Immunomodulatory effects of polysaccharopeptide in immunosuppressed mice induced by cyclophosphamide

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Received November 23, 2012; Accepted May 31, 2013

DOI: 10.3892/mmr.2013.1542

Abstract. Polysaccharopeptide (PSP) is well known for its immunoregulatory effects. In the present study, the effect of PSP on white blood cell (WBC) count, T lymphocyte subsets, B lymphocytes, Th1/Th2 balance and negative immune regulators was investigated using an immunosuppressed mouse model. The results demonstrated that the WBC count and the absolute number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells in the peripheral blood were increased in PSP-treated groups as compared with the cyclophosphamide (Cy) group. In addition, PSP reduced interleukin (IL)-4 and GATA binding protein 3 (GATA-3) mRNA relative expression levels and elevated the ratios of IL-2/IL-4 and the transcription factors, T-box-containing protein/GATA-3. The relative mRNA expression levels of the forkhead/winged-helix transcription factor box protein 3 (Foxp3), programmed death-1 (PD-1) and IL-10 were also downregulated by PSP. These observations indicate that the immunoregulatory effects of PSP are associated with restoration of WBC number, improving the absolute number of T lymphocyte subsets and B lymphocytes, inducing the Th1/Th2 response and downregulating the negative immune regulators, Foxp3, PD-1 and IL-10.

Introduction

Yun Zhi (*Coriolus versicolor*), a Chinese medicinal plant, is a macrofungi which belongs to the Basidiomycetes class and Polyporaceae family of fungi (1). Extracts from *Coriolus versicolor* have been previously demonstrated to represent a valuable adjuvant for the treatment of various forms of cancer in combination with chemotherapy or radiotherapy (2). Polysaccharopeptide (PSP) is extracted from the Cov-1 strain of *Coriolus versicolor* and is the main active ingredient, functioning as a biological response modifier (3). PSP is a proteoglycan of ~100 kDa and contains a polysaccharide and polypeptide portion (4,5). PSP is well known for its immunoregulatory, anticancer, anti-inflammatory and antiviral effects, and is used widely as an immune modifier in healthy and cancerous individuals in a number of Asian countries (4,6-9). PSP exerts its immunomodulatory actions by promoting the proliferation of the activation of macrophages, T lymphocytes and natural killer cells (10). However, the majority of studies are *in vitro* and the effect of PSP on lymphocyte proliferation, including T cells, is quite controversial (9,11). The efficacy of PSP on immunological effector cells under an immunosuppressive state *in vivo* is poorly understood. Therefore, the present study was designed to elucidate the immunomodulatory effects of PSP on immunological effector cells in immunosuppressed mice induced by cyclophosphamide (Cy).

Numerous studies have begun to dissect the pathways that may suppress immune responses, including effectors or regulators of T cell exhaustion (12,13). Three key negative regulatory pathways that have received particular attention are forkhead/winged-helix transcription factor box protein 3 $(Foxp3)^+$ regulatory T cells (Tregs), programmed death-1 (PD-1)/PD-L and interleukin (IL)-10/IL-10R (12). In the present study, the effect of PSP on the gene expression of negative immune regulators, Foxp3, PD-1 and IL-10, was also investigated in immunosuppressed mouse spleen tissues.

Materials and methods

Animals. Male Balb/c mice, obtained from the Animal Center of Nanjing Medical University (Nanjing, Jiangsu, China), were maintained in specific pathogen-free conditions and used at 6-8 weeks old. Mice were housed in an air-conditioned room at 25±2˚C with a relative humidity of 40-70% and a 12 h interval light/dark cycle. The mice were fed with tap water and a standard laboratory diet. All animal experiments were performed in accordance with institutional guidelines and the study was approved by the ethics committee of Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu, China.

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Key words: polysaccharopeptide, T lymphocyte subsets, Th1/Th2, immune

Drugs and chemicals. Pure polysaccharopeptide powder was provided by Jiangsu Nanjing Lao Shan Pharmacy Co., Ltd. (Nanjing, Jiangsu, China). Cyclophosphamide was provided by Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, China). Alexa Fluor 647 rat anti-mouse CD8a, PerCP-Cy 5.5 rat anti-mouse CD4, FITC hamster anti-mouse CD3e, PE rat anti-mouse CD19 and RBC lysis buffer were all obtained from BD Biosciences (Franklin Lakes, NJ, USA). TRIzol was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA); SYBR Premix *Ex Taq* (Tli RNase H Plus) and PrimeScript RT Master Mix were obtained from Takara Bio, Inc. (Shiga, Japan).

Experimental regimen. Balb/c mice were randomly assigned to four groups, including normal control, Cy and two PSP groups. The mice in the normal control and Cy groups were orally administrated with physiological saline. The two PSP groups were orally administered with PSP at a dose of 125 or 500 mg/kg/d body weight. All groups were administrated once a day for 25 consecutive days. The Cy and the two PSP groups were injected intraperitoneally with Cy at the dose of 150 mg/kg/d body weight on day 17 and 21 to generate an immunosuppressed animal model (14), while the mice in the normal control group were injected intraperitoneally with physiological saline as control. The solution of PSP and Cy was prepared by dissolving the compounds in physiological saline.

Detection of WBCs. Peripheral blood was collected from the retro-orbital plexus of each mouse on day 22 and 26 prior to being sacrificed. The blood was placed in a sterile EDTA-anticoagulated tube and WBCs were counted by a Sysmex XE-2100 hematology analyzer (Sysmex Corporation, Kobe, Japan).

Flow cytometry of lymphocyte subsets. EDTA-anticoagulated whole blood was collected on day 22 and 26. Whole blood (50 μl) was gently mixed with anti-mouse mAbs (Alexa Fluor 647 CD8a, PerCP-Cy 5.5 CD4, FITC CD3e and PE CD19) and incubated for 20 min at room temperature in the dark. After adding RBC lysis buffer for 10 min, the samples were centrifuged at 392 x g at 25˚C for 5 min and the supernatant was discarded. The samples were washed with 1 ml phosphate-buffered saline (PBS), centrifuged at 392 x g at 25˚C for 5 min and the supernatant was discarded. The cells were resuspended in PBS and then analyzed on a BD FACSCanto flow cytometer (BD Biosciences).

Calculation of absolute numbers of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells. The absolute number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells in the peripheral blood in each group was calculated by the percentage of each subset multiplied by lymphocyte absolute count (15). Lymphocyte absolute count was calculated by multiplying the proportion of lymphocytes by the WBC absolute number which was counted using a hemacytometer. To determine the proportion of lymphocytes, cells were treated with Wright's Giemsa stain and 200 cells/slide were manually counted using an oil immersion microscope (magnification, x100) (15).

Spleen and thymus indexes. On day 26, all mice were sacrificed. Prior to sacrifice, the body weight of the mice was recorded. The spleens and thymus glands were removed and the weights were recorded. The organ indexes of spleens and thymus were calculated according to the formula: organ index = weight of organ (mg)/body weight (10 g).

Total RNA isolation and real-time quantitative PCR. The relative expression of IL-2, IL-4, T-box-containing protein (T-bet), GATA binding protein 3 (GATA-3), Foxp3, PD-1 and IL-10 mRNA in the spleen tissues of the normal control, Cy and 125 mg/kg PSP groups was detected by real-time quantitative PCR. Total RNA was extracted from spleen tissues using TRIzol according to the manufacturer's instructions (Invitrogen Life Technologies). Concentration and quality of the extracted RNA were determined by measuring light absorbance at 260 nm (A_{260}) and the ratio of A_{260}/A_{280}. Reverse transcription reactions were performed using 0.5 μl RNA (1 μg/μl) in a volume of 10 μl according to the manufacturer's instructions. PCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). PCR programs were optimized and the primers and PCR product sizes are shown in Table I. The mRNA levels of different groups were normalized against levels of GAPDH. The relative differences in gene expression among study groups were determined using the comparative Ct ($\Delta\Delta$Ct) method and fold expression was calculated using the formula $2^{-\Delta\Delta Ct}$. $\Delta\Delta$Ct represents ΔCt values normalized against the mean ΔCt of control samples.

Statistical analysis. Data are presented as the mean ± SD. The results were statistically analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

General observations following Cy injection. All mice injected with Cy showed lethargy, dull pelage, fur piloerection and reduced food and water intake. In the Cy and 125 mg/kg PSP group, one mouse died one day following the first blood collection in each group.

Effect of PSP on peripheral blood WBCs. WBCs decreased on day 22 following the two Cy injections and then began to increase on day 26 in all groups except the normal control. In the two PSP groups, the peripheral blood WBCs counts were significantly higher than the Cy group and 125 mg/kg PSP was higher than 500 mg/kg PSP (Fig. 1)

Effect of PSP on absolute number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells. On day 22, absolute numbers of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells in the peripheral blood in the Cy group were significantly decreased compared with the normal control group. The reduction in the absolute number of $CD3^-CD19^+$ B cells was the most marked. The two PSP groups had significantly higher $CD3^+CD4^+$ T cell, $CD3^+CD8^+$ T cell, $CD3^-CD19^+$ B cell absolute numbers compared with

Table I. Sequences of primers used for qPCR.

Gene	Direction	Primer sequence (5'-3')	Product size (bp)
IL-2	Forward	CCCAAGCAGGCCACAGAATTGAAA	81
	Reverse	AGTCAAATCCAGAACATGCCGCAG	
IL-4	Forward	ATGGGTCTCAACCCCCAGCTAGT	399
	Reverse	GCTCTTTAGGCTTTCCAGGAAGTC	
T-bet	Forward	AACCAGTATCCTGTTCCCAGC	439
	Reverse	TGTCGCCACTGGAAGGATAG	
GATA-3	Forward	GAAGGCATCCAGACCCGAAAC	255
	Reverse	ACCCATGGCGGTGACCATGC	
PD-1	Forward	TGAACATCCTTGACACACGGC	170
	Reverse	GCCTTCTGGTTTGGGCGA	
IL-10	Forward	CCAGTTTTACCTGGTAGAAGTGATG	324
	Reverse	TGTCTAGGTCCTGGAGTCCAGCAGACTCAA	
Foxp3	Forward	ATTTACTCAACCCAAACCCT	156
	Reverse	TGTGTGATAGTGCCCGT	
GAPDH	Forward	CCCACAGTAAATTCAACGGCAC	564
	Reverse	CATTGGGGTTAGGAACACGGA	

IL, interleukin; T-bet, T-box-containing protein; GATA-3, GATA binding protein 3; PD-1, programmed death-1; Foxp3, forkhead/winged-helix transcription factor box protein 3.

Table II. Absolute number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells on day 22.

Group	n	$CD3^-CD19^+$ B cells ($x10^7$/l)	$CD3^+CD4^+$ T cells ($x10^9$/l)	$CD3^+CD8^+$ T cells ($x10^9$/l)
Normal control	8	148.03±36.39	0.86±0.24	0.24±0.08
Cy	8	0.67±0.40[a]	0.29±0.13[a]	0.06±0.03[a]
500 mg/kg PSP	8	1.97±0.79[a,b]	0.49±0.13[a,b]	0.10±0.03[a,b]
125 mg/kg PSP	8	2.19±2.05[a,b]	0.86±0.29[b]	0.18±0.08[b]

Data are expressed as the mean ± SD. [a]P<0.05 vs. normal control; [b]P<0.05 vs. Cy. Cy, cyclophosphamide; PSP, polysaccharopeptide.

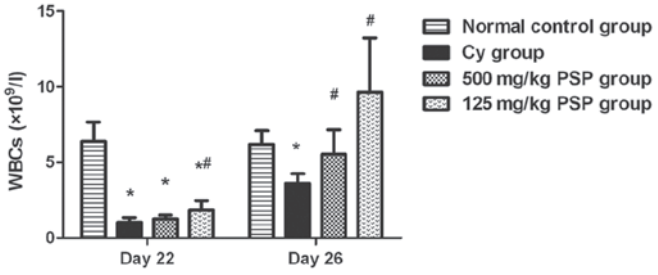

Figure 1. Levels of peripheral blood WBCs in each group. *P<0.05, vs. normal control; #P<0.05, vs. Cy. Cy, cyclophosphamide; PSP, polysaccharopeptide; WBCs, white blood cells.

the Cy group. The reduction in the 125 mg/kg PSP group was smaller than the 500 mg/kg PSP group (Table II). On day 26, the absolute numbers of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells in the Cy group, and the two PSP groups recovered significantly, but in the Cy group the absolute number of $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells was still lower than the normal control group. The absolute number of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in the 125 mg/kg PSP groups was superior to those in the 500 mg/kg PSP, Cy and the normal control groups. In addition, the absolute number of $CD3^-CD19^+$ B cells in the two PSP groups was higher than the Cy group, but still significantly lower than the normal control group (Table III).

Effect of PSP on spleen and thymus gland indexes. The spleen index in the Cy group was lower than in the normal control and the two PSP groups; however, this difference was not found to be statistically significant. The thymus index in the Cy group was significantly lower than the normal control group (P<0.05; Table IV).

Effect of PSP on Th1/Th2 balance. No marked differences were observed in the relative mRNA expression of IL-2 and T-bet in each group. However, IL-4 and GATA-3 mRNA expression in the Cy group was significantly higher than the normal control and 125 mg/kg PSP groups (both P<0.01 and P<0.05, respectively). There was no statistical difference between the normal control and 125 mg/kg PSP groups in

Table III. Absolute number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells on day 26.

Group	n	$CD3^-CD19^+$ B cells ($x10^7$/l)	$CD3^+CD4^+$ T cells ($x10^9$/l)	$CD3^+CD8^+$ T cells ($x10^9$/l)
Normal control	8	137.25±44.78	0.94±0.16	0.23±0.04
Cy	7	1.89±0.79[a]	0.89±0.19	0.17±0.04[a]
500 mg/kg PSP	8	5.38±2.63[a,b]	1.60±0.88	0.35±0.17[b]
125 mg/kg PSP	7	7.68±3.70[a,b]	3.06±1.27[a,b]	0.64±0.22[a,b]

Data are expressed as the mean ± SD. [a]P<0.05 vs. normal control; [b]P<0.05 vs. Cy. Cy, cyclophosphamide; PSP, polysaccharopeptide.

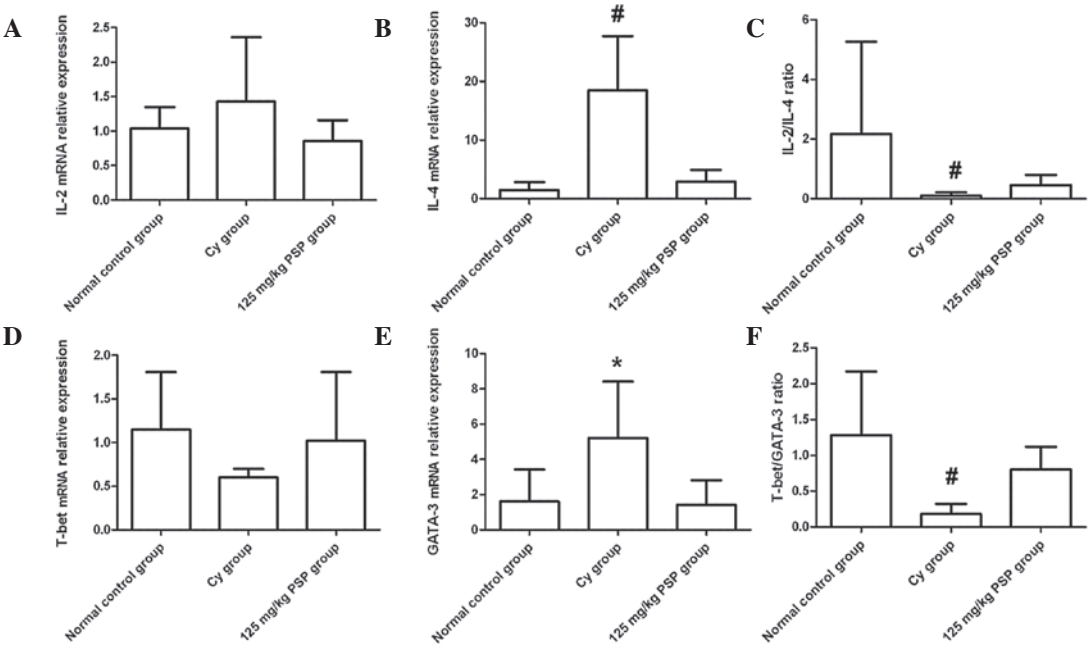

Figure 2. mRNA relative expression levels in spleen tissues of (A) IL-2, (B) IL-4, (D) T-bet and (E) GATA-3, and the ratios of (C) IL-2/IL-4 and (F) T-bet/GATA-3. mRNA levels were normalized against GAPDH expression in each sample and values are expressed as the mean ± SD fold increase over the normal control group (n=6, *P<0.05, vs. normal control and 125 mg/kg PSP; #P<0.01, vs. normal control and 125 mg/kg PSP). T-bet, T-box-containing protein; GATA-3, GATA binding protein 3; IL, interleukin; Cy, cyclophosphamide; PSP, polysaccharopeptide.

the ratios of IL-2/IL-4 and T-bet/GATA-3; however, the ratio in the two groups was significantly higher than the Cy group (P<0.01; Fig. 2).

Effect of PSP on the mRNA expression of negative immune regulators. The relative expression of Foxp3, PD-1 and IL-10 mRNA was significantly higher in the Cy group than the normal control (all P<0.01), and in the 125 mg/kg PSP group the relative expression of Foxp3 and IL-10 mRNA was lower than the Cy group (all P<0.01), but still higher than the normal control (P<0.01). PD-1 mRNA relative expression in the 125 mg/kg PSP group was lower than the Cy and normal control groups (P<0.01; Fig. 3).

Discussion

In the present study, the effects of PSP on the immune response of immunosuppressed mice induced by Cy were assessed by administering PSP to Balb/c mice for 25 days. The immunostimulatory effects of a drug or nutritional supplement are difficult to evaluate in healthy human individuals and animals (16). Cy is one of the most commonly used anticancer agents and immunodepressant drugs for preventing graft rejection, treating specific chronic autoimmune diseases and inducing experimental immunosuppression (17). Cy inhibits humoral and cellular immunity, and is most toxic to rapidly proliferating tissue, including the hematopoietic system, gastrointestinal epithelia, hair follicles and genital glands (18). Therefore, in the present study, Cy was selected as the immunosuppressive drug to induce the immunosuppressed mice model. This model is often used to evaluate the immunoregulatory effects of drugs (19-22). In the Cy-induced murine model, WBC counts were significantly decreased following Cy injection, then recovered spontaneously. In leukopenic mice orally administered with PSP, the decline in the WBC level was significantly alleviated and the WBC counts recovered to normal levels more rapidly.

Thymus and spleens are the most important immune organs. However, in this study, changes in spleen and thymus indexes of mice were not found to be significantly different from those of the two PSP groups and Cy group through the experimental period. This indicated that PSP treatment did

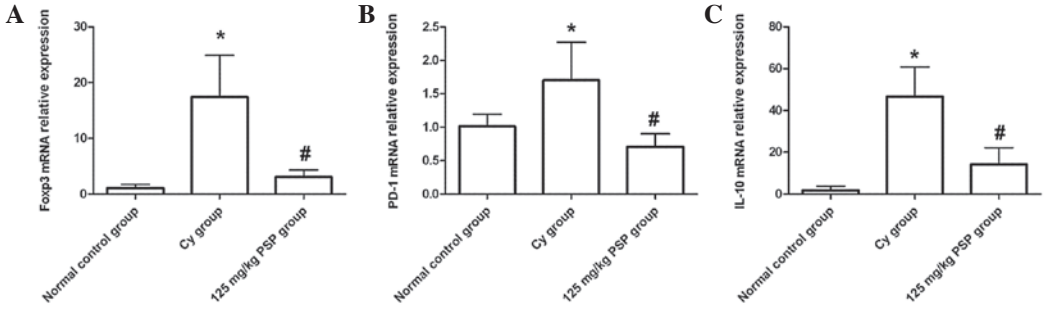

Figure 3. Relative mRNA expression levels in spleen tissues of (A) Foxp3, (B) PD-1 and (C) IL-10. mRNA levels were normalized against GAPDH expression in each sample and values are expressed as the mean ± SD fold increase over the normal control group (n=6, *P<0.01, vs. normal control and 125 mg/kg PSP; #P<0.01, vs. normal control). Foxp3, forkhead/winged-helix transcription factor box protein 3; PD-1, programmed death-1; IL, interleukin; Cy, cyclophosphamide; PSP, polysaccharopeptide.

Table IV. Spleen and thymus gland indexes.

Group	n	Spleen index (mg/10 g)	Thymus index (mg/10 g)
Normal control	8	64.49±43.46	10.91±2.49
Cy	7	39.11±16.51	7.71±3.70[a]
500 mg/kg PSP	8	52.88±29.67	10.04±1.45
125 mg/kg PSP	7	60.09±35.52	7.35±4.74

Data are expressed as the mean ± SD. [a]P<0.05 vs. normal control. Cy, cyclophosphamide; PSP, polysaccharopeptide.

not affect the body weight and spleen or thymus mass of mice.

T lymphocytes play a critical role in the development of acquired immune responses and B lymphocytes are important in the response to humoral immunity. $CD4^+$ and $CD8^+$ T cells are the main T lymphocyte subsets. In the present study, the proliferative responses to T- and B-cell mitogens were reduced markedly in all Cy-treated groups. PSP treatment promoted T- and B-cell proliferative responses and in specific cases the response was higher than normal levels. Of note, this response was higher in the 125 mg/kg PSP group compared with 500 mg/kg PSP. Previous studies have also indicated that PSP significantly increases the percentage of $CD4^+$ T lymphocytes, the ratio of $CD4^+/CD8^+$ and the quantity and percentage of the B lymphocytes, and enhances the immune system of cancer patients (23,24). However, in the present study, the absolute number of T lymphocyte subsets and B lymphocytes was investigated which has not been analyzed to date.

According to differences in corresponding cytokines, helper T cells (Th) may be subdivided into two cell subsets, termed Th1 and Th2. Th1 cytokines contribute to cell-mediated immunity while Th2 cytokines are responsible for humoral immunity (25,26). IL-2 is the main Th1 type cytokine and is important for promoting T-cell proliferation, cytokine production and the functional properties of B cells, macrophages and NK cells (27). IL-4 is a key regulator of the immune response and promotes differentiation of naive T cells into Th2 cells (28). T-bet and GATA-3 are specific transcription factors which have been hypothesized to serve as master regulators of Th1 and Th2 differentiation, respectively. Expression of T-bet and GATA-3 impose a complex programme of lineage restriction that facilitates preferential expression of the signature cytokines and, to varying degrees, silencing of the opposite differentiation limb (29). Th1/Th2 balance is a prerequisite for the functionality of immune system against infections. Ho *et al* found that PSP promotes the proliferation of mouse splenic lymphocytes and increases the expression of Th1 cell-associated cytokines, including IL-2, IL-12, IFN-γ and IL-18, in mouse splenic lymphocytes *in vitro* (30). An additional study showed that PSP exhibited suppressive effects on Th1 cytokines, including IL-2, but stimulated the production of the Th2 cytokine, IL-10, to inhibit T-cell proliferation in human PBMCs *in vitro* (31). The effect of PSP on the Th1/Th2 balance is controversial. In the present study, when the mice were treated with Cy, the relative mRNA expression of the Th2 type cytokine, IL-4 and its specific transcription factor, GATA-3, was significantly increased and the ratios of IL-2/IL-4 and T-bet/GATA-3 were reduced. However, no significant effect on Th1 type cytokines was found. These observations indicate an imbalance of Th1/Th2 with Th2 shift in the Cy model. PSP reduces the relative mRNA expression of Th2 type cytokine, IL-4 and its specific transcription factor, GATA-3, to moderate the Th1/Th2 balance.

More recently, research has begun to pay more attention to the pathways that may suppress immune responses. One key negative regulatory pathway is mediated by $CD4^+CD25^+$ regulatory T cells. Another two pathways that have also received particular focus are PD-1/PD-L and IL-10/IL-10R.

Tregs employ several mechanisms to suppress immune responses, including direct cell contact, indirectly by reducing the antigen-presenting capacity of antigen-presenting cells (32) or by suppressive cytokines, such as inhibitory cytokines IL-10 and TGF-β (33,34). FOXP3 is considered to represent the most reliable marker of Treg involvement in the formation and functioning of $CD4^+CD25^+$ T lymphocytes (35-38) and the level of FOXP3 expression has been shown to correlate with suppressive activity (35,38).

PD-1, which belongs to the CD28 family is mainly inducibly expressed on activated T cells, B cells, natural killer T cells and myeloid cells (39-42). PD-1 functions as an inhibitory co-signaling molecule and regulates immunity (43-46). PD-1 decreases T-cell receptor (TCR)-mediated cell proliferation,

cytokine production and cytolytic activity upon binding with its ligands, PD-L1 and PD-L2 (47-50). In addition, the PD-1 signaling cascade may inhibit $CD8^+$ T-cell effector function during chronic murine viral infections (51).

IL-10 was originally identified by Fiorentino *et al* (52,53). T-helper type 2 cells, subsets of regulatory T cells designated Tr1, Th1 and Th17 cells, are the four major T-cell sources of IL-10 (54). The main biological function of IL-10 is exerted on dendritic cells (DCs) and macrophages, and is a potent inhibitor of antigen presentation. IL-10 inhibits major histocompatibility complex class II expression as well as the upregulation of costimulatory molecules, CD80 and CD86, and inhibits the differentiation and maturation of DCs (55). In addition, the IL-10/IL-10R pathway plays a key role in the early events that determine whether an infection is rapidly cleared or progresses to chronicity with T-cell dysfunction (12).

The expression of negative immune regulators, including Foxp3, PD-1 and IL-10, in immunosuppressed mice and the effect of PSP on immunosuppressed mice is not clear.

In the present study, the expression of Foxp3, PD-1 and IL-10 mRNA was significantly higher in the Cy group, indicating that Cy exerts its immunosuppressive effect by a negative regulatory pathway. In the PSP group, the mRNA expression of Foxp3, IL-10 and PD-1 was lower than the Cy group, indicating that PSP may exert its immunomodulatory effects by downregulating the expression of negative immune regulators, Foxp3, PD-1 and IL-10.

In summary, the results of the present study demonstrate that PSP possesses immunoprotective effects and is capable of restoring Cy-induced immunosuppression, including depressed WBCs, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes and B lymphocytes, as well as reducing the expression of the Th2 type cytokine, IL-4 and its specific transcription factor, GATA-3 and negative immune regulators, such as Foxp3, PD-1 and IL-10. These observations show that PSP functions against the immune inhibition induced by Cy, indicating that PSP is a potent immunoenhancing and immunomodulating agent.

Acknowledgements

The present study was supported by the Medical Science Intensive Developing Project of Nanjing Government (No. ZKX10015), Jiangsu Province's Outstanding Medical Academic Leader Program (No. LJ201154) and Jiangsu Province's Clinical Medicine and Technology Special Program (No. BL2012034).

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