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Original Article

Bauduanjin exercise restores T cell function in man with heroin addicts by decreasing $CD4^+CD25^+Foxp3$ regulatory T cells

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Abstract: There is an increasing body of evidence that heroin addiction is associated with severe alterations in immune function, which might contribute to an increased risk to infectious diseases like hepatitis B and C or HIV. However, the impact of Baduanjin exercise on T cell function in heroin user is not well understood. Therefore, we analyzed the frequency of immune-suppressive $CD4^+CD25^+$FoxP3 regulatory T cells (Tregs) isolated from peripheral blood of heroin users before (n=30) and after (n=30) practicing Baduanjin exercise. Interestingly, we detected a significant decrease in the percentage of Tregs in PBMC of heroin addicted patients after practicing Baduanjin exercise for 3 months. The proliferative response of PBMCs decreased in heroin users, but elevated after practicing Baduanjin exercisers, whose mechanism might be related to the cytokine of TGF-β. These results suggest that the impaired immune responses observed in heroin users can be restored by Baduanjin exercise through decreasing Tregs or suppressing cytokine TGF-β.

Keywords: Qigong, baduanjin, immunity, addiction

Introduction

Heroin users have a high risk of developing infectious diseases such as HIV and hepatitis viruses [1] due to weak immunity function [2]. In the peripheral blood of heroin addicted patients the impaired lymphocyte proliferation and reduced natural killer (NK) cell activity were observed. The fragile immunity function will continue for a long time until drug addicts are under compulsory addiction treatment. The patients under compulsory addiction treatment are easily to resume heroines because of their weak will power and bad body conditions, which is also partly due to low immune function. Therefore finding an easy method to enhance the immune function is essential to reduce the high drug withdrawal rate for heroin users.

Baduanjin is derived from traditional Qigong and is created by State Sports General Administration in China in 2003. It has been proved that Baduanjin can improve body health. An Bingchen found that practicing Baduanjin is a helpful exercise for knee osteoarthritis [3]. Wang searched 13 databases for the role of qigong exercise on immunity and infections and found favorable results [4]. Byeongsang [5] found qigong could improve quality of life, mood and fatigue parameters, reducing inflammation, and improving immunity in cancer patients. Moreover, Qigong may be an alternative treatment for heroin users without side effects. Li and colleagues have analyzed the effectiveness of qigong therapy on detoxification of heroin addicts [6]. They found reduction of withdrawal symptoms in the qigong group occurred more rapidly than in the other groups. All the data suggested that qigong can reduce withdrawal symptoms and enhance heroin users' immunity. Baduanjin may enhance the immune function in heroin users, but further experiments should be conducted to prove the hypothesis.

Tregs, specialized T cells, are $CD4^+CD25^+$ and express the transcription factor Foxp3 [7]. Based on their immune-suppressive activity these Tregs play a pivotal role in the maintenance of peripheral immunological tolerance

Table 1. Characteristics of the study participants

	Healthy control	Heroin control	Baduanjin exercise inheroin use
Number of patients	30	30	27
Age (years old)	34.6 ± 5.4	35.6 ± 7.6	39.8 ± 8.5
Sex	male	male	male
Heroin exposure time (years)	-	5.6	5.9
Baduanjin exercise (months)	-	-	3

by limiting autoimmune processes and inflammatory responses [8], and also the criminal for immune deficient disease such as cancer [9]. However, until now the mechanisms underlying the immune effect of qigong on heroin remains unclear. Therefore Tregs were examined before and after practicing Baduanjin in heroin users in our study.

Thus, the aim of the present study was to analyze the effect of Baduanjinon heroin addiction. We determined the amount of Tregs in the peripheral blood of heroin users in comparison to healthy controls and patients before and after practicing Baduanjinqigong exercise for three months and investigated which effect these $CD25^{high}$ Tregs have on the functional phenotype of $CD4^+$ T cells.

Materials and methods

Patients

30 heroin users (all males) in control group, 30 heroin patients (all males) in Baduanjin exercise group, and 27 healthy donors(all males) without active infections or inflammatory diseases participated in this study after giving informed consent. There is no significant different between the age, sex, and heroin exposure time (as shown in **Table 1**). As for normal control, 27 healthy men were recruited. Heroin users were recruited at The Coercive Rehabilitation Center for Addicts Affiliated to The Public Security Bureau of Shanghai City for detoxification treatment. All patients have been routinely evaluated using a self-report questionnaire concerning addiction history. The study was approved by the local institutional review board.

Baduanjin qigong training program

The Baduanjin exercise objects at first learned Baduanjin for 2 weeks, and the performance of each object was corrected by the instructor. After that, the objects started to join a 12-week Baduanjin qigong training program, in which they practiced Baduanjin with the instructor for five times per week and no less than 1 hour per time. The entire set of Baduanjin includes 8 sections, which has been clearly stated elsewhere [10].

Cell extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from 15 ml heparin-treated blood of heroin user, objects in heroin control, exercise group and healthy control by density gradient centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Piscataway, NJ, USA). Isolated cells were counted and 3×10^6 cells were immediately analyzed by flow cytometry. Residual cells were cryopreserved in medium containing 10% fetal calf serum (FCS; PAA Laboratories, Austria) and 10% dimethyl sulfoxide (DMSO). $CD4^+$ and $CD4^+CD25^-$ T cells were isolated from thawed PBMCs using the $CD4^+$ T cell isolation kit II (Miltenyi Biotech, Germany) followed by negative selection on an AutoMACS separation system (Miltenyi Biotech, Germany) according to the manufacturer's recommendation. For $CD4^+CD25^-$ T cell preparation the same protocol was used except of adding 4 µl biotinylated anti-CD25 antibody (Miltenyi Biotech, Germany) per 1×10^7 cells to the cell/antibody cocktail mixture. Purity of cells was determined by flow cytometry. We observed no significant differences in the purity of MACS separated cells used for analysis of proliferative activity.

Flow cytometry

PBMCs were stained with fluorochrome-labeled anti-CD4 and anti-CD25 (both from MiltenyiBiotec, Germany). Intracellular staining was performed with the Foxp3 staining kit from eBioscience (NatuTec, Frankfurt, Germany) according to the manufacturer's recommendations. FACS analyses were performed with a FACSCalibur (BD Biosciences, Heidelberg, Germany) and FlowJo software (Tree Star, OR).

Lymphoproliferation

Microcultures of fresh PBMC were set up in triplicate for each sample (10^6 cells/ml) in

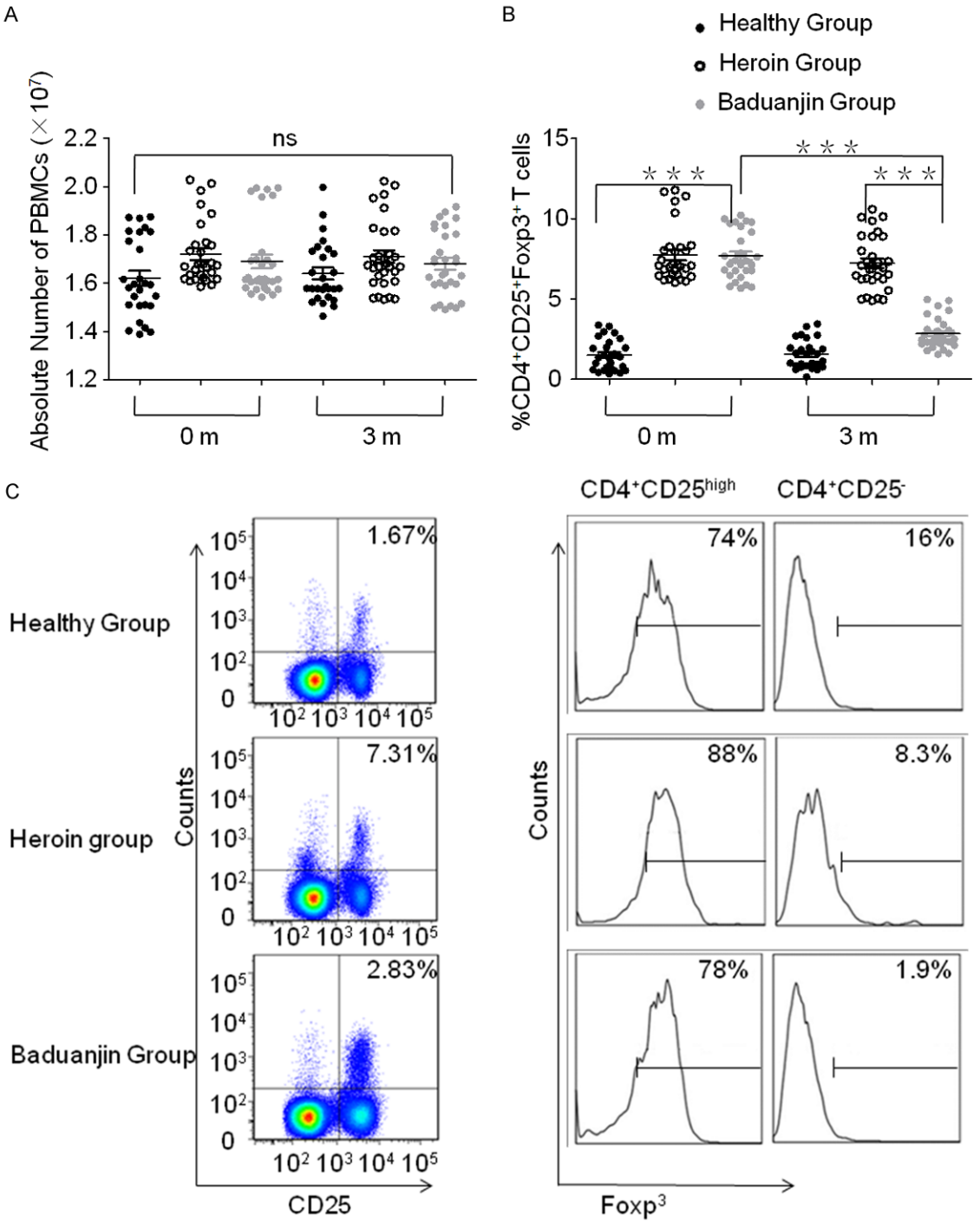

Figure 1. Decreased frequency of $CD4^+CD25^+FoxP3^+$ regulatory T cells in peripheral blood of Baduanjin exercise in heroin patients. PBMCs were isolated from heparinized blood samples and the absolute numbers of PBMCs isolated from 20 ml blood of healthy group (black circles, n=27), heroin group (white circles, n=30) and Baduanjin group (gray circles, n=30) was determined by cell counting (A). The percentages of $CD4^+CD25^{high}$ $Foxp3^+$ T cells (B) were analyzed by flow cytometry (control, n=27; heroin user, n=30, Baduanjin exercisers, n=30). The gating strategy for one representative probe of each group is depicted (C). ***P<0.001.

RPMI 1640, 10% FCS with or without PHA (1 μg/ml). After 48 h incubation at 37°C, 1.0 μCi of ^3H thymidine (specific activity 2 Ci/mmol, Amersham, UK) was added to all cultures. Eighteen hours later, cells were harvested by an automated cell harvester (Skatron) and

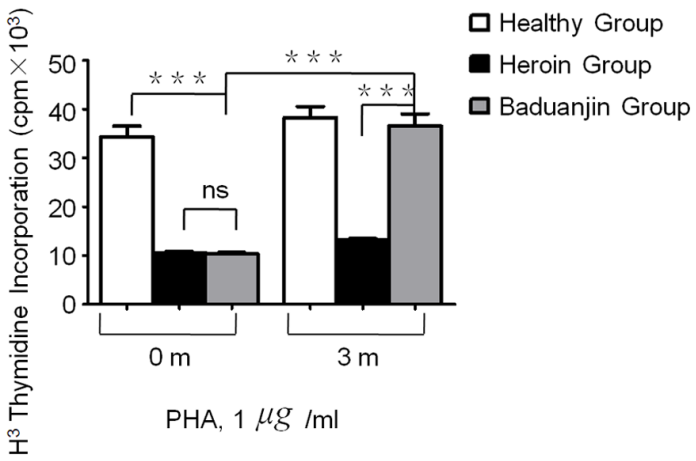

Figure 2. Increased proliferative activity of T cells from Baduanjin exercise in heroin patients. Proliferation was measured in PHA (1 μg/ml) stimulated PBMC cultures obtained from healthy control (black circles, n=27), heroin user (white circles, n=30) and Baduanjin exercisers (gray circles, n=30) treated subjects. Values are means ± SEM. **P*<0.01.

radioactivity was measured in a liquid scintillation counter (Packard, Downers Grove, IL, USA).

ELESA

PBMCs were treated as stated previously, and culture supernatants were collected from the top chamber of plates at 48 h after cultivation. The levels of TGF-β1 in culture supernatants were then assessed by ELISA. The TGF-β1 OptEIA ELISA set (PharMingen) was used to determine TGF-β1 concentrations after DAMGO or LPS stimulation of PBMCs. The absorbance of each well was measured by using an ELISA microplate reader at 490 nm. All the procedures were performed according to manufacturer's instruction.

Statistical analysis

Two-way-ANOVA was used for determination of statistical significance between experimental groups. Results were expressed as mean ± SEM. *P*-values <0.05 were considered statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001.

Results

Decrease of CD4⁺CD25⁺FoxP3⁺ regulatory T cells in peripheral blood of heroin users in Baduanjin group

To analyze possible effects of practicing Baduanjin exercise on the Treg cells in heroin users, we included 27 healthy control, 30 heroin patients in Baduanjin group, 30 patients in heroin control with similar age and gender (**Table 1**) in our study. We observed no significant differences in the number of PBMCs isolated from peripheral blood between the three different groups analyzed (**Figure 1A**). Interestingly, the percentage of $CD4^+CD25^+FoxP3^+$ T cells was increased in heroin control. However in Baduanjin group $CD4^+CD25^+FoxP3^+$ T cells were significantly decreased compared to heroin control ($P \leq 0.0001$), but still higher than healthy control (**Figure 1B**, $P \leq 0.0001$).

Increased proliferative activity of T cells from heroin users in Baduanjin group

It was described that heroin abuse influences T lymphocyte proliferation, we want to know whether qigong exercise can restore the T cell proliferation ability. Therefore, we isolated the T lymphocytes from the Baduanjin exercise group, control group, and normal healthy control. The T cells were stimulated with PHA at 1 μg/ml for 2 days. As control, isolated T cells from the same patient were left un-stimulated. As shown in **Figure 2**, T cells from Baduanjin exercises group showed significantly higher proliferative responses upon stimulation in vitro than T cells from heroin controls ($P \leq 0.0001$) and were comparable to T cells from normal healthy control.

Depletion of $CD4^+CD25^+$ T cells from T cells of heroin user restores their proliferative capability

Next, we asked whether the reduced proliferative activity of T cells was directly linked to the increased percentage of immune-suppressive Tregs. For this purpose, we depleted $CD4^+CD25^+$ T cells by using MACS technology with a mean purity of about 85% (**Figure 3A**), stimulated them in vitro or left them un-stimulated as control and analyzed their proliferative activity and TGF-β1 secretion. As shown in **Figure 3**, $CD4^+CD25^-$ T cells from healthy controls and Baduanjin exercisers exhibited elevated proliferative activity upon stimulation in vitro (**Figure 3B**). Strikingly, depletion of $CD25^{high}$ cells from T cells of heroin controls resulted in

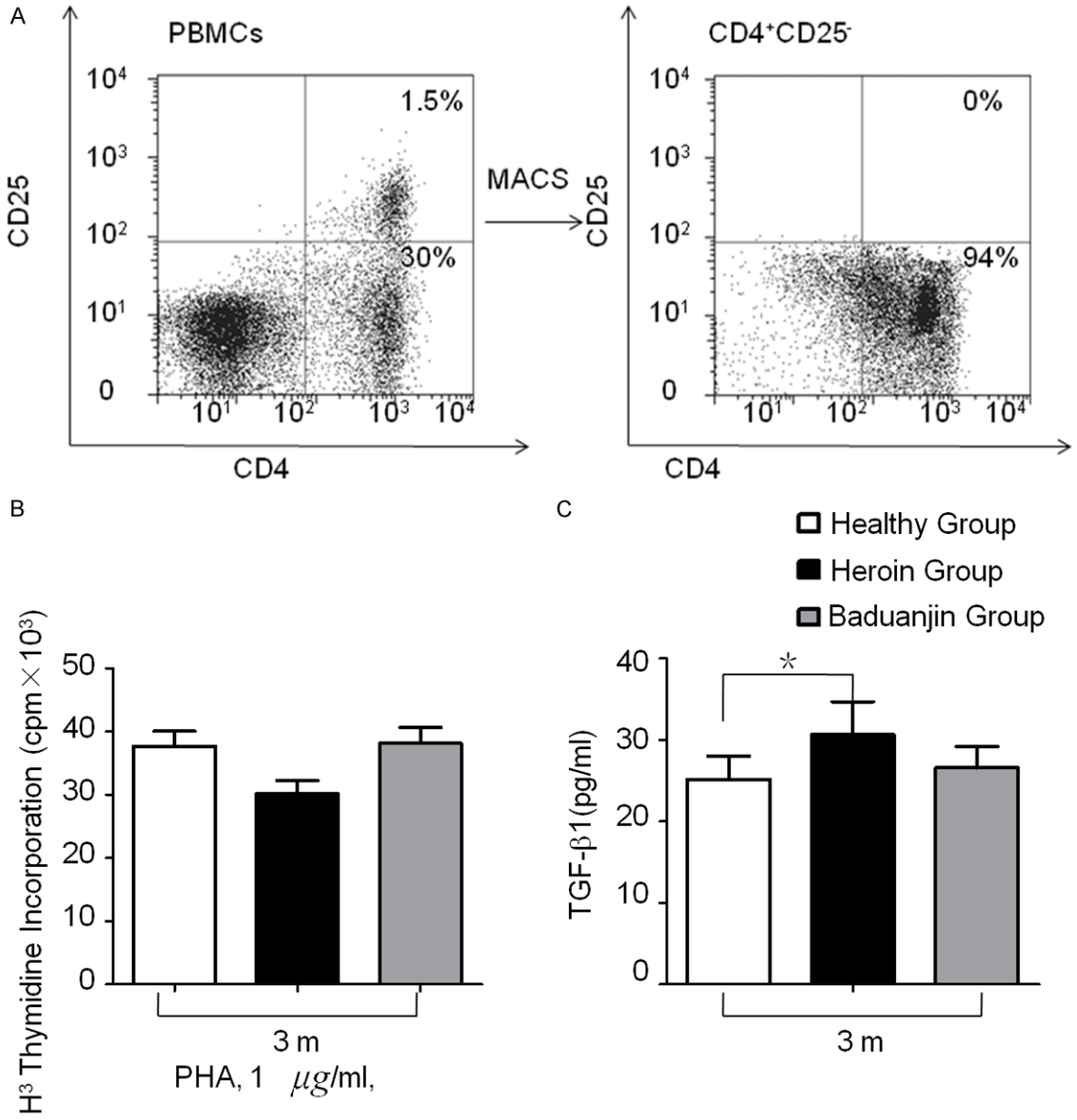

Figure 3. Similar proliferative activity of $CD25^{high}$ depleted $CD4^+$ T cells from healthy controls, heroin control and Baduanjin exerciser heroins. Five representative patients from each group were selected and their PBMCs were stained with anti-CD4 and anti-CD25 (A, left panel, representative for one experiment). $CD4^+CD25^-$ T cells were isolated from these PBMCs of healthy controls, heroin control and Baduanjin exerciser heroins by MACS technology. After separation cells were stained with anti-CD4 and anti-CD25 to verify depletion of $CD25^{high}$ cells (A, right panel, representative for one experiment). Percentages refer to gated lymphocytes. 6×10^5 isolated $CD4^+CD25^-$ T cells from healthy controls (black bars, n=5), heroin user (white bars, n=5) and Baduanjin exerciser heroins (gray bars, n=5) were stimulated with 1 μg/ml PHA for 2 days to determine proliferative activity (B) and analysis of TGF-β secretion in the cell-culture supernatants by Luminex technology (C). The percentage of proliferation was determined by gating on non-proliferated cells (mean CFSE signal of un-stimulated cells from one experiment) and calculating the percentage of proliferated cells. Values are means ± SEM. *P<0.01. One-way ANOVA was used for statistical analysis.

a significant increase in proliferative activity compared to whole T cells from heroin controls (**Figures 2** and **3B**), and nearly equal to that of the proliferative activity of healthy control and Baduanjin exercisers (**Figure 3B**). These results suggest that heroin abuse results in limited T cell function at least in part via expansion of Tregs. And Baduanjin exerciser can partly restore the T cell function. Moreover, we detected a slightly but not significantly increa-

sed TGF-β secretion of in vitro stimulated $CD4^+CD25^+$ depleted T cells from heroin user than of healthy controls or Baduanjin exerciser patients (**Figure 3C**).

Discussion

Previous studies have suggested that heroin addiction has multiple immune-suppressive effects including reduced proliferative responses of lymphocytes from the peripheral blood (Govitrapong et al, 1998), which might contribute to the increased risk for developing infectious diseases among heroin user. Moreover, several researchers have found that qigong could enhance immune functions. Many researchers [11, 12] proved that qigong could improve immunity and body health. Several systemic studies were launched for the role of qigong in improving immunity. Byeongsang [5] found qigong could improve quality of life, mood and fatigue parameters, and reduce inflammation and improve immunity in cancer patients. As a complementary and alternative modality of traditional Chinese medicine, qigong is often used in cancer patients to manage their symptom [12]. Wang searched 13 databases for the role of qigong exercise on immunity and infections and found positive results [4]. To verify the mechanism of qigong enhancing immune functions, Li ZQ and his group found that qigong practice may regulate immunity, metabolic rate, and cell death, possibly at the transcriptional level [13]. Interestingly, qigong may have therapeutic effect on heroin addicts [6]. Li M found that qigong may be an effective alternative for heroin detoxification without side effects, but the mechanism remains unknown. Therefore we hypothesis that qigong may enhance immune functions in heroin users.

Baduanjin exercise is smooth and relaxing qigong which is adaptable for each age, and Baduanjin exercise could also improve body health and immunity in different patients. Baduanjin exercise program can improve sleep quality for older adults [14]. Chen found Baduanjin exercise can promote and maintain the health status of middle-aged women by preventing their bone loss [10]. Mei found Baduanjin exercise could significantly decrease the levels of TC, TG, LDL-C levels in plasma and elevate plasma HDL-C level for the healthy people. In addition, Baduanjin exercise modulated the blood lipid metabolism for patients [15]. To access the feasibility and safety of Baduanjin in treating knee osteoarthritis, 28 patients were examined and the results showed Baduanjin exercise provided a safe and feasible treatment option for patients with knee OA, as well as offered reductions in pain, stiffness, and disability, which improved the patients' quadriceps strength and aerobic ability [16]. Hsu evaluated the effects of Baduanjin exercise on oxidative stress, antioxidant status and quality of life in middle-aged women, and found Baduanjin exercise has beneficial effects on improving quality of life, increasing antioxidant enzymes and reducing oxidative stress in middle-aged women [17]. Baduanjin exercise can improve immunity, but the underlining mechanisms remains unknown and until now there is no study was investigated on heroin addicts. In this study, Baduanjin exercise was used to analyze the immune effect on heroin users. As was reported, practicing qigong can improve the number and function of T cells, but the mechanism remains unknown. We therefore hypothesis qigong also restore heroin addicts' immune response.

Although $CD4^+CD25^+$ regulatory T cells only comprise 5%-15% of $CD4^+$ T cells, they inhibit both Th1 and Th2 response. And studies have reported that regulatory T cells play roles in cancer [18], immune deficiency disease, immune tolerance [19, 20], etc. Moreover, researchers also focus on the relationship between $CD4^+CD25^+$ regulatory T cell and heroin addicts. Liu WT [21] found impaired cellular immune function was found in heroin dependent with increased $CD4^+CD25^+FoxP3^+$ T cells and decreased $CD4^+$ T cells. The stress with the short-term withdrawal may be the cause of the autoimmune disease found in heroin users [22, 23]. Therefore, we suggested that qigong could also restore heroin addicts' immune response by decrease $CD4^+CD25^+$ regulatory T cells. To verify the hypothesis, 60 heroin addicts were recruited to analyze the relationship of $CD4^+CD25^+$ regulatory T cells and practicing Baduanjin exercise in heroin addicts.

In our studies, sixty heroin addicts were randomly divided into Baduanjin exerciser (practicing Baduanjin for 3 months) group and control group (not practicing Baduanjin). We found practicing Baduanjin could reduce $CD4^+CD25^+FoxP3^+$ T cells in peripheral blood mononuclear cells (PBMCs) in heroin addicts, with frequency was similar to normal healthy control. In Liu HT's group [21] $CD4^+CD25^+$ T

cells was increased in short-term withdrawal group compared to healthy control. Practicing Baduanjin could normalize $CD4^+CD25^+FoxP3^+$ Tregs in heroin addicts. We also found heroin could not change the whole number of PBMC but increase the proportion of Tregs (**Figure 1A** and **1B**). As was reported that impaired immune responses observed in heroin users were related to the expansion of Tregs [21].

Since Tregs can inhibit T cell response, lymphocyte proliferation was analyzed in three groups, and we found the lymphocyte proliferation was significantly decreased in heroin addicts. But practicing Baduanjin exercise can restore the lymphocyte proliferation response to normal range (**Figure 2**). Therefore, Baduanjin exercise could restore the immune function by decreasing the number of Tregs. Moreover, to further corroborate the suppressive effect of Tregs on T cell response in heroin users, lymphocyte proliferation was also analyzed after depleting $CD4^+CD25^+$ T cells. Interestingly, after depleting $CD4^+CD25^+$ T cells, lymphocyte proliferation in heroin group increased to the level similar to that in Baduanjin exercisers and healthy controls. The impaired immune function in heroin addicts was highly related to Tregs and we also found both the number and function of Tregs can be inhibited by practicing Baduanjin exercise. Thus, as an important complementary exercise, Badujian qigong can be used in heroin users without causing adverse side effects.

Interestingly, heroin addiction seems not only to modulate the frequency of peripheral Tregs, which might contribute to the immune-suppressive effect of heroin, but we also observed an intrinsic defect in $CD4^+CD25^+$ T cells. Depletion of $CD25^{high}$ Tregs from $CD4^+$ T cells of heroin user not fully restores the proliferative activity to values observed in Baduanjin exercisers and healthy controls. It was reported that heroin could inhibit immune response either directly or indirectly. A direct effect of heroin on immunity has been shown by Thomas [24], who reported that murine splenocytes treated with heroin in vitro, which exhibited decreased IL-2 production, B cell proliferation and NK-cell cytolytic activity upon stimulation.

Nevertheless, we demonstrated here for the first time that the frequencies of immune-suppressive Tregs significantly decreased after practicing Baduanjin exercise for 3 months in heroin users. $CD4^+CD25^+$ Tregs are well known key players in the maintenance of peripheral tolerance by controlling auto-reactive immune responses. However, an increase in Tregs' number and/or activity not only has unfavorable effects on tumor progression [25, 26] and the course of chronic infectious diseases [27], but also has effects on heroin addicts. It is also shown that TGF-β1 level increased slightly with the lower lymphocyte proliferation (**Figure 3B** and **3C**). It was reported that TGF-β1 was one important cytokine secreted by Tregs and has inhibitory results on T cells, B cells [28]. Our data suggested that practicing Baduanjin exercise can normalize the increase of $CD4^+CD25^+$ Tregs in heroin addicts, which in turn might contribute to the complementary treatment of invading pathogens in heroin addicted patients.

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Disclosure of conflict of interest

None.

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