. Comparing with uninfected group, * $P < 0.05$; ** $P < 0.01$; $n = 30$, $n = 30$.

to the effects of Treg cells, which inhibit an excessively powerful immune response, resulting in the escape of residual virus.

Naive CD4 T cells are activated by antigens of immature dendritic cells and then induced by TGF- β to express Foxp3⁺-Treg. Naive CD4 T cells that are activated by a mature dendritic cell produce IL-6, which acts in concert with TGF- β produced by Foxp3⁺-Treg to express Th17 cells. Subsequently, Th17 cells can produce IL-17A and IL-17F, and mature Th17 can produce GM-CSF to stimulate granulopoiesis and neutrophils. In

addition, the CD4 precursor can be induced by IL-10-conditioned dendritic cells to express Foxp3⁺-Treg, which is a marker of abundant IL-10 production. This process is displayed in Figure 5. Foxp3⁺-Treg and Th17 cells are closely associated with each other. Th17 cells are involved in the development of a variety of diseases through the secretion of inflammatory factors such as IL-17A, IL17F, and IL-6. Th17 cells are effector T cells that can clear extraneous pathogens. In contrast, Treg cells negatively regulate the body's immune response and tolerance by influencing

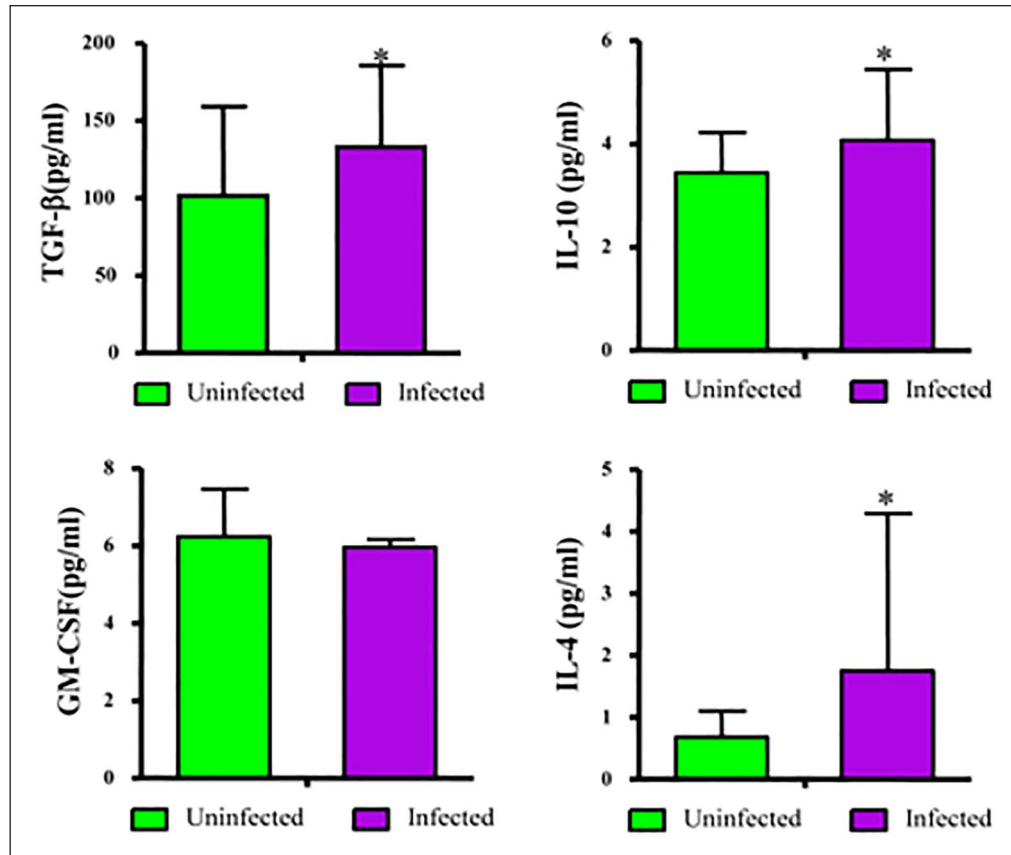


Figure 4. IL-4, IL-10, GM-CSF, and TGF- β levels in serum samples. Uninfected represented as healthy control and infected represented as patients with RHL. Comparing with uninfected group, * $P < 0.05$; ** $P < 0.01$; $n = 30$, $n = 30$.

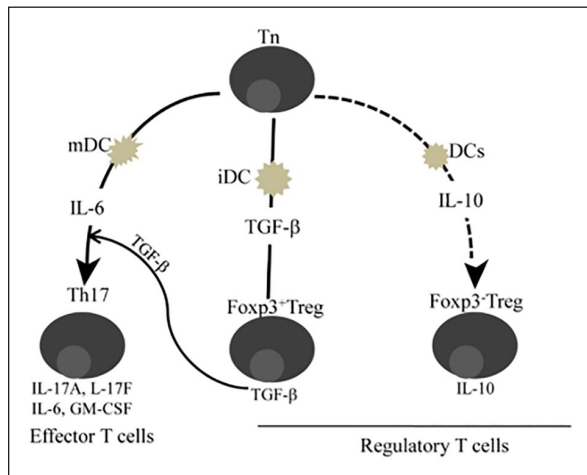


Figure 5. Divergent differentiation of Th17 and adaptive regulatory T-cell (Treg) lineages, emphasizing distinct pathways leading to Th17 effector cells, Foxp3⁺ adaptive Tregs, or Foxp3⁻ adaptive Treg.

the secretion of inhibitory factors such as IL-10 and TGF- β . Treg cells prevent tissue damage by inhibiting the strong immune response of T cells, but contribute to pathogen immune escape.

In our study, results of FCM revealed that the content of Foxp3⁺ Treg cells was significantly increased among patients with RHL, and ELISA demonstrated that the levels of TGF- β were also significantly increased. These findings were consistent with each other and indicated that patients with RHL feature by Treg immune activation. The content of IL-17A in peripheral blood lymphocytes was significantly increased in patients with RHL, but the content in plasma was not significantly higher than that in the control group. Thus, Treg cells play a key role in immune suppression and can reduce the inflammatory of IL-17A. The increase in Treg/Th17 levels indicates that the body undergoes negative feedback to inhibit the inflammatory immune response, thereby increasing the likelihood of HSV-1 escape.

IL-10 is a multi-functional negative regulator, mainly secreted by Th2 cells,²⁴ activated B cells,²⁵ monocytes, macrophages, and Treg cells.^{26,27} IL-10 is involved in the negative feedback regulation²⁸ of immune cells, inflammatory cells, tumor cells, and others and plays an important role in various

diseases such as autoimmune diseases²⁹ and severe infectious diseases.³⁰ When the inflammatory response is strong, IL-10 production is increased so that inflammation can be inhibited. The results of this study showed a significant increase in IL-10, and this increase may be the initiation of negative feedback regulation, which inhibits the inflammatory response of Th17, as well as other effects of T cells. In addition, the results of this study showed that the high levels of IL-4, TGF- β 1, and other anti-inflammatory cytokines. IL-4 is a multi-functional immune factor, and TGF- β 1 is a negative regulatory factor of immune response. They both can regulate the immune response and inhibit inflammation. These also demonstrate the activation of anti-inflammatory mechanisms in patients with RHL, suggesting that these results may also be the feature of self-healing of RHL.

In this study, we provide the original report of an imbalance of Th17/Treg cells in RHL caused by HSV-1 and definite evidence that Th17/Treg is an important pathogenic of the occurrence. Moreover, there are some limitations in our study. First, we exclusively deal with the specific Th17/Treg cells, neglecting other T-cell population or subpopulations. Second, we merely represent a phenomenon and have not well proved the exact mechanism of Treg/Th17 cells in HSV-1, which will be focused on in the future researches.

Conclusion

This study showed that HSV-1 infection is an important factor in RHL, and the recurrence of herpes is related to immune changes. The imbalance of Th17 and Treg cells and the upregulation of the expression of related immune factors indicated that RHL patients have the pro-inflammatory activation and anti-inflammatory inhibition, which may be one of the important mechanisms of HSV-1.

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Author contributions

S.-S.L., Y.-S.F., X.-X.P., and J.B. conceived and designed the experiments. X.-X.M. and J.B. wrote the paper. Other authors participated in the procedures of the experiment. All authors contribute to the study.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

Ethical approval for this study was obtained from IRB of Zhejiang Chinese Medical University (2014-zjtcu-001)*.

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Informed consent

Written informed consent was obtained from all subjects before the study.

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Supplemental material

Supplemental material for this article is available online.

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