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Research Paper

Regulatory properties of polysaccharopeptide derived from *Coriolus versicolor* and its combined effect with ciclosporin on the homeostasis of human lymphocytes

Cheuk-Lun Lee[a], Pingping Jiang[a], Wai-Hung Sit[a], Xiatong Yang[b] and Jennifer Man-Fan Wan[a]

[a]School of Biological Sciences, Kadoorie Biological Sciences Building, The University of Hong Kong, Pokfulam Road, Hong Kong SAR and [b]Institute of Microbiology and Immunology, Faculty of Life and Environmental Science, Shanghai Normal University, Shanghai, China

Abstract

Objectives Lymphocyte homoeostasis is essential in inflammatory and autoimmune diseases. In search of natural fungal metabolites with effects on lymphocyte homoeostasis, we recently reported that polysaccharopeptide (PSP) from *Coriolus versicolor* exhibited ciclosporin-like activity in controlling aberrant lymphocyte activation. This object of this study was to investigate its effect on lymphocyte homoeostasis. This was done by investigating the mechanistic actions of PSP in relation to ciclosporin by performing cell cycle and cell death analysis of human lymphocytes *in vitro*.

Methods We investigated the effect of PSP in the presence and absence of ciclosporin on cell proliferation, cell cycle, cell death, immunophenotype and cell cycle regulatory proteins in human lymphocytes.

Key findings The data showed that PSP exhibited homoeostatic activity by promoting and inhibiting the proliferation of resting and phytohaemagglutinin (PHA)-stimulated lymphocytes, respectively. PHA-stimulated lymphocytes exhibited G0/G1 cell cycle arrest that was accompanied by a reduction of cyclin E expression with PSP treatment. Both PSP and ciclosporin blocked the reduction of the CD4/CD8 ratio in stimulated lymphocytes. PSP did not induce cell death in human lymphocytes, but the suppression of the Fas-receptor suggested a protective role of PSP against extrinsic cell death signals. These homoeostatic effects were more potent with combined PSP and ciclosporin treatment than with either fungal metabolite alone.

Conclusions Collectively, the results reveal certain novel effects of PSP in lymphocyte homoeostasis and suggest potential as a specific immunomodulatory adjuvant for clinical applications in the treatment of autoimmune diseases.

Keywords ciclosporin; *Coriolus versicolor*; lymphocyte; polysaccharopeptide; proteoglycan

Introduction

Immune system homoeostasis requires a balance between activation and suppression so as to achieve normal immune responses.[1] The homoeostasis of the immune system has to be maintained such that it not only responds to infections but also stops self-damage. Immunoregulatory agents act by inducing the proliferation (by means of cell cycle control) or death (by means of apoptosis and necrosis) of particular cell types, and thus affect inflammatory and autoimmune diseases.

Ciclosporin (CsA, cyclosporine) is an immunosuppressive agent derived from the fungus *Tolypocladium inflatum* that has gained clinical application in inflammatory and autoimmune disorders.[2,3] The immune suppression mechanisms of ciclosporin involves its binding to calcineurin to prevent the activation of the nuclear factor of activated T cells (NFAT), which inhibits the subsequent activation of the inflammatory effectors.[4] However, despite its therapeutic benefit, long-term application of ciclosporin is associated with severe side effects and toxicity.[5,6] Current attempts are underway to reduce the dose of ciclosporin and attain therapeutic efficacy by using immunomodulatory adjuvants.[7–9]

Correspondence: Dr Jennifer Man-Fan Wan, School of Biological Sciences, Kardoorie Biological Science Building, The University of Hong Kong, Hong Kong, China.
E-mail: jmfwan@hkusua.hku.hk

In search of immunomodulatory agents with adjuvant potential with ciclosporin, our previous studies showed that polysaccharopeptide (PSP) derived from a Chinese medicinal fungus, *Coriolus versicolor*, possessed similar immunomodulatory mechanisms as ciclosporin in modulating human lymphocytes responses.[10,11] PSP is a proteoglycan of approximately 100 kDa produced from the mycelia of the fungus by fermentation technology.[12] The peptide contains 18 kinds of mainly neutral and acidic amino acids.[13] The heteropolysaccharide contains glucose, mannose, xylose, galactose, arabinose and rhamnose linked by alpha or beta 1–4, 1–2 and 1–3 glycosidic bonds.[12,14] PSP is well-known for its anticancer, anti-inflammatory, immunoregulatory and antiviral effects, attaining widespread usage as an immunomodifier in both healthy and cancerous individuals in many Asian countries.[13–16] PSP exhibits mechanistic actions similar to ciclosporin in the control of lymphocytic Th1 and Th2 cytokines by suppression of the MAPKp38 and STAT5 pathways.[11] Proteomic analysis has also revealed that the two medical fungal extracts share certain regulatory proteins in controlling lymphocyte activity.[10] Other studies demonstrated the immunomodulatory properties of PSP in a clinical trial[17] and animal studies.[13,18]

The resemblance of PSP to ciclosporin in its lymphocytic regulatory mechanism suggests that PSP is a potential immunomodulatory agent for controlling the aberrant lymphocyte proliferation involved in autoimmune and inflammatory diseases. However, little is known about its effect on lymphocyte homoeostasis. The objective of this study was to investigate the mechanistic actions of PSP in relation to ciclosporin by performing cell cycle and cell death analysis of human lymphocytes *in vitro*. The data show that PSP exhibits a cell cycle regulatory effect and homoeostatic control was more effective with PSP and ciclosporin in combination than with either fungal metabolite alone. Collectively, these results reveal novel aspects of PSP in human lymphocyte homoeostasis and suggest its development potential as a specific immunomodulatory agent and adjuvant for clinical application in autoimmune and inflammatory diseases.

Materials and Methods

Reagents

Polysaccharopeptide (PSP) was obtained from Winsor Health Products Ltd (Hong Kong). The product was endotoxin free according to the manufacturer. The preparation and HPLC analysis of PSP were reported previously.[10,19] Phytohaemagglutinin (PHA), ciclosporin, anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG and anti-alpha tubulin antibody were purchased from Sigma-Aldrich (St Louis, USA). Antihuman CD3-FITC, CD16-FITC, CD4-phycoerythrin (PE), CD8-PE, CD19-PE, CD56-PE, anti-cyclin D and mouse isotypical controls were purchased from BD Biosciences Pharmingen (San Diego, USA). Anti-cyclin A, anti-cyclin E, anti-cyclin-dependent kinase (CDK) 1, anti-CDK 2 and anti-CDK 4 were from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Bromodeoxyuridine (BrdU) cell proliferation assay was purchased from Roche Diagnostics Co (Basel, Switzerland).

Preparation of human lymphocytes

Buffy coat preparations from healthy humans were obtained from the Red Cross Blood Transfusion Service (Hong Kong). Human lymphocytes were separated by Ficoll-Paque. The remaining red blood cells were removed by red cell lysing buffer (NH^4Cl 0.83%, EDTA 0.003%, $NaHCO^3$ 0.084%) and macrophages were removed by polystyrene culture flask cell adherence. The lymphocytes were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin and 1% fungizone and incubated in a controlled atmosphere of 5% CO_2 at 37°C.

Analysis of cell proliferation by bromodeoxyuridine assay

The colorimetric immunoassay was adopted to measure the incorporation of BrdU during DNA synthesis in the proliferating cells. Briefly, BrdU at 10 μM was added to the culture 5 h before the end of the incubation period. The culture medium was removed and the cells were dried at 60°C. Cell fixative and 200 μl DNA denaturation solution was added for 30 min. The cells were incubated with 100 μl of peroxidase-conjugated anti-BrdU-monoclonal antibody for 2 h. After three washes with 300 μl washing buffer, 100 ml of TMB substrate solution was added for 20 min. The reaction was stopped by 25 μl 1 M H_2SO_4. The absorbance was measured at 450 nm with λ correction at 650 nm. The proliferation of cells was expressed as the stimulation index (SI) = (Absorbance of treated cells – Absorbance of blank)/(Absorbance of control – Absorbance of blank).

Cell cycle measurement by DNA/propidium iodide flow cytometry

Isolated lymphocytes (1×10^6) were fixed in 70% ethanol, washed three times with phosphate-buffered saline (PBS) and then stained with 0.4 ml propidium iodide (PI). The cells were analysed by flow cytometry (Beckman Coulter, Fullerton, USA) with the PI signals detected by a 625 nm band pass filter. The fluorescence signal was analysed by Winlist and Modfit (Verity Software House, Topsham, USA).

Western blot analysis

Protein was extracted from cells using 100 μl lysis buffer (25 mM Hepes, 150 mM NaCl, 1 mM $EDTANa_2$, 1 mM dithiothreitol, 1% Triton × 100 and 1% protease inhibitor fluid) at 4°C for 60 min. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, USA). Protein extracts were mixed with an equal volume of sample buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue, pH 6.8) and incubated at 95°C for 10 min. Protein was resolved on a 12% SDS-polyacryamide gel, electro-transferred to a PVDF membrane and blocked by 4% non-fat milk in PBS. The membrane was incubated with primary antibodies against alpha-tubulin, cyclin A, cyclin D, cyclin E, CDK1, CDK2, CDK4 and their corresponding secondary horseradish peroxidase-conjugated antibodies. The blots were washed five times with 0.05% Tween-20 in PBS between steps. Protein bands were visualized on an autoradiography film using an ECL reagent.

Bivariate cyclin/DNA content analysis

Lymphocytes (2×10^6) were fixed with 70% ethanol, washed with 1% BSA in PBS (PBS-BSA) twice and incubated with 100 μl of monoclonal anti-human cyclin E for 1.5 h. After another two washes with PBS-BSA, the cells were incubated with 100 μl of FITC-conjugated secondary antibody for 1 h. The cells were washed with PBS, stained with 0.5 ml PI and analysed by flow cytometry using 525 nm and 625 nm band pass filters. For data analysis, cells were divided into G0/G1, S and G2/M phases according to the DNA content. Quantitative analysis was performed on each individual phase, as shown by the relative number of cyclin-E-positive cells.

Apoptosis, necrosis and viability measurement

The cells were labelled with PI and annexin V conjugated with FITC for cell death analysis. Briefly, 2×10^5 cells were washed twice with PBS and once with binding buffer (100 mM HEPES, 1.5 M NaCl, 50 mM KCl, 10 mM $MgCl_2$ and 18 mM $CaCl_2$). Staining solution in 0.1 ml (0.5% annexin V, 10% PI and 89.5% binding buffer) was added to the cell suspension for 15 min. Binding buffer (0.4 ml) was added and the cells were analysed by flow cytometry using 525 nm and 625 nm band pass filters.

Immunophenotyping of surface markers

Cells (1×10^6) were washed with PBS and then with blocking buffer (1% BSA and 0.1% sodium azide in PBS). The cells were incubated with PE- or FITC-conjugated anti-human CD3, CD4, CD8, CD19 or CD25 antibodies in blocking buffer for 30 min at 4°C. The cells were analysed by flow cytometry and fluorescence signals were detected by 525 nm and 575 nm band pass filters.

Statistical analysis

The data were expressed as the mean ± SEM (standard error mean) and analysed using SigmaStat 2.03 (Jandel Scientific, San Rafael, USA). The data were compared by analysis of variance followed by a post-hoc test (Tukey's test) to discern differences between individual groups. $P < 0.05$ denoted significance.

Results

Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on DNA synthesis in lymphocytes

The DNA synthesis measured by BrdU uptake was expressed as the stimulation index (SI) in lymphocytes treated with PSP (Figure 1a) and ciclosporin (Figure 1b). In resting (nonstimulated) lymphocytes, PSP significantly enhanced the SI dose-dependently at 8 μM and 16 μM ($P < 0.05$). The SI of the proliferating (PHA-stimulated) lymphocytes was increased 16 fold as compared with control. Increasing the PSP dose steadily reduced SI and reached significance ($P < 0.05$) at 16 μM PSP. The treatment with ciclosporin exerted an inhibitory effect on DNA synthesis in both resting and proliferating lymphocytes ($P < 0.05$). The inhibitory effect of ciclosporin was more potent than that of PSP at the same dose.

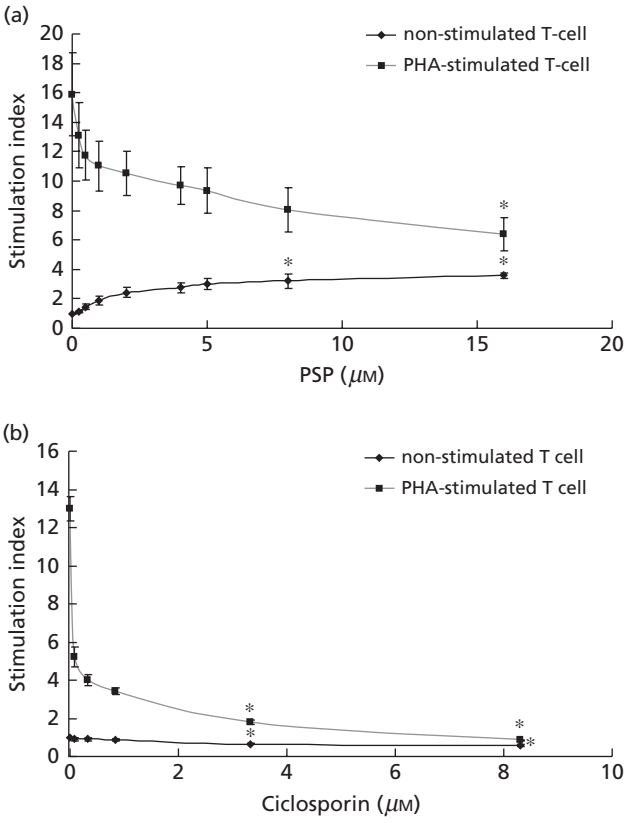

Figure 1 Effect of (a) *Coriolus versicolor* polysaccharopeptide and (b) ciclosporin on DNA synthesis in lymphocytes. Non-phytohaemagglutinin (PHA) and PHA (5 μg/ml)-stimulated human lymphocytes were incubated with polysaccharopeptide (PSP; 0, 0.25, 0.5, 1, 2, 4, 5, 8, 16 μM) and ciclosporin (0, 0.08, 0.33, 0.83, 8.31 μM) for 72 h. Cell proliferation was measured by bromodeoxyuridine incorporation and expressed as stimulation index (SI). SI = (Absorbance of sample – Absorbance of blank)/(Absorbance of control – Absorbance of blank). Data are mean ± SEM, $n = 6$). Dose effect was analysed by analysis of variance. *$P < 0.05$ vs control (0 μM PSP or ciclosporin).

Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on the cell cycle in lymphocytes

To investigate the combined effect of PSP and ciclosporin, 5 μM PSP and 0.83 μM ciclosporin were the doses used in this study. Table 1 and Figure 2 summarize the cell cycle pattern in PSP and ciclosporin-treated lymphocytes. In resting lymphocytes, the majority of the cells were detected in the quiescent G0/G1 phase, with only a few cells detected in the S and G2/M phases. PHA significantly stimulated DNA synthesis in lymphocytes by enhancing the S-phase cell population from 2.4% to 22.8% ($P < 0.001$) and this was accompanied by a reduction in G0/G1 cells from 94.5% to 73.5% ($P < 0.01$) as compared with non-PHA-stimulated control. Treatment with PSP and ciclosporin significantly increased ($P < 0.01$) the G0/G1-phase cells population of PHA-stimulated cells, from 73.5% to 82.9% and 91.1%, respectively. The ciclosporin inhibitory effect on cell proliferation was stronger than PSP, and the suppression of the S-phase cell population was even more pronounced (94.3%, $P < 0.05$ compared with PHA + PSP group) when the fungal metabolites were

Table 1 Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on the cell cycle distribution of lymphocytes

Treatment	G0/G1 (%)	S (%)	G2/M (%)
Non-PHA	94.49 ± 2.89	2.44 ± 1.22	3.08 ± 1.67
PHA	73.46 ± 0.80^{++}	22.78 ± 0.88^{+++}	3.76 ± 0.32
+ PSP	82.87 ± 0.82**	14.48 ± 0.90**	2.66 ± 0.14
+ ciclosporin	91.14 ± 2.37**	7.01 ± 1.97*	1.85 ± 0.46*
+ ciclosporin + PSP	94.27 ± 2.10**$^{,\#\#}$	3.95 ± 1.05	1.78 ± 1.06***$^{,\#}$

Non-phytohaemagglutinin (PHA) and PHA (5 μg/ml)-stimulated lymphocytes were incubated with polysaccharopeptide (PSP; 5 μM), ciclosporin (0.83 μM), or both, for 48 h. The cell cycle distribution was determined by flow cytometry with propidium iodide staining. Data are the mean ± SEM, $n = 4$, and compared by Student's *t*-test. $^{*,\ **,\ ***}P < 0.05$, 0.01 and 0.001 PHA + PSP, PHA + ciclosporin, PHA + ciclosporin + PSP vs PHA. $^{\#,\ \#\#}P < 0.05$ and 0.01 PHA + PSP vs PHA + ciclosporin + PSP. $^{++,\ +++}P < 0.01$ and 0.001 PHA vs non-PHA.

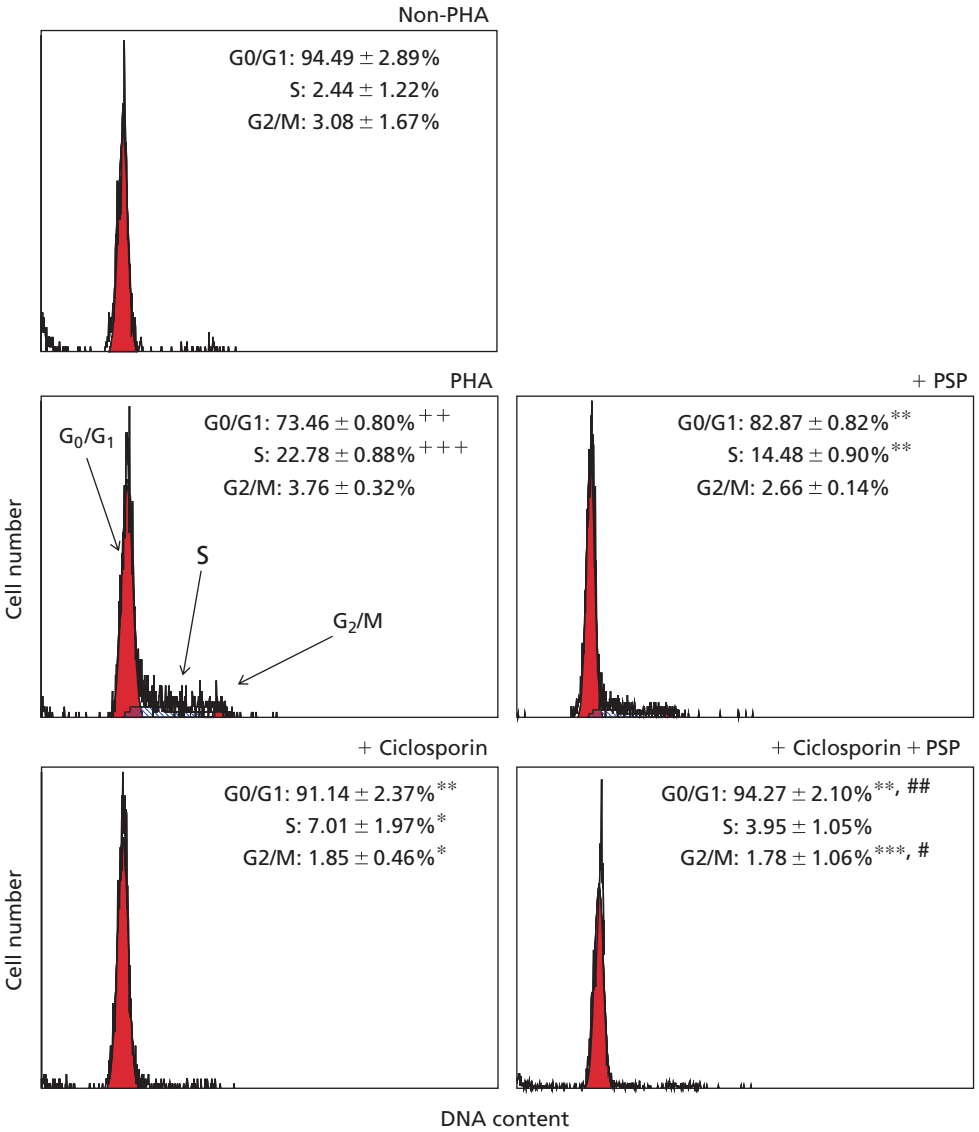

Figure 2 Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on the cell cycle in lymphocytes. Non-phytohaemagglutinin (PHA) and PHA (5 μg/ml)-stimulated lymphocytes were incubated with polysaccharopeptide (PSP; 5 μM), ciclosporin (0.83 μM), or both for 48 h. The cell cycle distribution was determined by flow cytometry with propidium iodide staining. Data are the mean ± SEM, $n = 4$, and compared by Student's *t*-test. $^{*,\ **,\ ***}P < 0.05$, 0.01 and 0.001 PHA + PSP, PHA + ciclosporin, PHA + ciclosporin + PSP vs PHA. $^{\#,\#\#}P < 0.05$ and 0.01 PHA + PSP vs PHA + ciclosporin + PSP. $^{++,\ +++}P < 0.01$ and 0.001 PHA vs non-PHA.

combined. The blockage at the G0/G1 phase was related to the reduced S-phase population, which was significantly reduced, from 22.8% to 14.5% ($P < 0.01$), 7.0% ($P < 0.05$) and 4.0% by PSP, ciclosporin and their co-treatment, respectively. Accordingly, the G2/M phase population was also significantly reduced by treatment with ciclosporin alone and the combination of the two fungal metabolites.

Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on cyclins and cyclin-dependent kinase expression in lymphocytes

PSP and ciclosporin were found to affect the G1–S phase checkpoint protein cyclin E (Figure 3). Cyclin E expression in resting lymphocytes was undetectable, which is consistent with the low population in S and G2/M on cell cycle analysis. Cyclin E was reduced by PSP or ciclosporin in PHA-stimulated cells. Co-treatment with PSP and ciclosporin further reduced the cyclin E expression in PHA-stimulated cells.

Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on cyclin E expression in the lymphocyte cell cycle

To further confirm that cyclin E was involved in the cell cycle effect of PSP and ciclosporin, bivariate cyclin E/PI flow cytometery was performed (Figure 4). The cyclin E expression level in all three phases was elevated after PHA challenge, with the strongest expression in the G0/G1 phase. Treatment with PSP and ciclosporin alone significantly reduced cyclin E expression in the G0/G1 phase, from 17.6% to 11.2% ($P < 0.05$) and 6.7% ($P < 0.05$), respectively. Co-treatment with the two metabolites further reduced the cyclin E expression to 4.3% ($P < 0.05$ vs PHA + PSP group). The expression level of cyclin E in both the S phase and G2/M phase was not affected by either PSP or ciclosporin, or their combination.

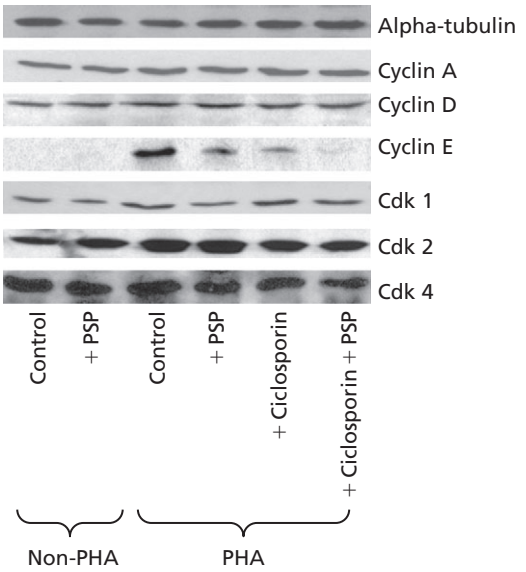

Figure 3 Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on cyclins and cyclin-dependent kinase expression in lymphocytes. Non-phytohaemagglutinin (PHA) or PHA (5 μg/ml)-stimulated lymphocytes in complete RPMI medium were treated with ciclosporin (0.83 μM), PSP (5 μM), or both, for 48 h. Cell lysates were examined by western blot analysis with antibodies to the corresponding cyclins and CDKs. The results are replicates from 4 individual experiments.

Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on the cell death of lymphocytes

Neither PSP alone nor co-treatment with ciclosporin had a significant influence on the viability, apoptosis and necrosis of both resting and PHA-stimulated lymphocytes (Table 2). Fas receptor ($CD3^+CD95^+$) expression in lymphocytes was increased from 8.2% to 54.4% upon PHA stimulation ($P < 0.05$). PSP significantly reduced the Fas receptor expression in resting lymphocytes (8.2% to 3.1%, $P < 0.05$). Treatment with ciclosporin significantly ($P < 0.05$) reduced the Fas receptor level in stimulated lymphocytes from to 54.4% to 39.5% ($P < 0.05$). Co-treatment of ciclosporin with 5 μM of PSP further suppressed the Fas receptor from 54.4% to 20.9% ($P < 0.05$ compared with the PHA + ciclosporin group).

Regulation of *Coriolus versicolor* polysaccharopeptide and ciclosporin on helper T-lymphocyte ($CD3^+CD4^+$), cytotoxic T-lymphocyte ($CD3^+CD8^+$) and B-lymphocyte ($CD19^+$) populations

Immunophenotyping of helper T-lymphocytes, cytotoxic T-lymphocytes, B-lymphocytes and natural killer cells is presented in Table 3. PHA stimulation significantly increased ($P < 0.001$) the percentage of cytotoxic T-lymphocyte marker CD8 and decreased the B-lymphocytemarker CD19 ($P < 0.05$). The increased percentage of $CD8^+$ lymphocytes by PHA was minimized by both PSP and ciclosporin treatment. Ciclosporin, but not PSP, significantly lowered the percentage of helper T-lymphocyte marker CD4 in the proliferating cells from 32.7% to 27.9% ($P < 0.05$). The CD4/CD8 ratio of the proliferating lymphocytes was significantly ($P < 0.01$) enhanced by the co-treatment with PSP and ciclosporin as compared with the PHA group. The percentage of B-lymphocytes was reduced from 14.7% to 12.1% ($P < 0.05$) under PHA stimulation. Ciclosporin treatment further reduced the B-lymphocyte population to 6.0% ($P < 0.001$), but this inhibitory effect was slightly antagonized (reduced to 8.3%) in the presence of PSP ($P < 0.05$).

Discussion

Polysaccharopeptide (PSP), a medicinal microbial metabolite derived from *Coriolus versicolor*, is known to be a modifier of the biological response via its ability to exert regulatory effects on the immune system.[10,11,13,17,18] In this study, we investigated the regulatory mechanisms of PSP on lymphocyte homoeostasis and compared its activity with that of ciclosporin. Our data show that ciclosporin inhibited both resting (non-PHA-stimulated) and proliferating (PHA-stimulated) lymphocytes. In contrast, PSP only suppressed the proliferation of PHA-stimulated lymphocytes. This suggests that PSP may have a distinctive balancing effect on human lymphocyte homoeostasis. The data also showed that homoeostatic activity in stimulated lymphocytes was more powerful with combined use of PSP and ciclosporin than using either fungal metabolite alone.

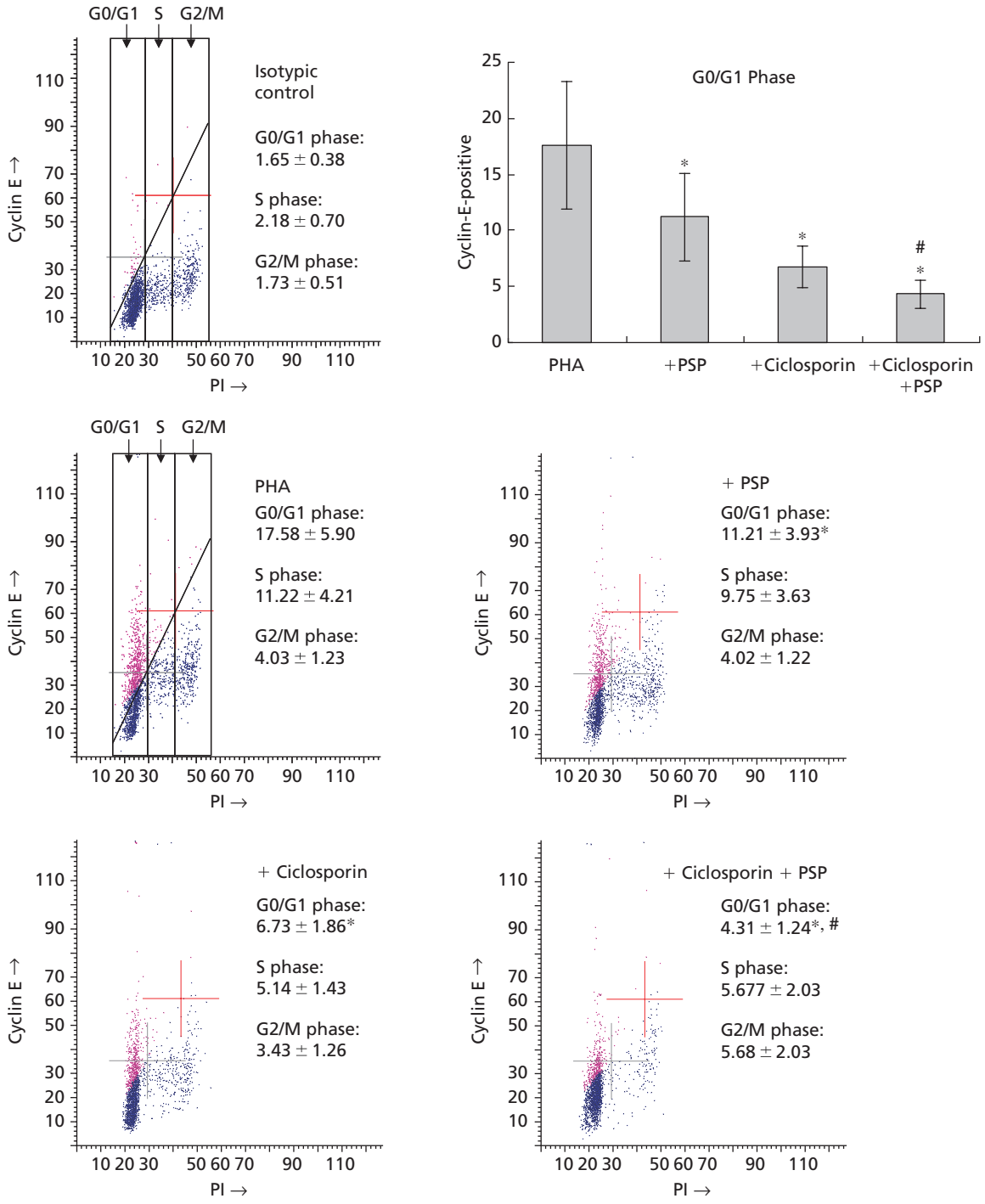

Figure 4 Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on cyclin E expression in the lymphocyte cell cycle. Lymphocytes were stimulated by phytohaemagglutinin (PHA; 5 μg/ml) and treated with polysaccharopeptide (PSP) (5 μM), ciclosporin (0.83 μM), or both, for 48 h by bivariate flow cytometry. The cyclin-E-positive threshold level was set based on the cyclin E isotypic control fluorescent level and calculates the percentage of cyclin-E-positive cells in different lymphocyte cycle phases. Data are the mean ± SEM, $n = 4$, and compared by Student's *t*-test. PI, propidium iodide. $^{*}P < 0.05$ PHA + PSP, PHA + ciclosporin, PHA + ciclosporin + PSP vs PHA. $^{\#}P < 0.05$ PHA + PSP vs PHA + ciclosporin + PSP.

Cell proliferation is often determined by the rate at which viable cells proceed through the cell cycle. Cell cycle analysis revealed that both PSP and ciclosporin are capable of reducing the aberrant proliferation of lymphocytes via arresting the G0/G1 phase transition to the S-phase, which is necessary for DNA synthesis. The G0/G1 phase arrest effect of PSP was additive on co-incubation with ciclosporin. Cell cycle progression is controlled by regulatory cyclins and their CDKs, which oscillate in an orderly manner during the course of cell cycle progression.[20] Western blot and flow cytometry analy-

Table 2 Effect of *Coriolus versicolor* polysaccharopeptide and ciclosporin on cell death and Fas receptor ($CD3^+CD95^+$) expression in lymphocytes

Treatment	Apoptosis	Viable	Necrosis	$CD3^+CD95^+$
Non-PHA	4.21 ± 1.39	87.85 ± 3.27	6.63 ± 1.69	8.21 ± 2.22
+ PSP (1 μM)	4.72 ± 1.59	85.47 ± 3.54	9.09 ± 2.01	7.45 ± 2.29
+ PSP (5 μM)	4.33 ± 1.37	83.30 ± 4.19	11.71 ± 2.86	3.12 ± 0.74*
PHA	9.74 ± 2.91$^+$	66.88 ± 6.23$^+$	19.37 ± 4.32$^+$	54.42 ± 0.82$^+$
+ PSP (1 μM)	9.69 ± 2.90	67.11 ± 5.50	19.49 ± 3.92	54.78 ± 3.46
+ PSP (5 μM)	12.47 ± 3.61	66.51 ± 5.30	17.84 ± 2.89	49.88 ± 4.36
PHA + ciclosporin	14.15 ± 4.56$^+$	66.02 ± 6.34$^+$	16.37 ± 2.88$^+$	39.51 ± 3.63$^{+,\#}$
+ PSP (1 μM)	11.16 ± 3.43	69.08 ± 5.10	17.14 ± 3.53	34.97 ± 3.30*
+ PSP (5 μM)	11.56 ± 3.42	68.33 ± 5.06	17.99 ± 3.30	20.94 ± 1.51*

Non-phytohaemagglutinin (PHA) and PHA (5 μg/ml)-stimulated lymphocytes were incubated with polysaccharopeptide (PSP; 1 and 5 μM), ciclosporin (0.83 μM), or both, for 48 h by bivariate annexin V/DNA-PI and CD3-PE/CD95-FITC staining. Data are the mean ± SEM, $n = 8$, and compared by analysis of variance. $^*P < 0.05$ PSP treated vs without PSP within the same group. $^+P < 0.05$ PHA, PHA + ciclosporin vs non-PHA. $^\#P < 0.05$ PHA vs PHA + ciclosporin.

Table 3 Regulation of *Coriolus versicolor* polysaccharopeptide and ciclosporin on helper T-lymphocyte ($CD3^+CD4^+$), cytotoxic T-lymphocyte ($CD3^+CD8^+$) and B-lymphocyte ($CD19^+$) populations

Treatment	$CD3^+CD4^+$ (%)	$CD3^+CD8^+$ (%)	$CD4^+/CD8^+$	$CD19^+$ (%)
Non-PHA	36.09 ± 1.80	22.54 ± 2.44	1.66	14.71 ± 0.40
+ PSP	39.41 ± 3.19	18.95 ± 1.88	2.17	14.23 ± 1.20
PHA	32.70 ± 0.83	49.46 ± 1.43^{+++}	0.66^{++}	12.11 ± 0.68$^+$
+ PSP	30.34 ± 1.99	37.69 ± 3.30**	0.84	10.85 ± 1.07
+ ciclosporin	27.92 ± 1.64*	24.83 ± 2.95***	1.21	5.97 ± 0.29***
+ ciclosporin + PSP	29.34 ± 1.82	21.15 ± 0.50***	1.40**	8.33 ± 0.18$^{***,\#}$

Non-phytohaemagglutinin (PHA) and PHA (5 μg/ml)-stimulated PBMCs were incubated with polysaccharopeptide (PSP; 5 μM), ciclosporin (0.83 μM), or both, for 48 h. Immunophenotypes of the lymphocytes were identified and quantified by bivariate staining of different CD markers. The positive threshold level setting was set based on the background fluorescent of isotypic control. Data are mean ± SEM, $n = 6$, and compared by Student's t-test. *, **, $^{***}P < 0.05$, 0.01 and 0.001 PHA + PSP, PHA + ciclosporin, PHA + ciclosporin + PSP vs PHA. $^\#P < 0.05$ PHA + PSP vs PHA + ciclosporin + PSP. $^{+,}$ $^{++,}$ $^{+++}P < 0.01$ and 0.001 PHA vs Non-PHA.

ses revealed that the influence of PSP on cell cycle progression shares a similar mechanism with ciclo-sporin, which involved a reduced expression level of the G1–S transitional cell cycle regulatory protein, cyclin E. Activation of cyclin E is required to signal proliferating cells to exit the G1 phase and enter the S phase for undergoing DNA replication, and the reduced expression of cyclin E at the G0/G1 phase would prolong the transition time at the G1–S checkpoint.[21] The present data reveal the regulatory roles of PSP on the cell cycle by controlling cyclin E expression in activated but not normal lymphocytes. PSP exhibited additive inhibitory effects with ciclosporin to suppress cyclin E expression. It has been demonstrated that the blocking of calcineurin by ciclosporin and other drugs also induces G0/G1 cell cycle arrest by reducing cyclin E expression.[22,23] Whether the regulation of the lymphocyte cell cycle by PSP involves calcineurin, however, has yet to be determined.

Our previous in-vitro study reported similar anticancer mechanisms of PSP on the G0/G1–S transition in human leukaemia.[24] However, PSP did not induce cell death in normal human lymphocytes as it does in cancerous cells. The data further supported its safe application as an anticancer chemotherapeutic adjuvant. On the other hand, it is not clear why PSP treatment alone suppressed Fas receptor expression and its co-treatment with ciclosporin enhanced the suppressive effect. The down-regulation of the Fas-receptor suggests a protective role of PSP against extrinsic cell death signals.

The reduction of the $CD3^+CD8^+$ cell population by PSP (Table 3) partially contributed to the suppression of the total lymphocytes. It has been reported that ciclosporin blocked the reduction of CD4/CD8 ratio in stimulated lymphocytes.[25] It is interesting to note that ciclosporin alone suppressed the population of $CD8^+$- and $CD19^+$-expressing lymphocytes, and exerted a selective additional effect on a decline of the inflammatory effector $CD8^+$ lymphocytes as well as a restoration of $CD19^+$-expressing lymphocytes when PSP was added. By promoting the population of $CD19^+$-expressing lymphocytes and inhibiting the population of $CD8^+$-expressing lymphocytes, PSP may influence the progression of the inflammatory response.

Cellular homoeostasis harmonizes the balance between cell proliferation and cell death.[26] Compared with ciclosporin, which suppresses both resting and proliferating lymphocytes, PSP seems to be more beneficial and less harmful in this regard. Our data demonstrated that PSP selectively controls the aberrant proliferation of the activated cells and stimulates the proliferation of the resting cells. The latter property explains the beneficial immunomodulatory function of PSP observed in both healthy subjects and cancer patients. Such selective properties of PSP can be explained by the structural

complexity of its proteoglycan.[14] The glycans in PSP are known to be involved in diverse intercellular, intracellular and cell-matrix recognition events, and it has been reported that modification of the glycan chains may affect the biological activity of a molecule.[27,28] Nevertheless, it is of interest that the structurally complex proteoglycans of PSP apparently allow it to agonistically or antagonistically interact with certain glycan-binding receptors, leading to diverse biological functions.

Conclusions

This study shows that PSP exhibits inhibitory mechanisms similar to ciclosporin in controlling the aberrant proliferation of activated human lymphocytes. PSP is currently undergoing a stage 4 clinical trial, thus far without exhibiting adverse physiological effects. Its selectivity in killing human leukaemic cells, but not healthy lymphocytes, supports its application as an anticancer agent alone or as an adjuvant.[16,24] Indeed, the data presented here show at least the possibility of applying PSP as an adjuvant to reduce the toxicity of ciclosporin. Since serious adverse effects of ciclosporin, such as de-novo cancers and nephrotoxicity, have been reported,[5,6] but no adverse effects of PSP even in very high dosage in both animals and human studies have been reported,[13,29,30] we suggest that further in-vivo studies and clinical trials should be undertaken to determine the role of PSP as an immunomodulatory agent, or adjuvant to ciclosporin, for the treatment of autoimmune and inflammatory diseases.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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