Low-dose IL-2 effectively restored decreased regulatory T cells in patients with Behçet's disease

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Abstract

Objective

Although Behçet's disease (BD) is associated with immune mechanisms, the changes in circulating lymphocytes, especially regulatory T (Treg) cells remain unclear. Furthermore, low dose IL-2 has been reported to promote the expansion of Treg cells. This study aimed to investigate the significance of these subsets in the pathogenesis and the effect of low dose IL-2 on BD.

Methods

Lymphocyte subsets and cytokines from 177 BD patients and 160 healthy controls (HCs) were characterised. Then the efficacy and safety of low dose IL-2 for refractory BD patients were explored.

Results

There was a decrease in the absolute number of Treg cells and IL-10 in patients, while no difference in Th1, Th2, Th17 cells or their related cytokines. Accordingly, the ratio of Th17/Treg cells in patients was greatly higher than those of healthy controls. Furthermore, circulating Treg levels were negatively correlated with erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and Behçet's disease current activity form (BDCAF), respectively. Lastly, after IL-2 treatment, all subsets were increased to some degree, while only Treg cells were amplified more dramatically, with a four-fold increase. Meanwhile, we found that the symptoms were mitigated without observed side effects.

Conclusion

BD might be triggered by the defect of immunotolerance with decreased Treg. Moreover, low-dose IL-2 proposes a potential treatment by restoring Treg and promoting rapidly remission.

Key words

Behçet's disease, low dose IL-2, regulatory T cells, autoimmune tolerance

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Introduction

Behçet's disease (BD) is an autoimmune disease characterised by recurrent oral ulcer, genital ulcer, uveitis or retinal vasculitis, skin lesion and other multi-system involvement. Although the aetiopathogenesis of BD has not yet been clarified, it is speculated that it might be related to genetics susceptibilities, trigger hypothesis, and immune system abnormalities (1-3). New insights into the perturbations of T cell homeostasis of BD recently emerged. Some study demonstrated that certain environmental factors activated $CD4^+$T cells, leading to the secretion of cytokines, stimulating the effector cells, and following the loss of immune tolerance (4).

$CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg), a subset of $CD4^+$T cells, have important function in the maintenance of peripheral tolerance. Treg cells inhibit the differentiation and function of Th17 cells and other effector T cells by secreting cytokines such as IL-10 and TGF-β (5), maintaining and restoring the immune balance. And a relative deficiency of Treg was considered to be a pivotal cause of autoimmune diseases, including diabetes mellitus, rheumatoid arthritis and lupus (6-8). The ability to achieve immunomodulation via infusion or amplification of Tregs cell is a promising therapeutic approach aiming at re-establishing the immunologic balance (9). But relevant studies were rarely reported in BD.

IL-2 was originally thought to be a strong cell growth factor of T cells and was essential to induce immune response against pathogens and tumours. In recent decades, IL-2 has been reported as regulatory cytokine to maintain self-immune tolerance (10). Treg cells express a high affinity IL-2 receptor alpha (IL-2Rα, CD25), which could combine with low concentration IL-2 in local microenvironment. Further, low dose IL-2 promoted the activation and amplification of Treg cells through JAK/STAT pathway (11). In recent clinical trials, administration of low-dose IL-2 has been shown to result in the selective expansion of Treg and clinical improvement for autoimmune and inflammatory disease (12-13). However, there are no reports of low dose IL-2 for BD patients.

In the present study, our goal was to evaluate the abnormal levels of peripheral T cell subpopulations and $CD4^+$T subsets as well as serum cytokines in BD patients. we also evaluated the correlation with disease activity. And the effect of low dose IL-2 on peripheral lymphocyte subsets was explored, which provide the basis to immunomodulatory therapy for BD.

Materials and methods

Subjects and experiments

A total of 177 patients (79 men, 98 women; 41.98±14.78 years of age) were included. The diagnosis of BD was made according to the International Study Group criteria (14). Clinical data on patients included age, sex, duration of disease and current symptoms. Furthermore, BD activity was analysed by erythrocyte sedimentation rate (ESR), C-reaction protein (CRP) or the Behçet's Disease Current Activity Form (BDCAF) (15). Patients with other inflammatory or autoimmune diseases, haematological diseases and malignant tumours were excluded. The 160 healthy adults were used as healthy controls (HCs) who were homogenous in age (40.70±12.60 years) and gender (41% men *vs*. 59% women).

Thirty-nine refractory BD patients were recruited into low-dose IL-2 group, who had failed to respond to conventional immunosuppressant therapy for more than half a year. These patients were administrated with rhIL-2 (recombinant human interleukin-2, Beijing Sihuan Biological Pharmaceutical co. LTD, China) at a dosage of 0.5 million IU subcutaneously per day for 5 days, combined with conventional therapy, with discontinued or decreased immunosuppressive agents. The IL-2 treatment protocol was based on our previous study for other autoimmune disease (16). Other patients only received conventional therapy as conventional group. Patients were evaluated one week after treatment, including physical examination and laboratory tests. This study was approved by the ethical committee of The Second Hospital of Shanxi Medical University,

China. An informed consent was obtained from each patients and control subjects before collecting material. The detailed demographic and clinical data of the patients are listed in Table I.

Flow cytometric analysis of lymphocyte subsets

Heparinised peripheral bloods were obtained from all patients and HCs. For analysis of Th1/Th2/Th17cells, identified as $CD4^+IFN\text{-}\gamma^+/CD4^+IL\text{-}4^+/CD4^+IL\text{-}17^+$ respectively, 80μl heparin-anticoagulated venous blood were stimulated by 10μl PMA, 10μl Ionomycin and 1μl GolgiStop in 37°C incubator for 5 hours and then stained with human anti-CD4-FITC antibodies in room temperature, away from light for 30 minutes. Using 1ml fresh Fixation/Permeabilisation to fix and permeabilise cells and then staine them by IL-4-PE and IFN-γ-APC in tube A and human anti-IL-17-PE in tube B. Washed cells with PBS and Tested on computer. For analysis of Treg cells, 80μl heparin-anticoagulated venous blood were surface-labelled with CD4-PE and CD25-PE-Cy5 followed by fixation and permeabilisation and intracellular staining with FOXP3-FITC. For analysis of T, B, $CD4^+T$, $CD8^+T$ and NK cells. Lymphocytes were incubated with fluorophore-conjugated monoclonal antibodies and analysed by flow cytometry. Absolute number of these cells were detected by Procount. The phenotypic characterisation of cells is T (CD3+CD19-), B (CD3-CD19+), $CD4^+T$ ($CD3^+CD4^+$), $CD8^+T$ ($CD3^+CD8^+$), NK (CD3-/$CD16^+CD56^+$). Labelled cells were washed and analysed with a FACSCalibur flow cytometer (Becton-Dickinson) using the Cell Quest software (Becton-Dickinson). In each case, staining was compared with that of the appropriately labelled isotype control antibody.

Cytometric beads array (CBA) analysis of cytokines

The basic parameters of FCM were set by BD FACSComp software. Cytokine standards were prepared using assay diluent by the method of serial dilutions. Capture bead was added into each tube that is samples, standards, and negative control and was incubated for 2 hours at room temperature in the absence of light. The captured data of the standard and the test specimen were transferred into BD FCAP Array software, and then the assay of IFN-γ, IL-4, IL-17, IL-6 and IL-10 were performed.

Table I. The demographic and clinical characteristic of the study population.

Clinical characteristic	Low-dose IL-2 Group (n=39)	Conventional Group (n=138)	*p*
Age (x±s), years	41.33 ± 14.23	42.43 ± 14.52	0.675
Sex (man/woman)	18/21	61/77	0.829
Duration (x±s), months	84.00 ± 90.51	116.55 ± 107.26	0.086
BDCAF	3.46 ± 1.35	2.59 ± 1.21	0.001
ESR	43.49 ± 38.57	22.47 ± 20.70	0.002
CRP	28.95 ± 33.10	14.05 ± 36.23	0.018
Symptom			
Mouth aphthosis	34 (87%)	100 (72%)	-
Genital ulcer	13 (33%)	44 (32%)	-
Erythema nodosum	9 (23%)	28 (20%)	-
Pustule	7 (18%)	35 (25%)	-
Arthralgia	18 (46%)	45 (33%)	-
Diarrhoea/haematochezia	7 (18%)	15 (11%)	-
Ocular symptom	16 (41%)	41 (30%)	-
Neurological symptom	4 (10%)	11 (8%)	-
Macrovascular symptom	3 (8%)	3 (2%)	-
Therapy (users/non-users)			
Glucocorticoids	36/3	109/29	0.056
DMARDs	9/30	42/96	0.370
Thalidomide	25/14	96/42	0.517
NSAIDs	18/21	64/74	0.980
Biological agents	1/38	1/137	0.919

BDCAF: Behçet's disease current activity form; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying anti-rheumatic drugs.

Statistical analysis

SPSS 21.0 was used in the statistical calculations. Data were presented as mean ± standard deviation or medians and interquartile ranges. Differences between the values were determined using Student's *t*-test. Grouped data were analysed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Mann-Whitney U-test was used for non-normal distribution. Spearman's rank test was used to describe the correlation. All tests were 2-sided at the significance level of 0.05.

Results

Circulating lymphocyte subsets in BD patients and healthy controls

The circulating levels of $CD4^+T$ subsets were analysed by flow cytometry. As compared to HCs, the number of Treg cells were significantly decreased in BD patients (median: 22.32 cells/μl vs. median: 33.12 cells/μl, $p<0.001$), while there was no difference about Th1, Th2 and Th17 cells between two groups (Fig. 1). At the same time, the median ratios of Th17/Treg cells in patients were greatly higher than those of HCs (0.38(0.24, 0.58) *vs*. 0.21(0.15, 0.34), $p<0.001$). Besides, we analysed each subset of other lymphocytes, and the results showed that the circulating NK cells in the patients with BD appeared to be lower than the proportion in the healthy donors (208(145, 326) *vs*. 282(185, 400), $p<0.001$). However, no difference was observed for that of T, B, $CD4^+T$, $CD8^+T$ cells between groups (Supplementary Table S1).

Decreased Treg cells were negatively correlated with the disease activity in BD patients

In view of the relation of immune cells to the assessment indicators of disease activity, the correlation analysis showed that circulating Treg levels were negatively correlated with ESR, CRP and BDCAF respectively (Table II). While no obvious correlation was seen in Th1, Th2, Th17 and NK cells.

The levels of serum cytokines in BD patients and healthy donors

To investigate the expression of the functional cytokines of T cells, plasma IFN-γ, IL-4, IL-17, IL-6 and IL-10 were measured by CBA. We found that the levels of IL-6 was higher in patients, but the difference was not statistically significant. Besides, the secretion of serum Th1 cytokine (IFN-γ), Th2 cytokine (IL-4) and Th17 cytokine (IL-17) showed no statistically significant difference between the two groups. There was, however, a statistically significant decrease in the secretion of IL-10 in the BD patients compared to the level of healthy donors [4.54(3.56, 8.73) *vs*. 12.73 (9.68, 14.56), p=0.004] (Fig. 2).

Rapid correction of imbalance between Treg and Th17 during IL-2 therapy

At baseline, T cells and their subsets in low-dose IL-2 group were all lower than conventional group. It showed that total T cells, B cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, Th1 cells, Th2 cells, and Th17 cells were increased after IL-2 treatment (Fig. 3A and Suppl. Fig. S1A). But only Treg cells were amplified more dramatically, with the four-fold increase (55.70 (34.07, 86.07) *vs*. 14.12 (9.85, 19.15), p<0.001). Accordingly, the ratio of Th17/Treg was decreased significantly in patients with IL-2 treatment (0.19 (0.09, 0.41) *vs*. 0.52 (0.23, 0.95), p<0.001), tended to balance and had no difference with HCs (p=0.275) (Fig. 3C). Conversely, single conventional therapy reduced NK cells and Th1 cells but had no effect on other lymphocytes. Hence, the ratio of Th17/Treg maintained at higher level (0.37 (0.25, 0.63), p<0.001) (Fig. 3 and Suppl. Fig. S1B).

IL-2 induced patients' clinical remission without severe adverse event

One week after IL-2 therapy, clinical assessments were performed again. We found that disease activity including ESR and CRP were both decreased distinctly (Fig. 4). Clinically, there were decreased severity and percentage of systemic involvements, such as erythema nodosum (5%), pustule (3%), arthralgia (5%), diarrhoea/haematochezia (5%), ocular symptom (18%) and neurological symptom (5%), without mouth aphthosis, genital ulcer and macrovascular symptom.

Although the white blood counts (WBC) and lymphocytes (LYMPH) were higher than that of pre-treatment, they remained at normal levels. Further, the results showed there was no significant change in haemoglobin (HGB), platelet counts (PLT), liver and kidney function (Fig. 5).

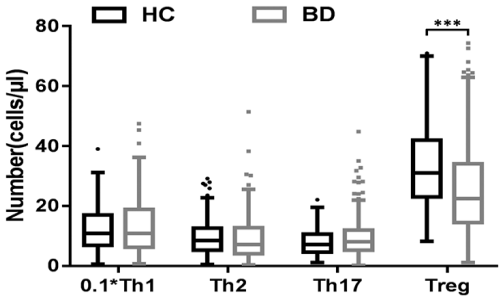

Fig. 1. Absolute number of $CD4^+$T subsets in patients with BD and healthy controls. *p<0.05, **p<0.01, ***p<0.001. BD: Behçet's disease; HC: healthy controls.

Table II. The correlation between disease activity and the number of lymphocytes.

Correlation	BDCAF		ESR		CRP	
	r	*p*	r	*p*	r	*p*
Th1	-0.106	0.146	-0.084	0.256	-0.134	0.070
Th2	-0.140	0.055	0.000	0.996	-0.065	0.384
Th17	-0.153	0.036	-0.132	0.072	-0.055	0.461
Treg	-0.757	<0.001	-0.318	<0.001	-0.388	<0.001
NK	-0.152	0.038	-0.038	0.609	-0.010	0.894

BDCAF: Behçet's disease current activity form; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

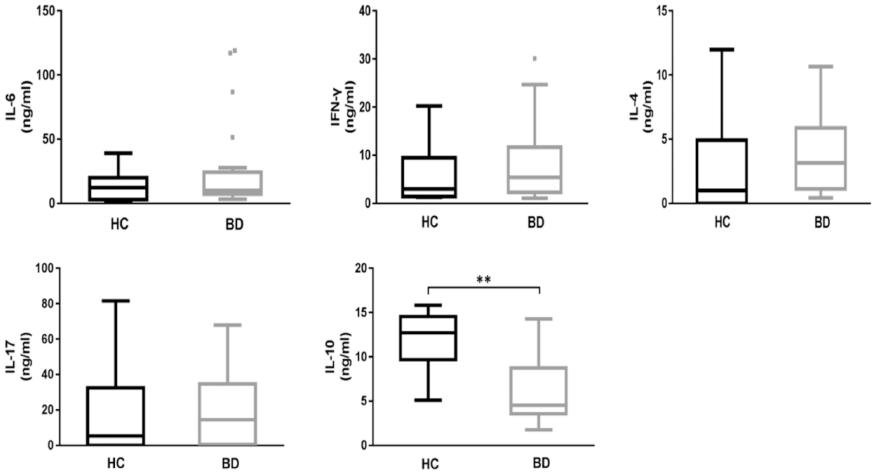

Fig. 2. The level of serum cytokines in BD patients and healthy controls. *p<0.05, **p<0.01, ***p<0.001. BD: Behçet's disease; HC: healthy controls.

Discussion

BD is a chronic multisystem inflammatory disease and the specific mechanisms remain unknown with limited options of therapeutic medicines. We started from the immunological differences between patients and healthy controls to further explore the immune regulation in the development and treatment of BD.

In the study, our results indicated that BD patients showed lower number of Treg cells than HCs, while there was no significant difference in the expression of either Th1, Th2 or Th17 cells. However, there were some controversial reports regarding the $CD4^+$T cell subsets in patients with BD. Some previous studies demonstrated the imbal-

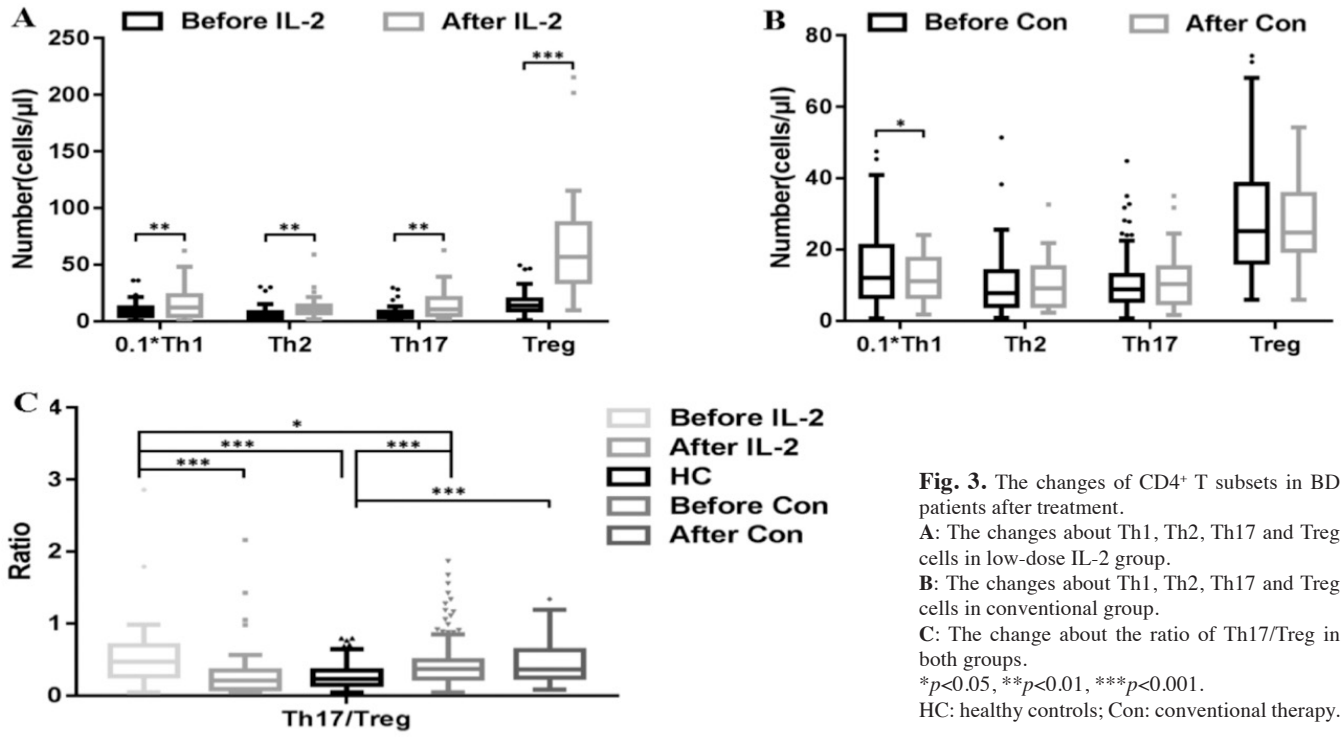

Fig. 3. The changes of CD4+ T subsets in BD patients after treatment.
A: The changes about Th1, Th2, Th17 and Treg cells in low-dose IL-2 group.
B: The changes about Th1, Th2, Th17 and Treg cells in conventional group.
C: The change about the ratio of Th17/Treg in both groups.
*p<0.05, **p<0.01, ***p<0.001.
HC: healthy controls; Con: conventional therapy.

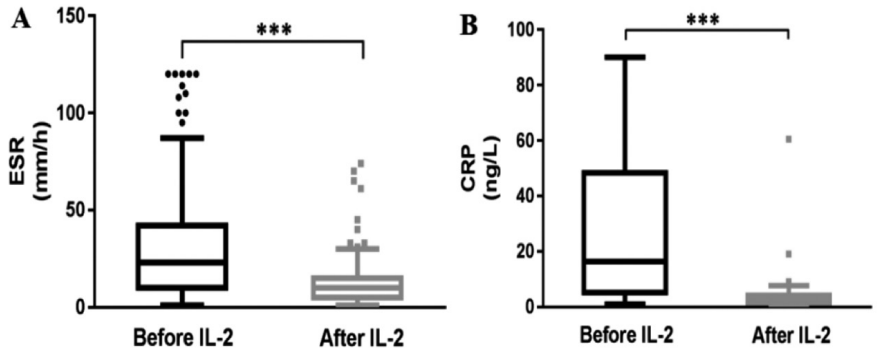

Fig. 4. The changes of disease activity in BD patients after low-dose IL-2 treatment.
A: The change of erythrocyte sedimentation rate (ESR). **B**: The change of C-reaction protein (CRP).
*p<0.05, **p<0.01, ***p<0.001.

ance of Th1/Th2 played a vital role in the pathogenesis of BD, which led to immunopathological impairment (17-18). But it was insufficient to explain the problem of conventional treatment strategy with poor and even higher side effects. With the deepening research on CD4+T cells, the novel T-cell subsets Th17, Treg cells and their cytokines were found intricately involved in autoimmune disorders (6, 19). Thl7 cells, named after the production of IL-17, which can induce the expression of inflammatory cytokines, chemokines and the matrix metal protease, caused histocyte destruction by inflammatory infiltration (20). Treg cells, characterised by the secretion of IL-10, play an important role in regulating immune balance, inducing immune tolerance and preventing the occurrence of autoimmune diseases and allergic diseases. The consumption of Treg cells in healthy individuals can trigger rapid autoimmune and inflammatory responses at any time, resulting in multi-organ immune damage (21, 22). Data on Treg subset among BD patients were scarce and conflicting. Direskeneli *et al*. reported a marked increase in Th17 (but not Th1) cell numbers and a decreased frequency of Treg cells in the peripheral blood of patients with active BD (23), while other authors reported elevated levels of Treg cells in such patients (24). These discrepancies might be due to the variations in age, gender, treatment program, race and ethnicity, or geographical parameters, especially different cell surface markers and flow cytometry methods. The majority of previous studies used CD4+CD25+T cells to measure the amount of CD4Treg cells between patients with autoimmune disease and healthy controls (25). Because different definitions for CD4Treg cells may lead to inconsistent results, we compared the status of CD4+CD25+T cells with that of CD4+CD25+Foxp3+T cells (CD4Treg) in peripheral blood of BD patients. The absolute number of CD4Treg cells were obviously lower in both conventional-treated BD and new BD patients than in healthy controls. It was noteworthy that no matter the alterations of the absolute number of CD4+CD25+ T cells or that of the percentage of CD4Treg cells were not significant. The expression of FOXP3 protein is considered as the most reliable marker of Treg cells and it is very important for the regulation function of Treg cells (26). So, the status of CD4+CD25+ T cells did not represent the real status of the CD4Treg cells in BD patients as well as healthy controls. Besides, percentage of CD4Treg also could not represent absolute number of CD4Treg cells. In this study, for the first time, advanced flow cytometry of

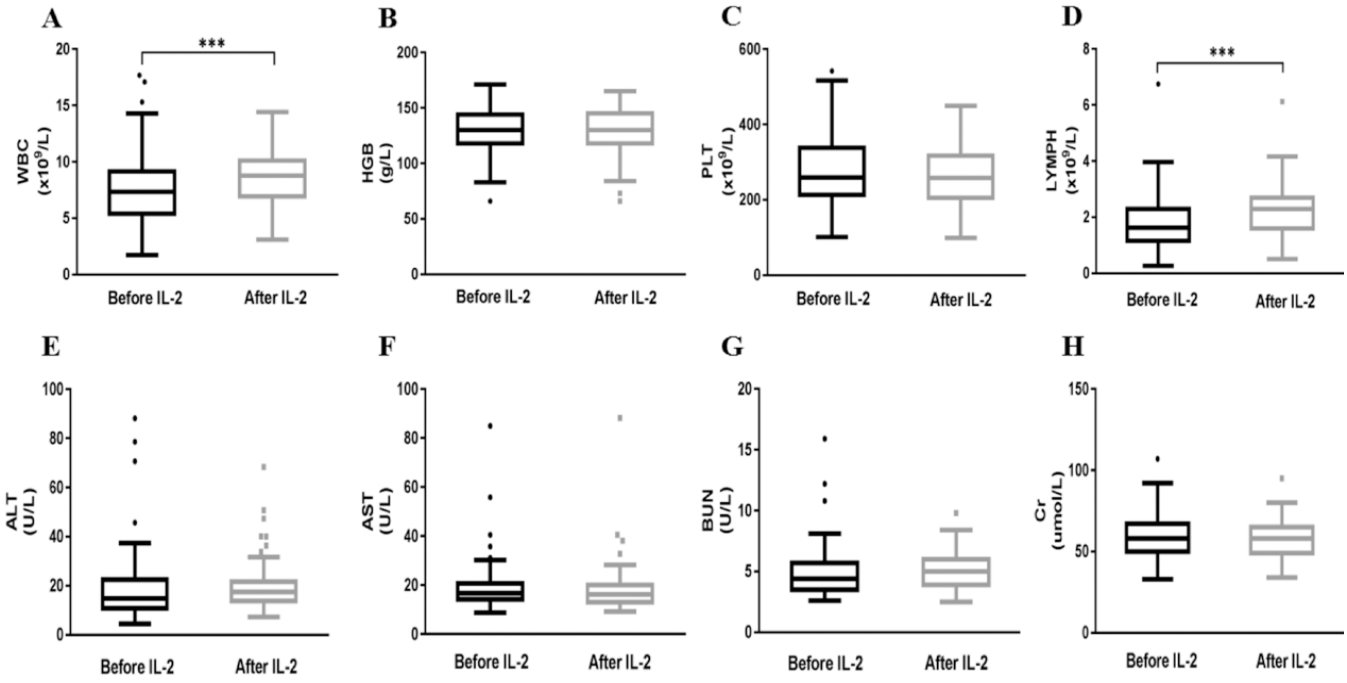

Fig. 5. The changes of clinical parameters in BD patients after low-dose IL-2 treatment. **A**-**D**: haematologic change; **E**-**F**: liver function; **G**-**H**: renal function. **p*<0.05, ***p*<0.01, ****p*<0.001.
WBC: white blood counts; HGB: haemoglobin; PLT: platelet counts; LYMPH: lymphocytes; ALT: alanine transaminase; AST: aspartate amino transferase; BUN: blood urea nitrogen; Cr: creatinine.

absolute counting was used to measure immune cells, which can more accurately reflect the true number of peripheral blood lymphocytes. In addition, the study found that the absolute number of NK cells was also decreased in patients, while T, B, $CD4^+$ T and $CD8^+$T cells showed no significant difference. Though in patients with BD, there are several conflicting reports on NK cell numbers (27-28). Our result was in accord with the opinion that defect of NK cells would result in diminished NK cell function and persistent inflammation response (29).

To further explore which lymphocyte subset plays a key role in the development of disease, we analysed their relationship with disease activity. This study indicated that only Treg cells showed significant negative correlation with disease activity index, while other cells did not. Meanwhile, compared with the healthy control, there were no changes in Th1, Th2 and Th17-related cytokines in the serum of BD patients, while the level of IL-10 was distinctly down-regulated, which was consistent with the expression of Treg cells. IL-10 is the main anti-inflammatory cytokine that acts through the induction of Treg cells and inhibition of Th1 response. Shahriar *et al*. also reported that low IL-10 expression was involved in the pathogenesis of BD, playing a central role in controlling inflammatory responses and regulating the immune response (30). It is proposed that decreased levels of this cytokine and the Th17/Treg cell imbalance caused by the reduction of Treg can contribute in the deficiency of immune tolerance and the progression of disease activity in BD patients. Therefore, promoting the proliferation of Treg cells might be the pointcut to prevent the progression of the disease and the key for precise treatment.

IL-2 is a pleiotropic cytokine and a key survival factor for Tregs. It maintains Tregs' function by promoting Foxp3 expression and subsequent production of immunoregulatory cytokines. Administration of low-dose IL-2 was shown to be a promising approach to prevent allograft rejection, treat acute coronary syndromes and systemic lupus erythematosus (31-33). In the study, the results turned out that all lymphocytes proliferated to some extent after low-dose IL-2 therapy. But only Treg cells were amplified more dramatically, 4 folds increase. And the ratio of Th17/Treg was decreased significantly, tended to balance and had no difference with healthy individuals. So low dose IL-2 is most suitable for over-treated BD patients with imbalanced Th17 and Treg cells, both of them at low cell levels, since the therapy can not only control the disease by expanding Treg but also reduce the infection rate by increasing properly effector T cells.

In addition, we have observed clinical improvement for refractory BD patients accompanied with glucocorticoid and disease-modifying anti-rheumatic drugs (DMARDS) reduction. No obvious abnormal indicators of liver function, kidney function or blood routine test were observed except some rashes around injection sites and flu-like symptoms. Of course, more multicentre studies and long-term efficacy are necessary to prove these ideas.

In conclusion, our study demonstrated that absolute number of circulating Treg cells and related cytokine in BD patients were reduced significantly and negatively correlated with disease activity. It was noteworthy that low dose IL-2 especially recovered Treg cells, which was helpful for the control of disease activity without obvious side effects.

Furthermore, our findings strengthen the concept of immunoregulation (maintaining a balance of Th17 and Tregs at proper levels) but not immunosuppressant in the treatment of BD.

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