The investigation of immunomodulatory activities of *Gloeostereum incaratum* polysaccharides in cyclophosphamide-induced immunosuppression mice

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Abstract. *Gloeostereum incarnatum*, a precious edible mushroom, displays anti-bacterial and anti-inflammatory activities; however, its immunomodulatory effect has not been studied yet. The present study aimed to investigate whether polysaccharide compositions of *G. incarnatum* polysaccharides (GIPS) possess immunomodulatory and immuno-enhancing effects in a Cyclophosphamide monohydrate (CTX)-induced BALB/c mice model. The 28-day GIPS administration at doses of 0.1, 0.3 and 0.9 g/kg remarkably reversed the bodyweight loss, increased the thymic index and promoted T lymphocyte proliferation in CTX-induced immunosuppressed mice. GIPS significantly raised the serum levels of immunoglobulin (Ig) A and IgG, promoted the production of interleukins (ILs), including IL-2, IL-3 and IL-6, interferons, including interferon (IFN)-α and IFN-γ, and monocyte chemotactic protein 1 in the spleen, which resulted in accelerating recovery of immunosuppression. Finally, GIPS showed anti-oxidative effects indicated by the increased superoxide dismutase levels in the serum and spleen, and the reduced level of reactive oxygen species in the spleen. The results of the current study demonstrated that GIPS positively adjusts the immune system, which may serve as a potential immunostimulatory agent.

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Introduction

The immune system, a complex self-regulation system, is responsible for the defense function of organism to prevent outside pathogens, remove the waste material and maintain the monitoring function for malignant cells (1). The immune system is highly sensitive (2), and shows impaired immune responsiveness to pathogen invasion, during which, immune cells are activated and secret pro-inflammatory mediators including interleukins (ILs) and interferons (INFs) are increased (3,4). And immunity closely related to oxidative stress, which could lead to aberrant expression of inflammatory cytokines and chemokines (5). Cyclophosphamide monohydrate (CTX), a cancer chemotherapeutic agent, facilitates cell apoptosis and decreases the homeostatic proliferation of regulatory T cells (6), which has been widely used in the establishment of immunosuppressive animal models (7-9).

Previous researchers focus their studies on searching immunomodulatory agents for years (10). However, the drugs performed in clinic, especially single-component chemicals, display severe adverse effects including general malaise and/or neurotoxicity (11,12), which is hard to meet the requirements of immunosuppressive patients. Dietary polysaccharides from natural products are getting more attention due to their health benefits and low toxicity (13,14), which have been confirmed to exhibit multiple immunomodulatory effects (15,16). *Gloeostereum incarnatum,* a precious edible mushroom, parasitizes broad-leaved wood in northern Japan and northern China (17). Traditionally, *G. incarnatum* preparation is widely used for the treatment for enteritis, dysentery and gastric ulcer (18). Polysaccharides obtained from *G. incarnatum* fruiting body have been reported to possess anti-tumor and anti-bacterial activities (17). However, the immunomodulatory effect of *G. incarnatum* has not been investigated yet.

The objective of this study aims to investigate whether polysaccharide compositions of *G. incarnatum* possess immunomodulatory and immuno-enhancing effects in a CTX-induced BALB/c mice model. Data demonstrated that *G. incarnatum* polysaccharides (GIPS) enhanced

immunologic function in CTX-induced immunosuppressed mice by promoting the production of immune-associated factors in spleen, elevating serum immunoglobulin (Ig) levels, and restoring the imbalance of anti-oxidation and oxidation.

Materials and methods

GIPS preparation. G. incaratum fruiting body (Yunnan, China) was smashed and extracted with 20-fold double distilled (D.D.) water at 90°C for 4 h twice. The water extracts were concentrated and deproteinated by Sevag agents (n-butanol:chloroform=1:4) (19). After centrifugation, the supernatant were collected, added 4-fold volume of ethanol, and placed at 4°C for overnight. The precipitation was dissolved in D.D. water and subjected to DEAE-52 cellulose anion exchange column (2.6x35 cm) (20). The fractions (fraction 1 to 4) of crude GIPS were collected together, freeze dried and stored at -80°C. The detailed information can be found in Fig. 1A and B.

Protocols for immunosuppression mice development and agents administration. The experimental protocol was approved by Animal Ethics Committee in Jilin University (20160402). 72 of Balb/c male mice (8-week old, 18-20 g) [SCXK (JI)-2014-0003] were housed in cages with a 12 h light/12 h dark period under a constant temperature (23±2°C) and humidity-controlled conditions (50-60%), and allowed free access to solid feed and tap water during the experiments.

After one-week acclimatization, mice were divided into six groups randomly as follows. Control group (n=12): 3-day normal saline injection following with 14-day normal saline gastric gavages. Cyclophosphamide (CTX)-injected group (model group; n=12): 3-day CTX (75 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) injection following with 14-day normal saline gastric gavages. GIPS-treated groups (n=12/group): 3-day CTX (75 mg/kg) injection following with 14-day GIPS (0.1, 0.3 and 0.9 g/kg; dissolved in normal saline) gastric gavages. Thymosin α1 (Tα1) (positive drug)-treated group (n=12): 3-day CTX (75 mg/kg) injection following with 14-day Tα1 (0.16 mg/kg; Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) injection. General health and body weights of mice were monitored during the whole experimental period. At 24 h after the last administration, all mice were weighted, and killed. Blood and organs (spleen and thymus) were sampled immediately. The thymus index was calculated according to the formula: Index (mg/10 g)=(Weight of thymus)/10 x (body weight).

Lymphocyte proliferation assay. Spleen of each mouse was aseptically removed and grounded into a single cell suspension (21), and seeded into a 96-well plate at density of $1x10^6/100$ μl mixed with 10 μl of RPMI-1640 (Control group) or 10 μl of 200 μg/ml concanavalin (ConA; Sigma-Aldrich; Merck KGaA). Then incubated under a humidified atmosphere containing 5%/95% CO_2/air at 37°C for 48 h similar as previous study (22), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich; Merck KGaA) assay were performed to analysis the changes of cell viability. The stimulation index (SI) of T lymphocyte

transformation was calculated as following equation: SI=OD$_{490}$ ConA/OD$_{490}$ control.

ELISA assay. The spleens were homogenized with D.D. water, and the protein concentrations were detected by a bicinchoninic acid protein assay kit (Merck KGaA). The levels of superoxide dismutase (SOD; 43125), ROS (43355), IgA (42791), IgG (42793) and IgM (42794) in serum and spleen, and the levels of ILs [IL-2 (42903), -3 (42902) and -6 (42912)], INFs [IFN-α (43102) and IFN-γ (42918)] and monocyte chemotactic protein 1 (MCP-1; 42818) in spleen were detected by commercial ELISA kits (Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China).

Statistical analysis. The statistical values are presented as the mean ± standard error of the mean (SEM). Statistically comparisons were analyzed using a one-way analysis of variance (ANOVA) followed by Dunn's test via SPSS 16.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of GIPS on bodyweight, thymus index and lymphocyte proliferation. CTX strongly reduced the bodyweights of mice after 3-day injection (P<0.05; Table I); in contrast, GIPS and Tα1 improved the weight gain in immunosuppressed mice after 7-day administration (P<0.05; Table I). Compared to normal mice, extremely low thymus index was observed in CTX-injected mice (P<0.001; Table I). GIPS at doses of 0.1, 0.3 and 0.9 g/kg and Tα1 at 0.16 mg/kg significantly enhanced the thymus index after 14-day treatment (P<0.05; Table I).

Lymphocyte proliferation is commonly used to reflect the immune function (23). Compared to CTX-induced immunosuppressant mice, GIPS at 0.9 g/kg, but not Tα1 promoted over 21.3% T lymphocyte proliferation (P<0.05; Fig. 2).

GIPS enhanced immune globulin levels in the serum and spleen of immunosuppressed mice. CTX injection suppressed the levels of IgA and IgG in serum (P<0.01; Fig. 3A and B), and reduced the levels of IgG and IgM in spleen (P<0.01; Fig. 3B and C) of mice. Compared to CTX-induced immunosuppressant mice, Tα1 at 0.16 mg/kg significantly enhanced the levels of IgA, IgG and IgM in serum (P<0.01; Fig 3), and the levels of IgG in spleen (P<0.01; Fig. 2B). Differently, GIPS failed to increase the spleen levels of immune globulin in CTX-induced immunosuppressant mice (Fig. 3). In contrast, GIPS administration resulted in >13.0% enhancement on IgA levels except for 0.1 g/kg of GIPS (P<0.001; Fig 3A) and >16.8% enhancement on IgG levels (P<0.05; Fig 3B) in serum of CTX-induced immunosuppressant mice.

Effects of GIPS on immune factors in the spleen of immunosuppressed mice. Compared to normal mice, the levels of ILs (IL-2, -3 and -10), INFs (IFN-α and IFN-γ) and MCP-1 in spleen were significantly lowered in CTX-induced immunosuppressant mice (P<0.05; Table II), which were all significantly restored by 14-day Tα1 administration at 0.16 mg/kg (P<0.05;

Table I. Regulatory effects of GIPS and Tα1 on body weight and organ indexes.

Measurement	CTRL	CTX (mg/kg) 75	GIPS (g/kg) 0.1	0.3	0.9	Tα1 (mg/kg) 0.16
Body weight (g)						
1st day	26.4±0.4	25.8±0.4	27.1±0.3	25.0±0.4	27.4±0.6	26.9±0.4
4th day	25.8±0.3	22.6±0.5[a]	22.4±0.4	22.8±0.7	22.8±0.6	22.5±0.4
11th day	27.9±0.4	24.6±0.3[a]	25.6±0.5	25.3±0.8	26.9±0.5[c]	24.8±0.6
18th day	27.3± 0.6	23.1±0.4[a]	25.1±0.2	24.7±0.9	26.0±0.8[c]	25.9±1.1[c]
Thymus index (mg/10 g)	0.23±0.02	0.09±0.01[b]	0.14±0.02[d]	0.13±0.03[c]	0.17±0.03[d]	0.15±0.03[d]

The changes of body weight were detected during the experimental period. After 14-day administration, thymus index was examined. Data were expressed as means ± SEM (n=12). [a]$P<0.05$ and [b]$P<0.001$ vs. control group, [c]$P<0.05$ and [d]$P<0.01$ vs. model group. CTX, Cyclophosphamide; GIPS, *G. incarnatum* polysaccharides; Tα1, Thymosin α1; CTRL, control.
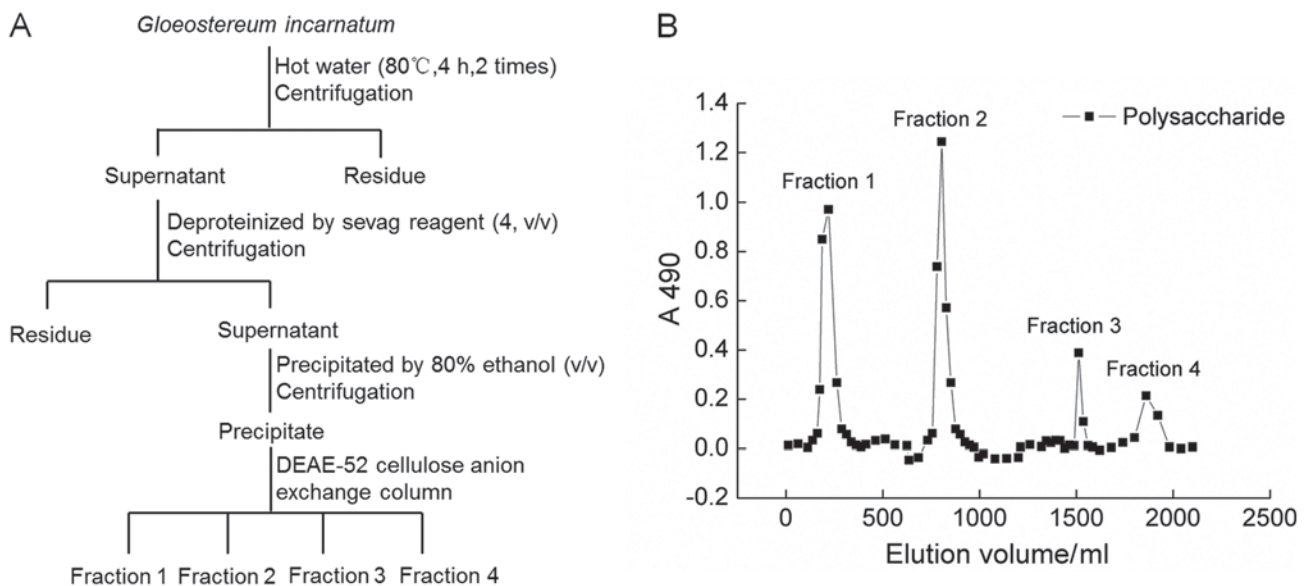

Figure 1. (A) The Scheme for extraction and isolation of polysaccharides from *G. incarnatum*. (B) DEAE-Sepharose fast flow chromatogram of the crude polysaccharides.

Table II). GIPS, especially at 0.9 g/kg, increase 191.1% of IL-2 (P<0.01), 94.7% of IL-3 (P<0.01), 136.1% of IL-6 (P<0.001), 68.7% of IFN-α (P<0.05), 50.6% of IFN-γ (P<0.05), and 77.8% of MCP-1 (P<0.001) in spleen compared to CTX-injected mice (Table II).

Effects of GIPS on the oxidative stress factors in serum and spleen of immunosuppressed mice. Compared to normal mice, CTX injection strongly reduced the levels of SOD in serum and spleen (P<0.001; Table III), but failed to significantly influence ROS levels (Table III). Tα1 only enhanced the levels of SOD (P<0.01; Table III), but not ROS, in serum and spleen of CTX-injected mice. Compared to immunosuppressed mice, 14-day GIPS administration resulted in >14.6% and >51.5% enhancement on SOD levels in serum (except for 0.1 g/kg GIPS) and spleen, respectively (P<0.05; Table III). GIPS showed no effects on serum ROS levels (Table III);

in contrast, GIPS at doses of 0.3 and 0.9 g/kg significantly reduced the levels of ROS in spleen compared to immunosuppressed mice (P<0.01; Table III).

Discussion

In this study, we evaluated the immunoregulation effect of GIPS in CTX-induced immunosuppressed mice, and this model is recognized as a well characterized model. There are reports that, CTX shows immunosuppressive effect mainly through lymphocytes toxicity via disturbing DNA and RNA function (24,25). GIPS significantly reversed the atrophy of immune organs, especially thymus, and enhanced T lymphocyte proliferation. Spleen, an important immune organ, is the primary site for the development of T cells and B cells (26). The proliferation of T and B lymphocytes in response to microbial antigen is known as a typical non-specific immune
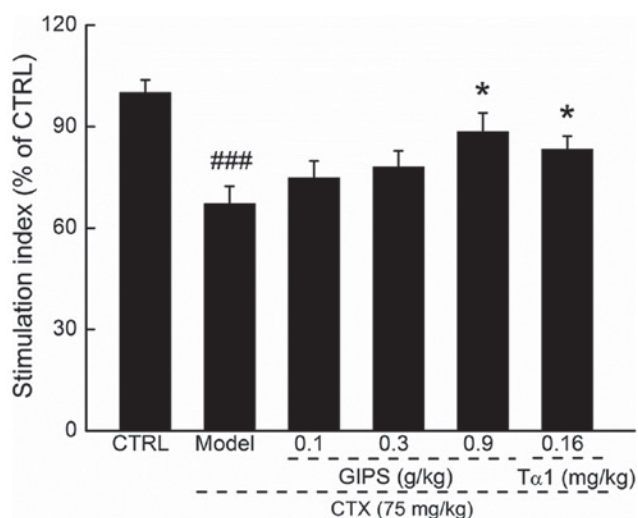
Figure 2. GIPS enhanced the lymphocyte proliferation of spleen in CTX-induced immunosuppressed mice. Data were expressed as means ± SEM (n=12). $^{###}P<0.001$ vs. control group, $^{*}P<0.05$ vs. model group. CTX, Cyclophosphamide; GIPS, *G. incarnatum* polysaccharides; Tα1, Thymosin α1.
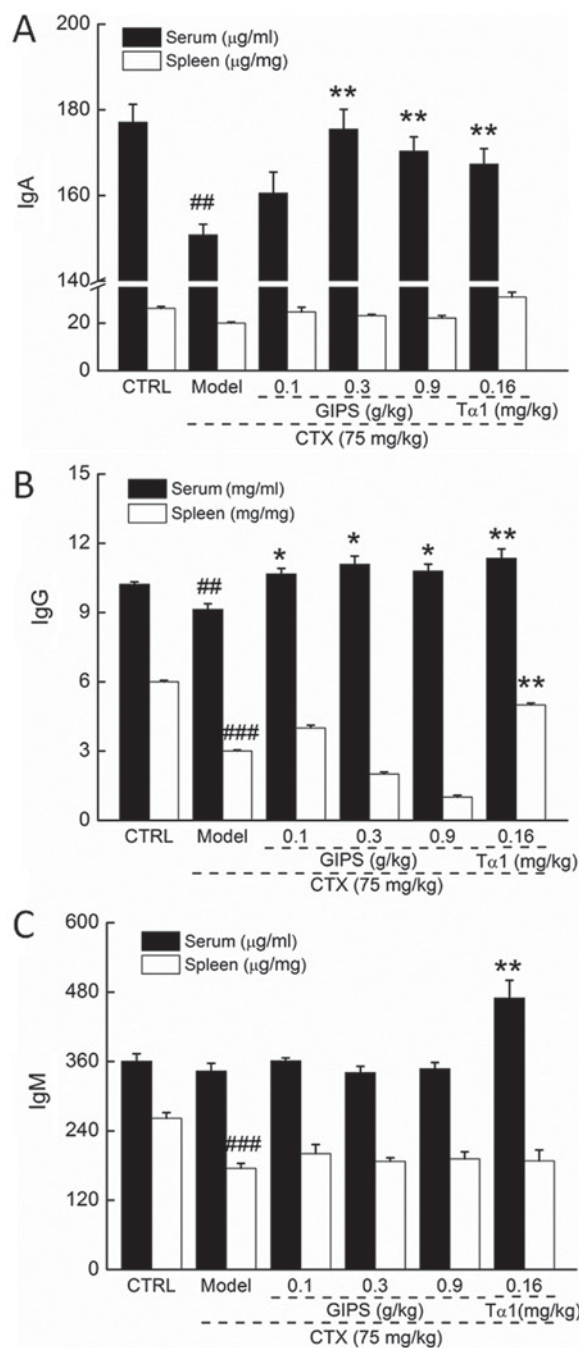

Figure 3. The regulatory effects of GIPS (0.1, 0.3 and 0.9 g/kg) and Tα1 (0.16 mg/kg) on the levels of (A) IgA, (B) IgG and (C) IgM in serum and spleen of CTX-induced immunosuppressed mice. Data were expressed as means ± SEM (n=12). $^{##}P<0.01$ and $^{###}P<0.001$ vs. control group, $^{*}P<0.05$ and $^{**}P<0.01$ vs. model group. CTX, Cyclophosphamide; GIPS, *G. incarnatum* polysaccharides; Tα1, Thymosin α1.

reaction. Thus, the determination of lymphocyte proliferation can reflect the immune status (23). Moreover, GIPS increased the serum Ig levels in CTX-induced immunosuppressed mice. Ig is the crucial component of organism immune system and plays a vital role in immune function (27). IgG is found to be the most abundant Igs and mediates natural passive immunity including antibacterial, antivirus and antitoxin (28); meanwhile, IgA shows bacteriolysis and promotive function in phagocytosis and aggregation (29). Immuno-stimulants, such as bacterial antigen lysate, significantly enhanced IgG and IgA serum levels in children with recurrent infections (30,31). IgM, one of the largest molecular weight Ig, accounts or 5-10% of the total serum Ig, which appears firstly in the humoral immune response of antibodies (32). The detection of serum IgM levels serves as an index of the early diagnosis of infection. Altogether, we successfully confirmed the immunoregulation activities of GIPS in the CTX-induced immunosuppressed mouse model.

GIPS successfully regulated the levels of ILs, INFs and MCP-1 in spleen of CTX-induced immunosuppressed mice. The immune response targets various cytokines, some of which directly influence immunity of organs. T helper (Th) cells, one of subsets of T cells, mediates the production of ILs, which can active immune cells during immune response evidencing by huper-levels of IL-2 activates pro-inflammatory CD4+ T cells (33). IL-3 participates in regulating myeloid cell expansion, autoimmunity and modulate human CD14+ monocyte response in short-term or long-term models of trained immunity (34). IL-6 is a critical factor for maturation, proliferation, differentiation and maintenance of B cells/plasma cells, which seems to enhance the development of pro-inflammatory cell and to inhibit the development T regulatory cells (35). IL-6 promotes the expression of IL-2 in T cells, which further interacts with IL-3 to promote the differentiation of T cells (36). Moreover, Th1 cells secretes IFN-α and IFN-γ, which promotes cytotoxic lymphocytes formation and activity of macrophage (37,38). IFN-γ, an

essential cytokine for host defense against pathogens, can stimulate macrophages to eliminate bacterial and tumor cell, activate neutrophil and natural killer (NK) cell (37). GIPS significantly enhanced the levels of MCP-1 in spleen of CTX-induced immunosuppressed mice. MCP-1, a member of the CC chemokine family, is responsible for recruiting monocytes, T cells and dendritic cells to inflammatory sites caused by infection (39). All the data obtained in our present experiment further confirmed the immunoregulation activities of GIPS in CTX-induced immunosuppressed mice.

Table II. Regulatory effects of GIPS and Tα1 on interleukins, interferons and MCP-1 in spleen of CTX-induced immunosuppressed mice.

Protein	CTRL	Model	GIPS (g/kg)			Tα1 (mg/kg)
			0.1	0.3	0.9	0.16
IL-2 (pg/mg)	21.2±1.0	6.7±0.8[b]	9.0±0.4	9.5±1.0	19.5±4.0[d]	12.0±0.7[c]
IL-3 (pg/mg)	6.4±0.37	3.8±0.4[a]	4.7±0.5	5.2±0.4	7.4±1.3[d]	6.0±0.7[c]
IL-6 (pg/mg)	9.7±0.7	3.6±0.5[b]	5.7±0.4	6.6±0.6[c]	8.5±1.1[e]	7.2±0.7[d]
IFN-α (pg/mg)	2.6±0.2	1.6±0.1[a]	2.4±0.1	2.6±0.3	2.7±0.1[c]	3.6±0.5[d]
IFN-γ (pg/mg)	15.4±0.8	7.9±0.6[b]	9.5±0.8	9.2±0.5	11.9±1.3[c]	12.4±1.2[d]
MCP-1 (pg/mg)	1.3±0.1	0.9±0.1[b]	1.2±0.1[d]	1.5±0.1[e]	1.6±0.1[e]	1.3±0.1[d]

Data were expressed as means ± SEM (n=12). [a]$P<0.05$ and [b]$P<0.001$ vs. control group, [c]$P<0.05$, [d]$P<0.01$ and [e]$P<0.001$ vs. model group. CTX, Cyclophosphamide; GIPS, *G. incarnatum* polysaccharides; Tα1, Thymosin α1; CTRL, control; IL, interleukin; IFN, interferon; MCP, monocyte chemotactic protein.

Table III. Regulatory effects of GIPS and Tα1 on the levels of SOD and ROS in serum and spleen of CTX-induced immunosuppressed mice.

Group	CTRL	Model	GIPS (g/kg)			Tα1 (mg/kg)
			0.1	0.3	0.9	0.16
Serum						
SOD (U/ml)	150.5±6.1	113.3±4.0[a]	119.0±3.1	129.8±1.6[b]	136.8±3.9[c]	138.0±3.1[c]
ROS (U/ml)	32.6±0.6	33.3±0.6	32.0±1.0	31.75±1.6	31.2±1.4	34.1±1.0
Spleen						
SOD (U/mg)	30.5±2.2	16.5±1.2[a]	25.0±2.3[b]	26.1±0.6[b]	27.8±2.5[c]	32.4±2.1[d]
ROS (U/mg)	39.8±2.7	42.7±7.1	30.7±1.9	28.2±2.9[b]	23.1±2.4[c]	30.4±2.9

Data were expressed as means ± SEM (n=12). [a]$P<0.001$ vs. control group, [b]$P<0.05$, [c]$P<0.01$ and [d]$P<0.001$ vs. model group. CTX, Cyclophosphamide; GIPS, *G. incarnatum* polysaccharides; Tα1, Thymosin α1; CTRL, control; SOD, superoxide dismutase; ROS, reactive oxygen species.

On the other hand, ooxidative stress is identified as one of the pathogenic factor of the immune system in inflammatory diseases (40), which has an effect on human T cell differentiation and polarization (41). The immune cell functions are mainly associated with the imbalance of the antioxidant/oxidant, especially the over-generation of ROS (42). Therefore, it is beneficial to prevent the injury of immune cells and maintain normal immune system function with adequate amounts of antioxidants (43). The balance between production and consumption of ROS is a key factor that determines the the physiological activities and function of T cells (44). SOD, the endogenous anti-oxidant, is the recognized as the first line of defense of oxidative stress, especially helping to scavenging ROS (45). GIPSsignificantly enhanced the levels of SOD in serum and spleen, and reduced the levels of ROS in spleen of CTX-induced immunosuppressed mice. However, more experiments need to be performed to investigate the roles of anti-oxidation of GIPS during its immunoregulation effects.

To our knowledge, we first confirmed the immunomodulatory effects of *G. incaratum* in the CTX-induced immunosuppressed mouse model, and provided the experimental evidence for the use of GIPS as a potential immunostimulatory agent. Although we found that GIPS can regulate the levels of ILs, INFs and oxidative stress factors, the underlying mechanism still need further investigation.

Acknowledgements

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