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Thymic Stromal Lymphopoietin promoted CD4$^+$CD25$^-$ T cells differentiation isolated from the thymus of patients with Myasthenia Gravis to CD4$^+$CD25$^+$ Regulatory T Cells *in vitro*

Running Title: Correlation of Intestinal Flora and Myasthenia Gravis

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Abstract: Thymic stromal lymphopoietin (TSLP) is a cytokine and is closely related to Interleukin (IL) - 7, and hTSLP can activation through the human thymus dendritic cell in thymic to indirectly promote the differentiation of natural Regulatory T cells (Tregs) of the thymus. In this study, we focused on recombinant TSLP to determine its effects on the differentiation of CD4+CD25-T cells separated from the thymus of myasthenia gravis (MG) patients. Our results demonstrated that exogenous TSLP could increase CD4+CD25+T/CD4+T cells ratio, up-regulate the expression of Foxp3 mRNA and protein expression in CD4+CD25+Treg cells. Furthermore, we found that CD4+CD25+ Treg cells induced by exogenous TSLP could secrete IL - 10, Transforming growth factor (TGF) - β and the ability to inhibit CD4+T cell proliferation improved. These results indicate that TSLP may promote the differentiation of thymic CD4+CD25-T cells of MG patient to CD4+CD25+Foxp3+ regulatory T cells and enhance the function of immune suppression.

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Keywords: myasthenia gravis; thymic stromal lymphopoietin; regulatory T cells; TGF- β; IL-10

1 INTRODUCTION

Myasthenia Gravis (MG) is an acquired autoimmune syndrome in which pathogenic autoantibodies attack vital proteins at the neuromuscular junction, most often the muscle nicotinic acetylcholine receptor (AChR)--disrupting synaptic transmission. Usually, the result is severe muscle weakness, fatigability, and disability in patients, which may be life threatening[1,2]. The precise origin of the autoimmune response in MG is unknown, but abnormalities of the thymus gland (hyperplasia or neoplasia) almost certainly play a role in patients with anti-AChR antibodies, and genetic predispositions also likely to influence which patients develop the disorder. High-affinity, anti-AChR antibodies bind to the neuromuscular junction, activate complement, and accelerate AChR destruction while causing failure of neuromuscular transmission and the resulting myasthenic symptoms. However, autoreactive AChR specific CD4+ T cells, which can be detected in most MG patients, likely play an essential role in MG, through cognate interactions with B cells lead to the synthesis of anti-AChR antibodies[3-5].

Immune tolerance to self-antigens is initially achieved during thymic development by the clonal deletion of potentially autoreactive T cells[6,7]. However, some of these pathogenic cells, including some with reactivity to skeletal muscle AChR, survive clonal deletion in healthy individuals and are kept in check by peripheral tolerance mechanisms, most notably by a specialised subset of CD4+ T cells called regulatory T cells. Treg cells are a subpopulation of T cells that act to suppress activation of other immune cells and thereby maintain immune system homeostasis and self-tolerance. Current theories regarding the pathogenesis of autoimmune disease hypothesise that a functional deficiency in Treg cells could fail to suppress autoreactive T cell responses[8-9, 11].

Thymic abnormalities associated with myasthenia gravis, ten percent of patients with myasthenia gravis have a thymic tumour and 70% have hyperplastic changes, but the nature of

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the association is uncertain[12-14]. Thymic stromal lymphopoietin (TSLP) is a cytokine and is closely related to interleukin-7, secreted from thymic epithelial cells. Preliminary studies have shown that TSLP can activate CD11c+ dendritic cells (DC) in normal thymus, to indirectly promote the differentiation of thymus Treg cells[15-17]. And we have found that the expression of TSLP and the level of Treg cells in the removed thymus of the MG patients are decreased[18].

In this study, CD4+CD25- T cells and CD11c+ DC successfully separated from the removed thymus of MG patients. Recombinant hTSLP performed the induction of cell differentiation in vitro, the expression of Foxp3, the secretion of IL-10 and TGF-β and the immunological function of the induced cells tested. Which to evaluate whether the TSLP can promote the differentiation of thymic CD4+CD25-T cells of MG patient to CD4+CD25+Foxp3+ regulatory T cells, and improve the function of immune suppression.

2 MATERIALS AND METHODS

2.1 DC Cells and CD4+ CD25-T cells separation and viability Determination

12 Patients have diagnosed MG with thymoma, from July 2012 to December 2014 in Taihe Hospital, Affiliated Hubei University of Medicine, five males and seven females. They had not taken immunosuppressive drugs before thymectomy and excluded other autoimmune diseases and new Infectious disease, and no family history of autoimmune diseases. The research program authorised by the ethics committee of Taihe Hospital and signed the informed consent by patients.

Collected the MG patients thymic tissue, mechanically disrupted, filtered with a metal mesh, gradient centrifuged. The mononuclear cells were prepared, suspended these cells with PBS. Then purification was done through separation of the CD4+CD25- Treg cells and CD11c+ DC with sorting beads kit (Germany Miltenyi Biotec Inc.) respectively. The viability of the sorted cells was tested by trypan blue. And the viability was determined by counting 200 cells in the microscope. In our research, the cell viability was excellent and met the needs of the next experiments.

2.2 DCs activation and CD4+CD25-T Cells induction with TSLP

The separated DCs seeded in 96-well plates, each well 100uL, added different concentrations of recombinant hTSLP (ProSpec-Tany Co.) and PBS control group, incubated at 37°C in a humidified 5% CO2 incubator for 24 hours, and collected these DCs (TSLP activated DC). Then added the activated DCs into the separated CD4+CD25-T cells in 24-well plates, incubated at 37°C, 5% CO2 for seven days. Cells and the culture supernatants collected for the next test.

2.3 Tested the percentage of CD4 + CD25+Treg / CD4+T by Flow cytometry

The cultured cells collected by centrifugation and washed three times with PBS adjusted the cell concentration to10^6 / mL. Took 100uL cell suspension, added CD4-FITC antibody and CD25-PE antibody 1uL respectively (Purchased from Beckman Co.), incubated at 4°C in the dark box for 30 min, and washed three times with the buffer. Then the labelled cell suspension was tested by flow cytometry, CD4+CD25+ / CD4+ percentage calculated, which represent the expression levels of CD4+CD25+Treg cells.

2.4 Detection of Foxp3 mRNA expression by RT-PCR and western blotting

Trizol (Takara Co.) was added to each group cells, incubated for 5 min at room temperature, vigorously mixed with 100 ml chloroform and centrifuged to separate the aqueous phase from the organic phase. Isopropanol added to the aqueous phase (1: 1), and samples incubated at -20°C for two hours and centrifuged to precipitate the RNA. The precipitate was washed with 75% ethanol and dissolved in diethylpyrocarbonate-treated double-distilled water. First-strand cDNA was synthesised via reverse transcription using total RNA Transcript II reverse transcriptase. RT-PCR was carried out following the specific operating instructions (Takara Co.), and the housekeeping gene β-actin used as the internal standard. All primers synthesised by Huda Gene Ltd (Beijing, China), and their sequences are as follows: β-actin sense 5'-CCTTGGTAG TGGATAATGGGTC -3’, and antisense 5’- CATAACGCCCTGGTGTCG-3’. Foxp3 sense 5’-TGACCAAGGCTTCATCTGTG-3’, and antisense 5’-GAACTCTGGGAATGTGCTGTT-3’.

After collecting each group cells, the cells were lysed with cell lysis buffer and extracted total protein Protein concentration was measured using the Bradford method. Protein resolved via SDS-PAGE and then transferred to a nitrocellulose membrane, blocked with 5% BSA at room temperature for 1h, and incubated with Foxp3 antibodies (SIGMA Co.) overnight at 4°C, washed the membrane with TBST. The membranes were incubated with the appropriate HRP-conjugated goat anti-rabbit IgG at room temperature for one hour, followed by detection with ECL (PerkinElmer Co) and X-ray film.

2.5 Measurement of IL-10 and TGF-β cytokine levels

The supernatants of each group cells were collected, and stored at -80°C. ELISA measured the level of IL-10 and TGF-β in the supernatant following the operating instructions (see table 1 and table 2).

2.6 Immunosuppressive function of the Treg cells by flow cytometry

CD4+T in the healthy control group was isolated, and 1 ml of the culture medium without FBS was re-suspended. After adding five μl/ml carboxyl fluorescein succinimide ester (CFSE) (Biyuntian, China), 37 ℃ avoid light incubation for 10 min, 1 ml of FBS (Gibco, USA) was added to stop the reaction for 10 min. Then, washed twice with PBS and resuspended in complete medium (RPMI1640 medium + 10% FBS + penicillin, streptomycin). CD3, CD28, IL-2 (R&D Systems, USA), and CD4+CD25+Treg added to with the CD4+CD25+T: CD4+T = 1:1 cells for 4 days, and washed with PBS for 2 times. Then they were detected by flow cytometry (With the proliferation of CD4+T cells, decrease in the average fluorescence intensity, so the average fluorescence intensity is directly proportional to the proliferation inhibition of Treg).

2.7 Statistical analysis

Statistical analysis results are presented as the mean±s.d. of triplicate samples. Statistical testing was conducted using the Student's t-test or ANOVA followed by Tukey's test using SPSS13.0 statistical software. Protection results were evaluated using one-way ANOVA followed by Dunnett's test. For all tests, $P<0.05$ was considered statistically significant.

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3 RESULTS

3.1 TSLP can induce CD4+CD25+Treg Cell production, increase CD4+CD25+Treg Cells/CD4+T cells ratio.

DCs were cultured in different TSLP concentrations (0.1, 1, 10, 20 ng / mL) for 24 h, next the TSLP-induced DCs were co-cultured with the CD4 + CD25-T cell separating from the thymus of myasthenia gravis patients for 7d. The FCM results found that the ratio of CD4 + CD25 + Treg cells / CD4 + T cells in 10 ng / mL TSLP group (9.79 ±1.73) %, 20 ng / mL TSLP group (10.80 ±1.39) %, increased significantly ($p<0.05$). And was no significant difference among the PBS control group (3.01 ± 0.61) %, 0.1 ng / mL TSLP group (3.00 ± 0.56) % and 1 ng / mL TSLP Group (3.01±0.52) % ($p>0.05$).

3.2 TSLP can upregulate Foxp3 mRNA Expression in the CD4+CD25+Treg cells

The level of Foxp3 mRNA expression has calculated the ratio of Foxp3 and β–actin. The study suggested that the expression was rare in PBS control group (1.31 ± 0.02), 0.1 ng / mL TSLP group (1.48 ±0.12), and 1 ng / mL TSLP group (1.66±0.11), there was no significant difference among these groups ($p> 0.05$). But the expression in 10 ng / mL TSLP group (4.48 ± 0.33) and 20 ng / mL TSLP group for (6.41± 0.56) were higher than that in PBS control group significantly ($p<0.01$). see the Figure 1.

3.3 TSLP can increase Foxp3 protein expression in CD4+CD25+Treg cell

Foxp3 protein Expression in PBS control cells were in a very low level, relative quantification was (0.290±0.006). Compared with the PBS control group, the expression in 10 ng / mL TSLP group (0.610 ± 0.003), 20 ng / mL TSLP group (0.680 ± 0.002) were increased significantly ($p<0.01$), but there was no statistically difference among 0.1 ng / mL TSLP group (0.330 ± 0.006), 1 ng / mL TSLP group Foxp3 protein expression Levels were (0.390 ± 0.003) and PBS control group ($p> 0.05$). See the Figure 2.

3.4 TSLP can promote discretion IL-10, TGF-β in CD4+CD25+Treg cell

IL-10, TGF-β content of each group supernatant were detected the using ELISA method. The

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result suggested that IL-10, TGF-β content of 10 ng / mL group, 20 ng / mL group increased significantly (P <0.01), while 1 ng / mL group, 0.1 ng / mL group and the control group had no significant difference (P> 0.05).

3.5 TSLP can improve the ability of the induced CD4+CD25+Treg cells to inhibit T cell proliferation.

The inhibitory to T cell proliferation observed Using ***flow cytometry***. The results found that the average fluorescence intensity of control group was 15.36 ±1.13 (picture A), 10 ng / mL TSLP absorbance group was 19.69±1.19 (picture B), 20 ng / mL group was 20.23±0.89 (picture C), compared with the control group, with a significant difference (P <0.05), and 0.1 ng / mL group was 16.53±1.65 (picture D), 1 ng / mL group was 16.65±0.96 (picture E), and the control group had no significant difference (P> 0.05). See the Figure 3(A-E).

4 DISCUSSIONS

Regulatory T cells (Tregs) play an important role in maintaining immune homeostasis. Tregs suppress the function of CD4+ T cells to limit the immune response[19-21]. Alterations in the number and function of Tregs has involved in several autoimmune diseases including multiple sclerosis, active rheumatoid arthritis, type 1 diabetes, systemic lupus erythematosus, inflammatory bowel disease, and sepsis, some of which are antibody-dependent autoimmune disorders[8,22-27]. In human, two major classes of Tregs have been identified to date: CD4 and CD8 Tregs. CD4 Tregs consist of two types, natural Tregs (nTregs) that constitutively and inducible Tregs (iTregs). Natural Tregs originate from the thymus as CD4+ cells expressing high levels of CD25 together with the transcription factor FoxP3. nTregs represent approximately 5-10% of the total CD4+T cell population. Inducible Tregs originate from the thymus as single- positive CD4 cells. They differentiate into CD25 and FoxP3 expressing Tregs (iTregs) following adequate antigenic stimulation in the presence of cognate antigen and specific immunoregulatory cytokines such as TGF-β, IL-10, and IL-4[20,29,30]. Functional defects in Treg cells and a decreased expression of Foxp3, a key transcription factor in the development and function of Treg, have also been demonstrated in the thymus of MG patients, and some reports also describe lower numbers of CD4+CD25+ GITR+ T regulatory cells in PBL of MG

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patients[10]. It also reported that after thymectomy or effective immunosuppression in MG patients, $CD4^+$ $CD25^{high}$ cell numbers increased and reached normal or elevated values compared to healthy controls. Our study has found that MG peripheral blood CD4+ CD25+ Foxp3+ Treg / CD4+ T cell ratio was significantly lower than the control group, the immune depression function of CD4 + CD25 + Treg flawed[9, 11,18]. These observations have triggered the attempts to target Treg cells for the treatment of MG.

Thymic stromal lymphopoietin (TSLP) supports the growth and differentiation of B cells and the proliferation of T cells. The TLSP receptor (TLSPR) is heterodimeric, consisting of the IL-7R–α chain and a standard γ receptor–like a chain (TSLPR-γ). Human TSLP and TSLPR were cloned in 2001 by computational analyses of human genomic data[31-34]. Yong-Jun Liu found that hTSLP instead potently activated immature CD11c+ myeloid dendritic cells (mDCs). TSLP-activated DCs induced robust proliferation of naive allogeneic CD4+ T cells[35-36]. In vivo, TSLP was shown to be highly expressed by keratinocytes in atopic dermatitis lesions, and its expression was associated with the migration and activation of Langerhans cells, suggesting for the first time that TSLP might be an early trigger for DC-mediated allergic inflammation. Human TSLP was later found to be expressed by epithelial cells in peripheral mucosal-associated lymphoid tissue, where it activates mDCs to induce homeostatic proliferation of naive and memory CD4+ T cells in the periphery[37-40]. Hassall's corpuscles also produce TSLP in the human thymus, where it instructs thymic DCs to convert high-affinity self-reactive T cells into CD4+CD25+Foxp3+ regulatory T cells[41]. Our studies have shown that there are a lot of TSLP expression in Hassall’s corpuscles, and the levels decreased naturally in MG patients with thymoma. The low expression of TSLP is positively related to phenotype deficiency of the CD4+ CD25+ FoxP3+ during Treg cell growth[18].Thymic microenvironment change may be involved in the pathogenesis of MG. It found that the TSLP expression decreased in patients with thymoma or hyperplasia, but can TSLP induce Treg cells in MG patients with thymoma or hyperplasia? Liev et al[42] found that TSLP produced by intestinal epithelial cells was involved in tolerogenic DC generation. The differentiation of Foxp3 + Treg cells induced by DC was decreased from 16.4% to 12.5% and could recover by supplying exogenous TSLP. In this study, we focused on recombinant TSLP to determine its effects on the differentiation of CD4+CD25-

T cells separated from the thymus of MG patients. Our results demonstrated that exogenous TSLP could increase CD4+CD25+T/CD4+T cells ratio, upregulate the expression of Foxp3 mRNA and protein expression in CD4+CD25+Treg cells. Furthermore, we found that CD4+CD25+ Treg cells induced by exogenous TSLP could secrete IL-10, TGF-β and the ability to inhibit CD4+T cell proliferation improved. These results indicate that TSLP may promote the differentiation of thymic CD4+CD25-T cells of MG patient to CD4+CD25+Foxp3+ regulatory T cells, and enhance the function of immune suppression.

Disclosure of Conflicts of Interests

None.

Author Contributions

JY, NW, MRK, QS, YS: protocol development, sample collection, performing research, analysis and writing, manuscript review. LZ, HYZ, YFW: protocol development, manuscript review.

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FIGURES:

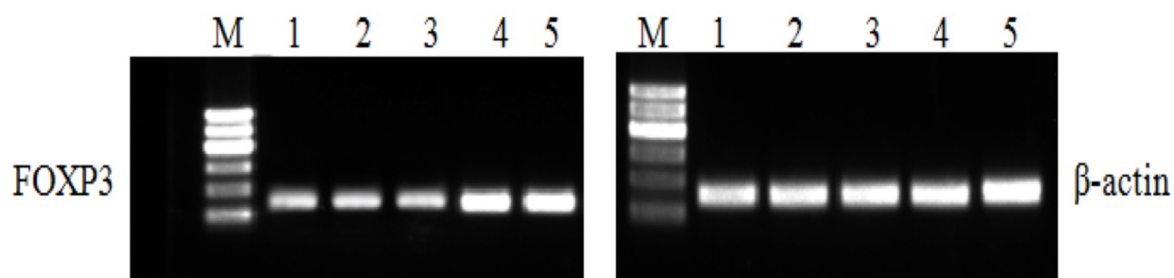

Fig 1. (n=12) RT-PCR for the Foxp3 mRNA of every group

M， Marker I； 1 to 5 is： control group、0.1 ng/mL TSLP group、1 ng/mL TSLP group、10 ng/mL TSLP group、20 ng/mL group

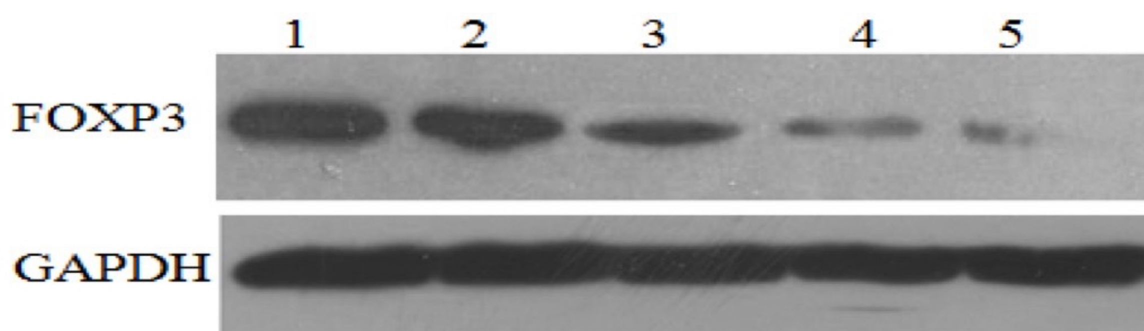

Fig2. (n=12) Western-Blot for the Foxp3 protein expression of every group cells

1 to 5 is： 20 ng/mL TSLP group、10 ng/mL TSLP group、

1 ng/mL TSLP group、0.1 ng/mL TSLP group、control group

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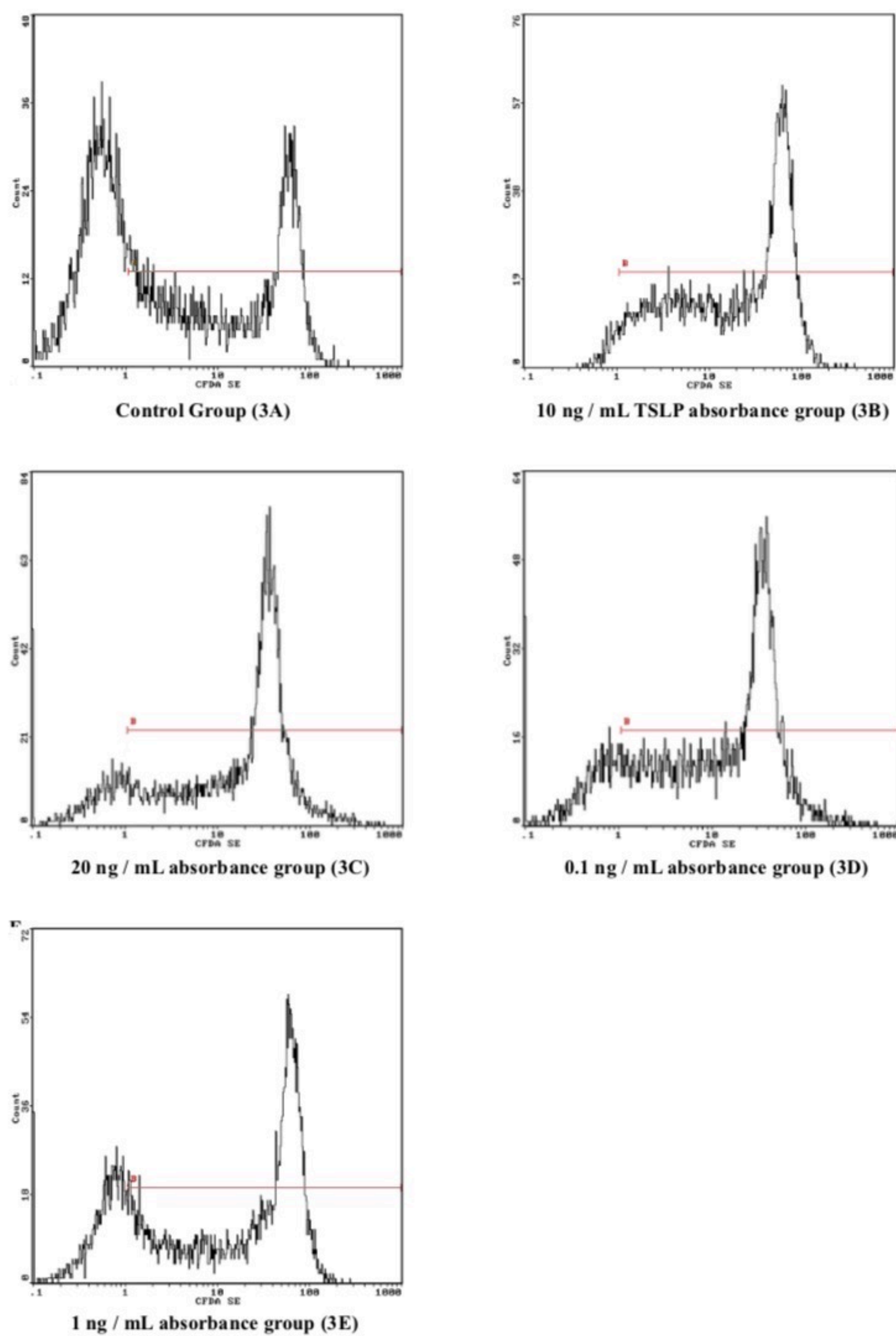

Fig 3 (A-E). The inhibition of Treg cells on the proliferation of normal $CD4^{+}CD25^{-}$ T cells

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TABLES:

Table 1: TGF-β of co-culture supernatant in each group (n=14)

	Control group	TSLP group			
		0.1 ng/mL	1 ng/mL	10 ng/mL	20 ng/mL
TGF-β（pg/mL）	134.57±8.34	136.70±11.92	136.16±9.18	392.34±38.01	395.80±37.08
F	228.54				
P		0.874	0.906	0.000	0.000

Table 2: IL-10 of co-culture supernatant in each group (n=14)

	Control group	TSLP group			
		0.1 ng/mL	1 ng/mL	10 ng/mL	20 ng/mL
IL-10（pg/mL）	21.11±5.87	21.92±4.62	22.67±5.16	58.89±15.84	62.07±15.75
F	42.66				
P		0.860	0.736	0.000	0.000