



Inactivated *P. aeruginosa* restores immune imbalance of chronic idiopathic urticaria

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Abstract

The main pathology involved in chronic idiopathic urticaria (CIU) is immunological dysfunction which mainly adapts to the immune system of body. *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin (PA) is an inactivated *Pseudomonas aeruginosa* biological product which displays a broad immune regulatory effect. The current study was designed to explore the protective nature of PA as an immune regulator in CIU. The participants were randomly divided into CIU + PA, CIU, control + PA and control group. Ig E, anti FcεRI, anti IgE antibody, IL-4, IL-17, TGF-β1 and interferon-γ in the sera were assayed by ELISA. Then CD4⁺ T cells and CD19⁺ B cells were isolated from peripheral blood of patients with CIU (*n* = 10) and healthy control (*n* = 10). CD4⁺ T cells and CD19⁺ B proliferation and apoptosis were analyzed through CCK-8 and flow cytometry respectively. T helper cells differentiations were assessed by real-time PCR. The results revealed that compared with the control group, the curative effect of CIU + PA group was more effective than that of the CIU control group. There was a hyper proliferation of CD19⁺ B cells in the CIU patients. Moreover, it was also discovered that presence of Th1 decreased while Th2 cells increased in CIU patients. PA significantly inhibited the proliferation of CD19⁺ B and Th2 cells but at the same time promoted the proliferation of Th1 compared to healthy control. The conclusion arrived at from this study is that the PA displayed a remarkable regulatory effect in CD4⁺ T cells and CD19⁺ B cells function by promoting Th1 but inhibited Th2 and the hyperfunction of B cells of CIU patients.

Keywords Chronic idiopathic urticaria · Cytokines · PA-MSHA · T cells

Introduction

Chronic Idiopathic Urticaria (CIU) is defined as a skin lesion consisting of a weal that keeps swelling for more than six weeks. This localized intracutaneous edema (weal) is mostly surrounded by red patches (erythema). Itchy, red and swollen weal in CIU can occur as a result of a skin mast cell activation, local vasodilatation, and increased vascular permeability which are the cardinal features of this disease. There are various factors that trigger CIU. Over half of all the cases of chronic idiopathic hives were as a result of an autoimmune trigger [1, 2]. According to statistics at least 30% of patients

with CIU were detected with serum autoantibody and intra-dermal injection of autologous serum-induced weal and erythema reaction. The severity of urticaria is directly related to the amount of autoantibody present in the serum. Anti IgE or anti high-affinity IgE receptor antibodies can stimulate the release of histamine and blood eosinophil [3, 4].

The pathogenesis of CIU is still a mystery but the major cells it attacks are the mast cells and basophil [5, 6]. It is well-recognized that 50% of CIU is autoimmune in nature. It is caused by anti-high affinity IgE receptor antibodies (anti-FcεRI) and less frequently by anti-IgE autoantibody that lead to mast cell and basophile activation thereby giving rise to the release of histamine and other proinflammatory mediators. The activation of a classical complement pathway and formation of C5a are important in dermal mast cell activation. There is enough evidence to prove that there is anti-IgE receptor or anti-IgE antibody in CIU patient's which make skin mast cells or functional basophile degranulation. The autologous serum skin test is used as a screening test for CIU and has a sensitivity and specificity of about

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70 and 80%, respectively. In the acute stage, about 58% of the patients have IgE molecular antibodies in vivo and 25% patients have anti-IgE receptor antibody. Moreover, post-binding intracellular activation pathways following stimulation by anti-IgE and anti-IgE receptor are the main cause of the release of histamine and other inflammatory mediators regardless of very little serum IgE [6, 7]. Our studies highlighted the fact that the immune imbalance is the primary cause of CIU characterized by B cell hyperfunction induced autoantibody production regardless of the result of the autologous serum skin test [8]. Therefore, it is important to control the autoimmune basis in CIU treatment. IFN- γ is a promoting factor of Th1 cell differentiation [9]. Similarly, IL-4, IL-6 and TGF- β play crucial roles in the regulation of T cells differentiation. IL-4 promoted the differentiation of Th2 and IL-6 promoted the generation of Th17 whereas TGF- β had a direct influence in the differentiation of Treg cells [10]. T-bet, GATA-3, ROR- γ t and Foxp3 mRNA are thought to be the key regulators of Th1, Th2, Th17 and Treg cells respectively [11].

Pseudomonas aeruginosa-mannose-sensitive hemagglutinin (PA) is an inactivated *Pseudomonas aeruginosa* biological product that has been combined with mannose sensitive hemagglutination to chemically inactivate *P. aeruginosa*. It has been proved by related experiments that it has broad-spectrum immunomodulatory effect and has an obvious anti-tumor effect on tumor cells [12]. For this reason, it has been used in the adjuvant treatment of various cancers such as lung cancer and breast cancer [13, 14]. But it has not yet been reported on the study of CIU.

The current ongoing studies investigated the effects of PA on the lymphocytes activation and differentiation in CIU patients and found that PA markedly inhibited the proliferation of CD19⁺ B and Th2 cells whereas it promoted the proliferation of Th1. Similarly, CD19⁺ B cells hyperfunction was also inhibited by the PA-MSHA in CIU patients. Hence, PA-MSHA restores immune imbalance in CIU.

Materials and methods

Participants

All participants with CIU ($n=60$; 27 males and 33 female with an average age of 39.66 ± 16.4 years from 18 to 62 years) between Jan 2016 and Jan 2017 were selected from No.3 Hospital of Shandong, Jinan, China. They were randomly divided into two groups: CIU + PA ($n=30$) and CIU group ($n=30$). These patients have had to deal with weal daily or at least four times a week for 6 months or longer. Patients with physical urticaria, urticarial vasculitis, food allergies or autoimmune diseases were excluded. None of the patients took corticosteroids or immunosuppressive drugs.

Twenty healthy participants (10 males and 10 females with an average age of 40.16 ± 12.75 years, from 18–62 years) were selected. They were then randomly divided into control + PA ($n=10$) and control group ($n=10$). The presence of immune-mediated or other relevant disorders was evaluated and eliminated. All individuals signed a written informed consent, and the study protocol was approved by the institutional ethics committee of No.3 Hospital of Shandong (3SDH-2016–190).

The method of treatments

The patients in the CIU + PA group were medicated with calcium gluconate tablets (1 tablet/ time, oral 1 time a day); PA (intravenous 10^7 cfu/time /day); Vitamin D3 (intramuscular 1 time per week) while the patients in the CIU group were medicated with same drug and dosage excluding PA. All medications were administered to both groups for 4 weeks with the exact amount of dosage and on time. Liver, kidney, blood and urine routine examination were checked before and after the treatment. At the end of the treatment, the clinical curative effect was evaluated and adverse reactions were recorded. These patients were monitored for 2 months after treatment and relapses were recorded.

Curative effects assessment

The curative effects of CIU + PA were assessed according to the three features of urticaria including itching, erythema number and erythema size: (1) Itching: No, mild (does not affect the normal life and work), medium (tolerable) and severe (significantly affect the normal life and work). Patients were asked to grade the itching on a scale of 0, 1, 2, 3 respectively; (2) Erythema number: no, skin lesion < 10, 10 ~ 25, > 25 were scored as 0, 1, 2, 3, respectively; (3) Erythema size: no, diameter < 1.5 cm, 1.5 ~ 2.5 cm, > 2.5 cm were scored as 0, 1, 2, 3, respectively. Total scores were obtained according to the three items above. The symptom score reduction index (SSRI) was calculated using the following formula: (Total scores before treatment—total scores after treatment)/ total scores before treatment $\times$ 100%. Efficacy was classified on the basis of SSRI (90% or higher, cure; 60% ~ 89%, powerfully; 20 ~ 59%, effectively; < 20%, void). Total scores fell below 60% after conspicuous improvement and or relapse [15].

ELISA

Serums from different groups were collected and IgE, anti-IgE receptor, anti-IgE antibody (Rapid bio, CA, USA), IFN- γ , IL-4, IL-6 and TGF- β (Rapid bio, CA, USA) were examined with rat anti-human ELISA kits according to the manufacturer's instructions. Secretions were assayed using

ELISA kits (R&D Systems, Shanghai, China). The absorbance value was at a 450 nm read and the antibodies levels were determined according to a standard curve.

Lymphocytes cells isolation

Peripheral blood mononuclear cells (PBMCs) (10 mL) were isolated using Ficoll-Hypaque solution (Haoyang biotech, Tianjing, China), and cultured in RPMI-1640 at 37 °C in 5% CO₂ for 2 h until the monocytes adhered to the bottom of culture flasks. Then the lymphocytes suspended in middle were isolated. CD4⁺T cells were isolated using EasySep™ Human CD4⁺ T Cell Isolation Kit (STEMCELL, Shanghai, China). The B cells were then isolated using EasySep Human CD19⁺, 10e9 cells (STEMCELL, Shanghai, China). The purity was more than 90% by using flow cytometry assay and the viability was more than 90% by using Trypan blue staining. Cells were cultivated at 37 °C in RPMI 1640 medium, supplemented with penicillin–streptomycin prior to the analysis.

CCK-8 assays of cell viability

The effects of PA on the proliferation of CD4⁺ T cells and CD 19⁺ B cells were examined using cell counting kit-8 assays (CCK-8, Dingguo, Beijing, China). CD4⁺ T cells or CD 19⁺ B (1000 cells/ well) from healthy control ($n = 10$) and CIU ($n = 10$), were treated with or without 0, 0.01, 0.1, 1, 10×10^8 cfu/mL PA and then maintained at 37 °C for 24 h. PA was supplied by Wanter Bio Pharmaceutical Co., Ltd, Beijing, China (2.0×10^9 cfu/mL) [16].

Real-time polymerase chain reaction (PCR)

CD4⁺ T cells or CD 19⁺ B (10^6 cells) from healthy control ($n = 10$) and CIU ($n = 10$), were treated with or without 1×10^7 cfu /mL PA and total RNA from CD4⁺T cells of different groups were extracted using Trizol (Invitrogen, CA, USA) and a reverse transcription was carried out with a Reverse Transcriptase kit (Takara, Dalian, China). Real-time PCR was performed with the following primers: T-bet (sense 5'-GGACCCAAGTGTCAACTGC-3', anti-sense 5'-TGTCGCCACTGGAAGGA-3'); GATA-3 (sense 5'-GCCATTTCGTACATGGAAGC-3' anti-sense 5'-CGGAGGGTAAACGGACAG AG-3'); RORC- γ t

(sense 5'-GCAGCAACAGGAACAAGTGG-3', anti-sense 5'-GCTTTGCCTCGTTCTGGACT-3') and Foxp3 (sense 5'-GCAGCAACAGGAACAAGTGG-3', anti-sense 5'-GCTTTGCCTCGTTCTGGACT-3'); β -actin (sense 5'-GTGGACATCCGCAAAGAC-3', anti-sense 5'-AAAGGGTGTAACGCAACTAA-3'). PCR was carried out in a 7300 real-time PCR System (Applied Biosystems, CA, USA) using general SYBR green fluorescence detection for 10 min at 94 °C followed by 45 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative quantitative expression was calculated as $2^{-\Delta\Delta CT}$ methods.

Statistical analysis

The whole data were analyzed using SPSS 16 statistical software, and qualitative data were compared through the use of χ^2 test. The data was checked using the normality test and the homogeneity test of variance. The variance of single factor was used if the data showed a normal distribution and homogeneity of variance analysis. On the other hand, Wilcoxon rank-sum test was used if the data was not consistent with normal distribution and homogeneity of variance test. *P* values less than 0.05 were considered significant.

Results

PA promoted curative effects

After treatment with the combination of PA, the curative effects significantly improved. The effectiveness (%) was 90% in the experimental group, but only 60% in the control group ($p = 0.002$, Table 1). There were no serious adverse reactions. Both groups of patients experienced general adverse reactions such as drowsiness, dizziness, dry mouth, nausea. Five cases (16.7%) in the experimental group and 10 cases (33.3%) in the control group showed adverse reactions but returned to normal when they quit taking the medication. The incidence of the adverse events had statistical significance between the two groups ($p = 0.021$) (Table 1). Moreover, as shown in Table 1, the recurrence rate was 16.7% (5 cases) in the treatment group and 20.0% (15 cases) in the

Table 1 Evaluation of curative effects

Groups	Cure	Powerful	Effective	Void	Effectiveness (%)	Adverse reaction (%)	Relapse (%)
CIU Treatment group ($n = 30$)	19	4	4	3	90.0	16.7	16.7
CIU control group ($n = 30$)	10	2	6	12	60.0	33.3	50.0

control group after 4 weeks of withdrawal ($p=0.001$). The difference was statistically significant.

PA inhibited CD19⁺B cells function

Next, the Ig E, anti FcεRI and anti IgE antibody in the serum from 4 groups were carefully examined. The results revealed that the Ig E, anti FcεRI and anti IgE antibody in the CIU group were significantly higher than those in the healthy control group ($p<0.01$ respectively), and the level of IgE was lower than that of the healthy control group ($p<0.05$). The PA (10^7 cfu/mL) treatment significantly decreased anti FcεRI and anti IgE antibodies in CIU patients but it had no effect on IgE level (Table 2). PA had no effect on CD19⁺ B cells secretion from healthy control.

Effects of PA on cytokine secretion from CD4⁺T cells

Then the modulatory effect of PA on cytokine secretion was evaluated (Fig. 1). The IL-4 increased while IFN-γ was

decreased significantly in CIU patients when compared with those in healthy control. The increased IL-4 and decreased IFN-γ were abrogated through a PA-MSHA treatment. These results showed that PA is able to inhibit IL-4 and promote IFN-γ secretion through CD4⁺T cells. Besides, the TGF-β was increased significantly in CIU patients when compared with healthy control.

Immunomodulatory effects of PA on the lymphoproliferative response

Next, we isolated CD4⁺ T Cell and CD19⁺ B cells from 4 groups including control ($n=10$) and CIU ($n=10$) and observed the effect of different concentrations of PA (0, 0.01, 0.1, 1, 10×10^8 cfu/mL) on lymphocyte proliferation. The results showed that CD4⁺ T cells had no significant differences among the 4 groups and PA promoted the proliferation of CD4⁺ T Cell ($p<0.05$) from 1×10^7 cfu/mL. However, CD19⁺ B cells significantly increased in CIU group, and PA inhibited the proliferation of CD19⁺ B cells of patients of CIU

Table 2 Anti-FcεRI, anti-IgE antibody and Ig E in CD19⁺ cells

Groups	IgE	Anti-FcεRI antibody	Anti-IgE antibody
Control ($n=10$)	85.32 ± 6.33	2.04 ± 0.57	50.32 ± 5.87
CIU ($n=30$)	$44.14 \pm 6.52^{**}$	$6.88 \pm 0.94^{**}$	$104.63 \pm 18.46^{**}$
Control + PA ($n=10$)	82.45 ± 5.87	1.87 ± 0.78	$44.37.3 \pm 4.09$
CIU + PA ($n=30$)	55.36 ± 5.18	$1.63 \pm 0.55^{##}$	$48.95 \pm 3.95^{##}$

Samples are from 20 healthy controls and 60 CIU patients $^{**}p<0.01$ vs. control and $^{##}p<0.01$ vs. CIU

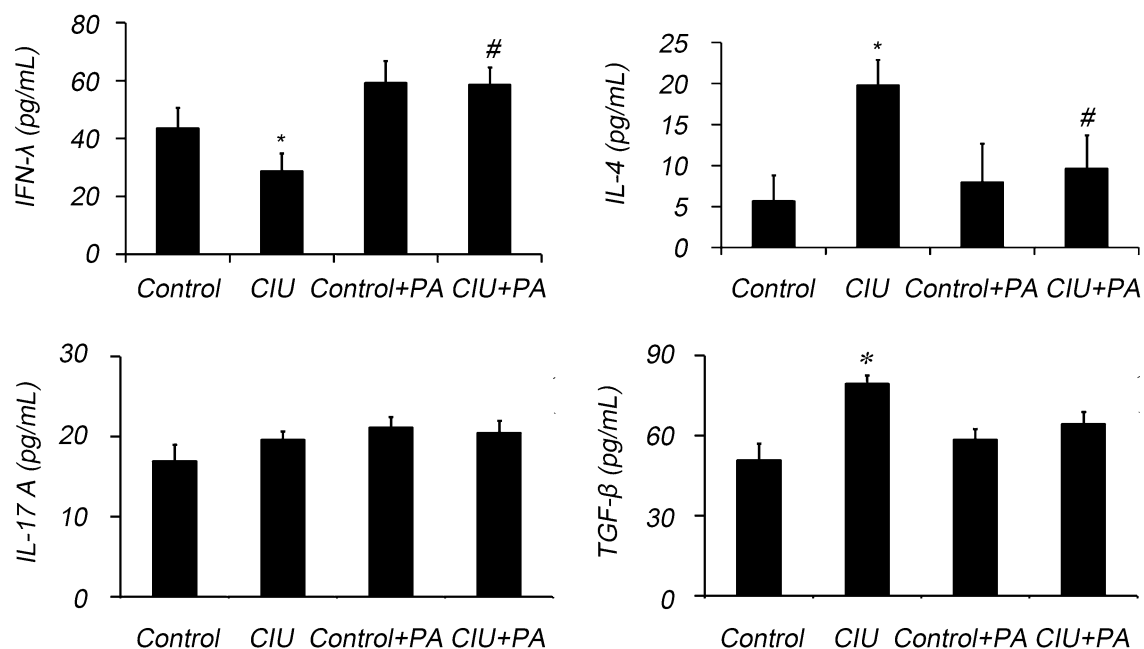


Fig. 1 Effects of PA on cytokine secretion in sera from 20 healthy controls and 60 CIU patients were assayed by ELISA. $^{*}p<0.05$ vs. control and $^{#}p<0.05$ vs. CIU

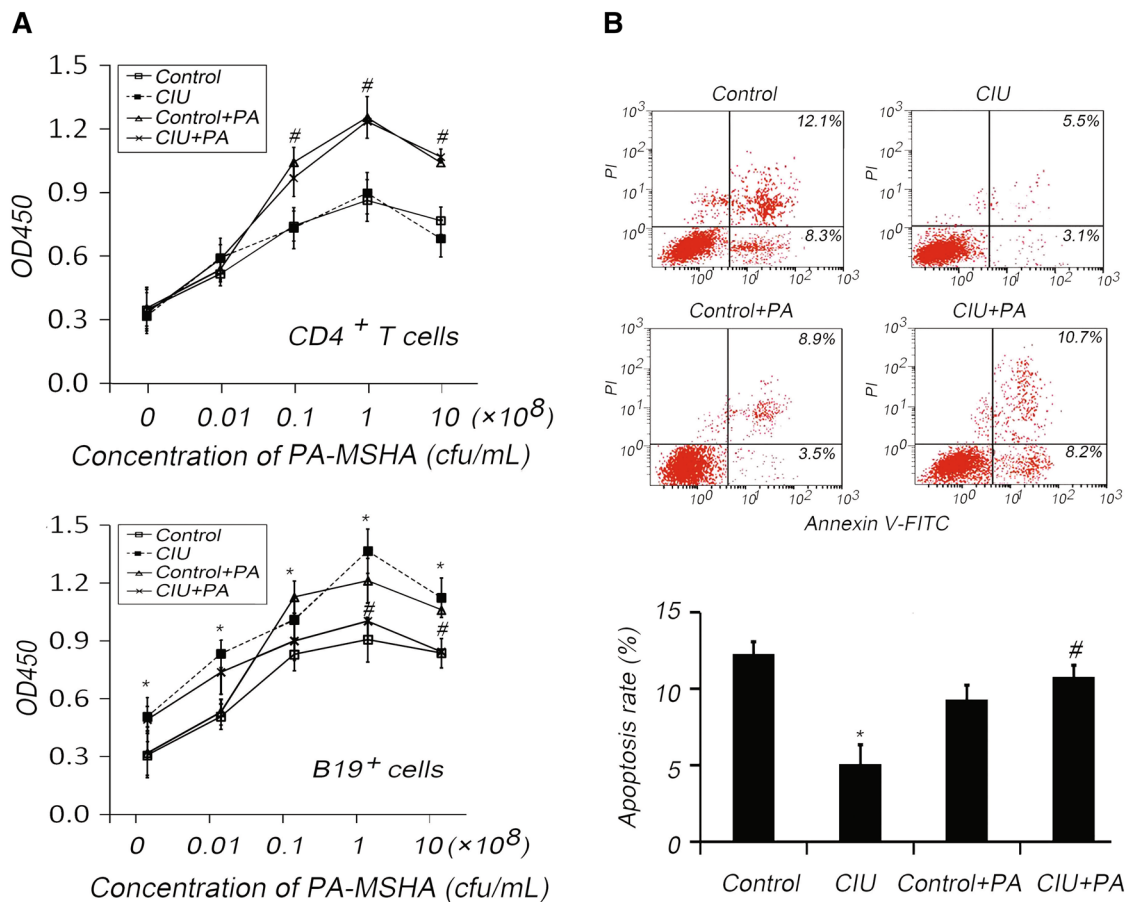


Fig. 2 Immunomodulatory effect of PA on the lymphoproliferative response was assayed by CCK-8 assay. CD4⁺ T cells or CD 19⁺ B cells were from healthy control ($n=10$) and CIU ($n=10$) and treated with or without 0, 0.01, 0.1, 1, 10×10^8 cfu/mL PA-MSHA, and

then maintained at 37 °C for 24 h. **a** Effect of PA on the proliferative response of CD4⁺ T cells or CD19⁺ B cells. * $p < 0.05$ vs. control and # $p < 0.05$ vs. CIU. **b** PA induced CD19⁺ cells apoptosis in CIU patients. * $p < 0.05$ vs. control and # $p < 0.05$ vs. CIU

(Fig. 2a). We then evaluated whether the marked inhibition of proliferation of CD19⁺ B cells was due to an increase in cellular apoptosis. The results showed that PA (10^7 cfu/mL) were able to induce apoptosis of CD19⁺ B cells in CIU group (Fig. 2b).

Effects of PA on the expression of transcription factors in CD4⁺T cells

T-bet, GATA-3, ROR- γ t and Foxp3 mRNA are thought to be the key regulators of Th1, Th2, Th17 and Treg cells respectively. Next, we examined the expression of transcription factors using real-time PCR. The results showed that the

expression levels of GATA-3 increased while T-bet decreased in CIU patients. The dysfunction of Th cells was reversed by PA (10^7 cfu/mL). Besides, PA promoted the expression of T-bet mRNA from the healthy control group (Fig. 3).

Discussion

CIU is defined as itchy, red and swollen weal on the skin. This condition can persist in a patient daily for more than six months. It is autoimmune in nature. It is caused by anti-Fc ϵ RI and less frequently by anti-IgE autoantibody that lead to mast cell and basophile activation. This gives

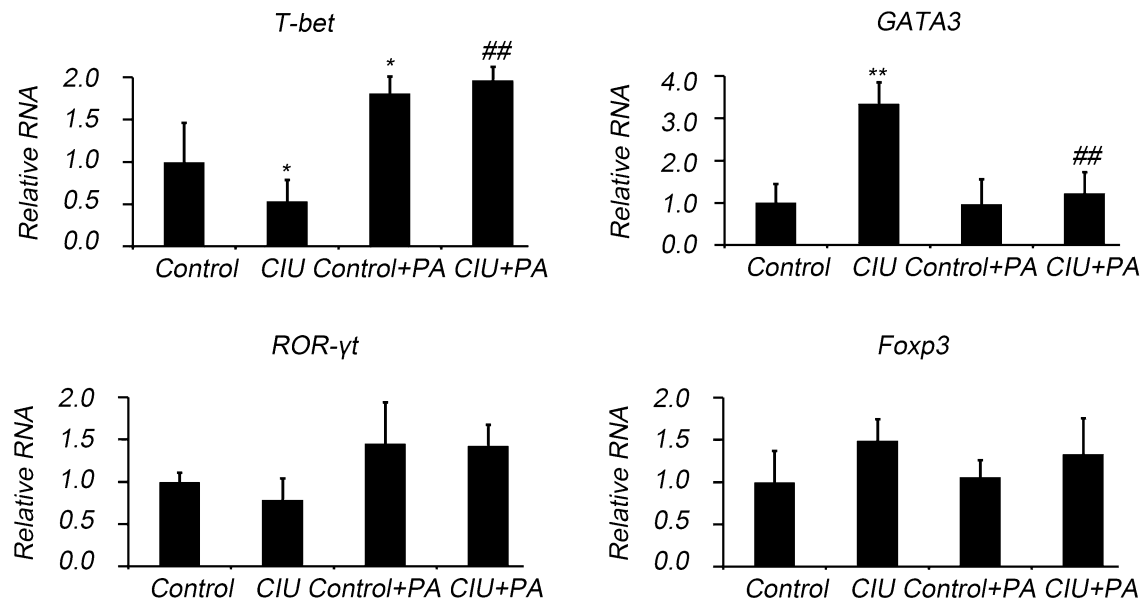


Fig. 3 Effects of PA on transcription factors expression in CD4⁺T cells were assayed by real-time qPCR. CD4⁺ T cells (10^6 cells) were from healthy control ($n=10$) and CIU ($n=10$) and treated with or

without 1×10^7 cfu /mL PA and total RNA from CD4⁺T cells of different groups. * $p < 0.05$ and ** $p < 0.01$ vs. control and ## $p < 0.01$ vs. CIU

rise to the release of histamine and other proinflammatory mediators. Currently, the treatment of CIU includes corticosteroids, cyclophosphamide, methotrexate, cyclosporine and other immunosuppressive agents but these drugs are not applicable to all patients due to its severe side effects. A new biological agent called monoclonal antibody (Omalizumab) was discovered to treat CIU but it also caused serious side effects even allergic shock in some patients [17]. Therefore, it has become crucial to understand the pathogenesis of CIU and find a more specific treatment. Although immune dysregulation is generally attributed to the cause of CIU, there is no clear evidence to date linking it to the unbalanced immunoregulatory or proinflammatory factors for the occurrence of this disease. Therefore, in the present study we aimed to prove the effects of PA on proliferation and apoptosis, Th1, Th2, Th17 and Treg differentiation and B cells function.

There was an obvious immune imbalance mainly T in helper cells subgroup imbalance as well as B cell in CIU individuals. However, many studies on T helper cells immune imbalance are contradictory. Kay reported that there were elevations in Th2 initiating cytokines such as interleukin-33, interleukin-25 in CIU patients [18]. Interleukin 33 is a member of the IL-1 family that potently drives production of Th2-associated cytokines. Interleukin-25 is secreted by Th2 and mast cells. However, Gao reported that IFN- γ receptor and interleukin-10 receptor were statistically increased while IL-4R showed no significant differences between the CIU and control [19]. Chen reported that there is a defective function of Treg cells in CIU patients [20]. As far as this study is concerned,

our findings showed that PA-induced marked promotion on the proliferation of CD4⁺ T cells both in control and CIU patients. However, it had a marked inhibition of CD19⁺ B cells which is mediated by apoptosis in CIU patients when compared with control. Furthermore, the IL-4 was increased while IFN- γ was decreased significantly in CIU patients when compared with healthy control. Similarly, PA-MSHA effectively promoted IFN- γ secretion and inhibited IL-4 secretion. Moreover, the expression levels of GATA-3 increased while T-bet decreased in CIU patients after the administration of PA. PA promoted Th1 differentiation and inhibited Th2 differentiation highlighting that PA could have immunoregulatory effects.

A variety of antibodies are involved in the pathogenesis of CIU but the main pathogenic antibodies are anti Fc ϵ RI (the high-affinity IgE receptor) and anti IgE antibodies [21]. Various previous studies revealed that the pathogenesis of anti Fc ϵ RI antibodies and anti IgE antibodies depend on the level of IgE in the body. When the level of IgE in a serum is lower than anti Fc ϵ RI antibodies or anti IgE which could not be neutralized by IgE in the blood binds with IgE on the mast cell membrane and activate the intracellular tyrosine kinase pathway to release histamine [22]. In the present study, we discovered that the anti Fc ϵ RI and anti IgE antibody in the CIU group were significantly higher whereas the level of IgE was lower than those in the healthy control group. The PA treatment significantly decreased anti-Fc ϵ RI and anti IgE antibodies in CIU patients while it had no influence on IgE level indicating that it was effective in regulating B cell hyper function in CIU patients.

Conclusion

The immune dysregulation may play an important role in the pathogenesis of CIU. PA exerts a broad immune regulatory effect on T cell and B cell functions such as proliferative responsiveness and cytokine production, disclosing a role for pharmacological agents particularly related to CIU patients. Thus, from this study it was concluded that PA restores immune imbalance in CIU.

Author contributions All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; XJ, SC, ZX and WA conducted the experiment. SC wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no conflict of interest.

Ethics approval The study was approved by the Institutional Ethic Committee of No.3 Hospital of Shandong (3SDH-2016-190).

Informed consent Informed consent was obtained from all individual participants included in the study.

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