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Polysaccharopeptide mimics ciclosporin-mediated Th1/Th2 cytokine balance for suppression of activated human T cell proliferation by MAPKp38 and STAT5 pathways

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Abstract

The activation of T helper (Th) cell subsets plays an important role in the human immune system. Uncontrolled Th1 and Th2 responses lead to autoimmune and inflammatory diseases, respectively. The identification of agents that modulate the Th1/Th2 cytokines is therefore essential for controlling these diseases. We recently reported that polysaccharopeptide (PSP) from *Coriolus versicolor* exhibited ciclosporin-like activities to control aberrant T lymphocyte activation. Here, we compared the properties of PSP with ciclosporin on cell proliferation, $CD25^+$ expression, secretion of Th1/Th2 cytokines and activation of mitogen-activated protein kinase (MAPK)p38 and signal transducers and activators of transcription 5 (STAT5) on T cells. The data show that PSP alone suppresses the proliferation of activated T cells. PSP exhibited similar and additive inhibitory effects to ciclosporin to suppress activated T cell proliferation, Th1 cytokines and reduce $CD3^+/CD25^+$ cell expression, but not Th2 cytokine expression, which helps the cytokine balance shift towards Th2 dominance. These suppressive actions of PSP involved the MAPKp38 and STAT5 pathways. These findings refine our understanding of the effects of PSP on T lymphocytes and its adjuvant properties with the immunosuppressant ciclosporin for possible control of autoimmune diseases.

Introduction

The immune system involves regulation of homeostasis between cellular and humoral immune activity by cytokines produced from T-helper 1 (Th1) and T-helper 2 (Th2) cells (Mosmann et al 1986). Immunosuppressive drugs with the ability to alter the Th1/Th2 cytokine production profiles are essential in the control of autoimmune and inflammatory diseases. Ciclosporin is a potent immunosuppressive drug found in *Tolypocladium inflatum*. It is useful in the treatment of graft versus host, autoimmune and inflammatory diseases via its ability to control the activation of T cells (Sigal & Dumont 1992). The immunosuppressive effect of ciclosporin is mediated primarily through inhibition of IL-2 from T cells through calcineurin to prevent dephosphorylation of nuclear factor of activated T cell (NFAT) and thereby IL-2 expression (Kronke et al 1984; Agarwal et al 2000). However, ciclosporin is not specific, and long-term use is associated with significant side-effects and toxicities (Sheil 1998; Baran et al 2004). There is thus a need for new immunosuppressants with no or fewer side-effects.

In the search for a new immunomodulatory agent, we have discovered that polysaccharopeptide (PSP) from the medicinal fungus *Coriolus versicolor* suppresses human T cell activities in a similar manner to ciclosporin (Lee et al 2007). Proteomics analysis revealed that both ciclosporin and PSP exhibited inhibitory effect on the expression of proteins that are important for T cell homeostasis (Lee et al 2007). PSP has a molecular size of 100 kDa and contains a polysaccharide and a polypeptide portion (Ng 1998; Cui & Chisti 2003). The structural variability of PSP is thought to provide flexibility in its interaction with different cell constituents and affecting cell–cell interactions in higher organisms. The therapeutic properties of PSP include antiviral, immunomodulating and anticancer activities (Collins & Ng 1997; Ng 1998; Kidd 2000; Ho et al 2004; Yang et al 2005). PSP increases levels of cytokines such as tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2 and IL-6, and IL-2 receptors in non-stimulated immunity, and this

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has been postulated to be beneficial for patients with depressed immunity (Ooi & Liu 2000; Hsieh et al 2002; Ho et al 2004). The diverse mechanisms of PSP in modulating the normal, activated and depressed immunity observed in these studies and in previous studies remain unclear, but imply a potential regulatory role in T cell proliferation.

This study aimed to investigate the ciclosporin-like immunosuppressive mechanisms of PSP on human T cells. This was achieved by measuring the levels of Th1 (IL-1β, IL-2, IL-12, IFN-γ, TNF-α) and Th2 (IL-4, IL-10) cytokines, which PSP and ciclosporin induce similarly, and the additive inhibitory effect on the production of Th1 cytokines. The present data also revealed the involvement of the mitogen-activated protein kinase (MAPK)p38 and signal transducers and activators of transcription 5 (STAT5) pathways in suppressing formation of Th1 cytokines by PSP. The data suggest the potential use of PSP as an adjuvant immunomodulator with ciclosporin for the prevention of T cell related autoimmune disorders.

Materials and Methods

Reagents

Phytohaemagglutinin (PHA), ciclosporin and anti-beta tubulin antibody were purchased from Sigma (St Louis, MO, USA). Anti-human CD3 FITC-conjugated and PE-conjugated CD25 antibodies, STAT1, phospho-STAT1, STAT5 and phospho-STAT5 antibodies were from BD Biosciences Pharmingen (San Diego, CA, USA). Anti-human p38 and phospho-p38 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Human IL-2, IFN-γ and β-actin primers were obtained from the Genome Research Center (The University of Hong Kong); the dNTP mixture and DNA standard marker were purchased from Invitrogen Life Technology (Carlsbad, CA, USA). *C. versicolor* PSP was obtained from Winsor Health Product Ltd (Hong Kong), which is endotoxin free as stated by the manufacturer. The extraction, purification, isolation and HPLC analysis of PSP have been reported previously (Hui et al 2005; Yang et al 2005; Lee et al 2006, 2007; Wan et al 2008). The doses of PSP used in this study were 100 μg mL^{-1} (1 μM) and 500 μg mL^{-1} (5 μM); doses of ciclosporin were 100 ng mL^{-1} (83.1 nM), 1000 ng mL^{-1} (0.831 μM) and 4000 ng mL^{-1} (3.32 μM).

Purification and activation of T lymphocytes

Human lymphocytes were prepared from the buffy layer of blood from healthy individuals, obtained from the Red Cross Blood Transfusion Service (Hong Kong). The T lymphocytes were isolated by Ficoll-Paque density-gradient centrifugation, red cell lysing buffer (NH_4Cl 0.83%, $NaHCO_3$ 0.084%, ethylenediaminetetraacetic acid 0.003%) and cell adherence to polystyrene culture flasks to remove the adhesive monocytes. CD3$^+$ T cell populations were 85% with about 5% variation as measured by flow cytometry. The isolated T lymphocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin and 1% fungizone, and were incubated at 37°C in 5% carbon dioxide.

5-Bromo-2-deoxyuridine (BrdU) cell proliferation assay

Cell proliferation was measured by the incorporation of BrdU (Roche Diagnostics Corp., Indianapolis, IN, USA). Cells (2×10^5) were cultured for 66 h and incubated with BrdU for 6 h. The cells were then dried at 60°C. FixDeant solution (200 μL) was added and incubated for 30 min. Peroxidase-conjugated anti-BrdU-monoclonal antibody (100 μL) was added and incubated for 2 h. The plate was then washed with washing buffer, and incubated with 100 μL TMB for 15 min, after which the reaction was terminated by addition of 25 μL 1 M H_2SO_4. The absorbance was measured at 450 nm with λ correction at 650 nm. Cell proliferation was expressed as: stimulation index = (absorbance of sample − absorbance of blank) ÷ (absorbance of control − absorbance of blank).

Measurement of cytokine production by ELISA

The level of Th1 (IL-1β, IL-2, IL-12, IFN-γ, TNF-α) and Th2 (IL-4, IL-10) cytokines were measured from the culture supernatant of 2×10^5 cells using commercial ELISA kits (BD Biosciences Pharmingen, San Diego, CA, USA). Briefly, the ELISA plate was coated with capture antibodies in sodium bicarbonate buffer (0.1 M, pH 9.5) overnight at 4°C. The wells were washed fives times with 0.05% Tween-20 in phosphate-buffered saline (PBS) between steps. The wells were blocked with 200 μL 10% FBS in PBS for 1 h and then with culture supernatant (100 μL) for 2 h, then incubated with 100 μL detection antibody and avidin horseradish peroxidase conjugate for 1 h. Colour development was achieved by incubation with 100 μL TMB; the reaction was terminated by addition of 50 μL 2 M H_2SO_4. The absorbance was measured at 450 nm with λ correction of 570 nm.

Immunophenotyping of CD25 and CD3 surface markers

T lymphocytes (1×10^6) were washed with PBS and with blocking buffer (1% bovine serum albumin and 0.1% sodium azide in PBS). The cells were incubated with FITC-conjugated anti-human CD3 and PE-conjugated CD25 antibodies in blocking buffer for 30 min at 4°C. Cells positively stained for CD3 (T cell receptor) and CD25 (IL-2 receptor α chain) were analysed at 525 nm and 575 nm using a flow cytometer (Coulter Epic Elite ESP; Beckman Coulter, Inc., Fullerton, CA, USA). The fluorescence signals were analysed by Winlist software (Verity Software House, Topsham, ME, USA).

mRNA analysis by RT-PCR

Total RNA was isolated from 1×10^7 T lymphocytes using the Trizol reagent (Invitrogen) and the RNA concentration was measured by UV spectrometry. Reverse transcription was carried out with 1 μg template RNA in a final volume of 20 μL containing the RT reaction mix and RT enzyme mix (Invitrogen). The contents were incubated at 25°C for 10 min, 42°C for 50 min and 85°C for 5 min. The RT products were incubated with 1 μL (2U) *E. coli* RNase H at 37°C for 20 min.

PCR was preformed in a final volume of 25 μL containing 1 μL RT product, 2 mM $MgCl_2$, 0.25 mM dNTP, 0.2 μM specific

sense and antisense primers, tag polymerase buffer and 1 IU Tag polymerase (Promega, Charbonnieres, France) in PCR buffer. The reaction mixtures were incubated in a thermocycler (icycler, Bio-Rad, Hercules, CA, USA) at 94°C for 3 min to denature the DNA template. PCR amplification consisted of 24–30 cycles of 45 s denaturation at 95°C, 45 s annealing at 59–62°C and 1 min of elongation at 72°C. The sequences of the primers are: IL-2, forward: AGAGGGATTTCACCTA-CATCCA; reverse, AGTTGCATCCTGTACATTGTGG; IFN-γ, forward: AGACATGGCAACAGGTCTCC; reverse, GCCCAGTTCCTGCAGAGTAG; beta-actin, forward, GAT-CAAGATCATTGCTCCTCCT; reverse, ATAGTCCGCCTA-GAAGCATTTG. The PCR products were analysed by electrophoresis in 2% agarose gel, labelled with ethidium bromide and visualized by UV illumination.

Western blot analysis

Whole-cell protein lysate was extracted from 1×10^7 cells per sample with 100 μL lysing buffer (25 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Triton 100 and protease inhibitor cocktails). The cells were lysed at 4°C for 60 min and the cell debris was removed by centrifugation at 13 000 *g* for 15 min. The protein concentration was determined using the Bio-Rad protein assay. For electrophoresis, protein extract was mixed with sample buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) and boiled for 5 min. Proteins in the extract were resolved by 12% SDS-PAGE running with a constant current at 15 mA. The gels were electroblotted on a polyvinylidene difluoride membrane and incubated with primary antibodies specific for beta tubulin, p38, phospho-p38, STAT1, phospho-STAT1, STAT5 and phospho-STAT5 and corresponding horseradish peroxidase-conjugated secondary antibodies. The ECL reagent was used for visualization of the membrane-bound proteins on an autoradiography film. The protein bands were measured and quantified by densitometry and the concentrations expressed as densitometry units (DU) (Quantity One, Bio-Rad).

Statistical analysis

All values were expressed as mean ± s.e.m. The data were analysed statistically using two-way analysis of variance (ANOVA). The effect of different treatments with time on cytokine levels was measured by repeated-measures two-way ANOVA. Following these, a post-hoc test (Tukey's test) was used to discern differences between individual groups. The data were analysed using SigmaStat 2.03 (Jandel Scientific, San Rafael, CA, USA). A P value below 0.05 was taken as significant.

Results

Effect of PSP on proliferation of resting and activated T cells

The effects of PSP on normal T cells and those treated with PHA, ciclosporin or PHA + ciclosporin are summarized in Figure 1. PSP had no effect on the proliferation of resting T cells whereas ciclosporin at 1000 ng mL^{-1} significantly

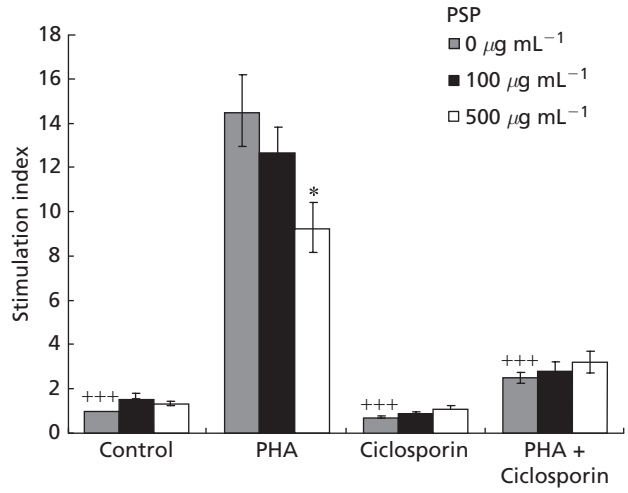

Figure 1 The combined effect of PSP and ciclosporin on proliferation of T lymphocytes. Normal T lymphocytes and cells treated with ciclosporin (1000 ng mL^{-1}), PHA (5 μg mL^{-1}) or PHA + ciclosporin were incubated with PSP (0, 100 and 500 μg mL^{-1}) for 72 h. Cell proliferation was measured by incorporation of BrdU and expressed as stimulation index (SI). Data are mean ± s.e.m. (n = 8). *$P < 0.05$ vs. without PSP; $^{+++}P < 0.001$ for control and PHA + ciclosporin vs. PHA (without PSP).

suppressed the proliferation of resting T cells by 31.2% ($P < 0.001$). PHA stimulation increased the stimulation index of the T cells by 14.46 fold ($P < 0.001$). PSP at 500 μg mL^{-1} suppressed 36.23% ($P < 0.05$) and ciclosporin suppressed 82.7% ($P < 0.001$) of the proliferation of activated T cells. In contrast to stimulated T cells, PSP had no effect on ciclosporin-treated resting or PHA-stimulated cells.

Temporal effect of PSP on Th1 and Th2 cytokine production by proliferating T lymphocytes

Table 1 shows the temporal production of cytokines by proliferating T cells without (control) and with treatment with PSP (500 μg mL^{-1}). In the absence of PSP, the proliferating T cells produced peak amounts of IL-1β and TNF-α at 12 h, IL-10 at 18 h, IL-2 at 24 h, and IFN-γ and IL-4 at 48 h after PHA activation. PSP suppressed the production of Th1 cytokines (i.e. IL-1β, IL-2, IFN-γ and TNF-α), but had no effect on the production of Th2 cytokines (IL-4 and IL-10) by PHA-treated T cells. Production of TNF-α was reduced by 66.47% at 12 h ($P < 0.01$), 79.83% ($P < 0.01$) at 18 h and 69.76% ($P < 0.05$) at 24 h. The level of IL-2 was significantly decreased by 47.75% ($P < 0.01$), 34.44% ($P < 0.01$) and 48.39% ($P < 0.05$) at 18, 24 and 48 h, respectively. Production of IFN-γ was reduced by 83.4% ($P < 0.001$) and 76.67% ($P < 0.01$) at 48 and 72 h, respectively. There was also a significant decrease (40.18%, $P < 0.05$) in IL-1β production at 6 h. Production of IL-12 was undetectable.

Effect of PSP and ciclosporin on Th1 and Th2 cytokine production by T lymphocytes

The combined effect of PSP and ciclosporin on Th1/Th2 cytokine production during the control of T cell proliferation

Table 1 Time profile of the effect of PSP on cytokine secretion by T lymphocytes

Time (h)	Th-1 cytokines (pg mL^{-1})				Th-2 cytokines (pg mL^{-1})	
	IL-1β	IL-2	TNF-α	IFN-γ	IL-4	IL-10
Control						
6	1253.59 ± 162.00	40.00 ± 1.15	1165.60 ± 353.63	9.21 ± 3.39	37.17 ± 1.27	19.03 ± 2.04
12	1993.72 ± 180.94^{+}	72.16 ± 6.13	1992.85 ± 524.17	14.10 ± 4.34	35.98 ± 3.89	81.86 ± 20.84
18	1789.61 ± 184.21	121.05 ± 9.64^{+}	1941.44 ± 529.72	90.86 ± 63.39	33.89 ± 3.39	532.38 ± 175.74^{+++}
24	1727.33 ± 173.67	162.97 ± 25.02^{++}	1341.05 ± 451.20	267.08 ± 176.97	31.08 ± 2.83	421.24 ± 107.87^{++}
48	1462.31 ± 157.32	101.41 ± 8.51	358.48 ± 99.52	999.68 ± 391.46^{+++}	29.86 ± 2.05	332.17 ± 65.28^{+}
72	1205.33 ± 356.57	61.22 ± 7.51	252.81 ± 48.60	699.57 ± 351.52^{+}	30.41 ± 1.75	284.48 ± 71.31
PSP						
6	687.87 ± 219.35*	41.33 ± 6.41	605.72 ± 238.92	16.72 ± 10.52	34.58 ± 1.29	32.22 ± 4.28
12	1555.63 ± 225.23^{++}	50.19 ± 3.45	668.03 ± 77.71**	12.77 ± 7.16	31.78 ± 4.13	208.83 ± 95.16
18	1454.45 ± 197.47^{+}	63.25 ± 6.86**	543.62 ± 65.33**	17.73 ± 8.29	33.90 ± 3.25	538.84 ± 102.12^{+++}
24	1501.60 ± 206.90^{++}	106.84 ± 44.88**	405.43 ± 39.87*	33.15 ± 18.19	34.98 ± 1.62	443.21 ± 81.24^{+++}
48	1408.50 ± 156.44^{+}	52.34 ± 4.71*	246.21 ± 34.20	165.81 ± 116.48***	40.47 ± 2.81	468.15 ± 135.42^{+++}
72	1076.22 ± 189.89	67.80 ± 5.81	193.34 ± 48.17	163.22 ± 110.84**	35.21 ± 4.25	376.88 ± 124.04^{++}

PHA (5 μg mL^{-1})-stimulated human T lymphocytes were incubated with or without PSP (500 μg mL^{-1}) in supplemented RPMI for 6, 12, 18, 24, 48 and 72 h. Cytokines in culture supernatants were measured by ELISA. Data are mean ± s.e.m. (n = 5).
$^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs 6 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control at same time point.

Table 2 The combined effect of PSP and ciclosporin on secretion of Th1 and Th2 cytokines by PHA-stimulated T-lymphocytes

PSP (μg mL^{-1})	Th-1 cytokines (pg mL^{-1})				Th-2 cytokines (pg mL^{-1})	
	IL-1β	IL-2	TNF-α	IFN-γ	IL-4	IL-10
Control						
0	1041.48 ± 239.81	193.33 ± 15.62	2609.38 ± 56.71	631.44 ± 37.70	90.32 ± 4.30	876.17 ± 69.92
100	1085.65 ± 250.45	108.59 ± 11.91	2098.16 ± 54.40***	423.41 ± 96.41***	126.17 ± 27.63	1037.07 ± 81.86
500	1239.27 ± 290.03	67.99 ± 9.53*	633.66 ± 83.70***	141.46 ± 15.79***	87.63 ± 17.51	1224.55 ± 102.53**
Ciclosporin 100 ng mL^{-1}						
0	1165.81 ± 263.47	89.15 ± 14.28	1669.40 ± 27.91^{+++}	310.50 ± 18.23^{+++}	36.47 ± 6.28^{+}	602.39 ± 62.96^{+}
100	841.72 ± 217.79	71.84 ± 7.05	1110.42 ± 54.27***	210.11 ± 29.44	27.72 ± 3.61	746.88 ± 51.37
500	1002.20 ± 230.12	56.80 ± 9.46*	506.25 ± 25.94***	72.82 ± 6.37***	21.32 ± 2.90	993.60 ± 107.82***
Ciclosporin 1000 ng mL^{-1}						
0	1154.32 ± 264.22	70.48 ± 12.47^{++}	2048.65 ± 71.70^{+++}	327.77 ± 14.87^{+++}	31.85 ± 4.98^{++}	417.76 ± 60.84^{+++}
100	980.30 ± 258.21	49.84 ± 7.47	1735.41 ± 61.00***	241.70 ± 26.08	25.36 ± 3.49	589.18 ± 70.88
500	1041.41 ± 250.95	39.64 ± 6.58*	769.17 ± 33.52***	70.35 ± 9.14***	22.84 ± 5.85	734.41 ± 80.58**
Ciclosporin 4000 ng mL^{-1}						
0	1116.09 ± 276.58	32.04 ± 4.58^{+}	2686.10 ± 45.57	266.21 ± 21.94^{+++}	27.19 ± 3.24^{++}	99.61 ± 18.03^{+++}
100	1174.53 ± 296.82	26.04 ± 3.54	2453.42 ± 50.59**	174.07 ± 19.92	19.12 ± 2.08	185.18 ± 33.99
500	1071.25 ± 298.53	24.56 ± 3.03	1296.95 ± 67.14***	50.04 ± 7.89***	16.13 ± 1.93	230.09 ± 43.56

PHA (5 μg mL^{-1})-stimulated human T lymphocytes were incubated with ciclosporin (0, 100, 1000 and 4000 ng mL^{-1}) and/or PSP (0, 100 and 500 μg mL^{-1}). Cells were incubated in supplemented RPMI for 18 h for IL-1β and TNF-α, 24 h for IL-2 and 48 h for IFN-γ. Cytokines in culture supernatants were measured by ELISA. Data are mean ± s.e.m. (n = 12).
$^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ for ciclosporin without PSP treatment vs control (without PSP treatment); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs treatments without PSP under same group (control and ciclosporin at different dose).

with PSP (0, 100, 500 μg mL^{-1}) or ciclosporin (0, 100, 1000 and 4000 ng mL^{-1}) (Table 2) was examined. The IL-1β level was unaffected by PSP or ciclosporin at all the concentrations tested, either singly or in combination. PSP at 500 μg mL^{-1} suppressed production of IL-2 by 64.83% ($P < 0.05$), IFN-γ by 77.60% ($P < 0.001$) and TNF-α by 55.03% ($P < 0.001$) (Th1 cytokines). Ciclosporin suppressed the production of IL-2 and IFN-γ in a dose-dependent manner ($P < 0.05$ and $P < 0.001$, respectively, at 1000 ng mL^{-1}). Unexpectedly, TNF-α was suppressed at low doses ($P < 0.001$) but not high doses of ciclosporin. The suppressive effect of ciclosporin on IL-2, IFN-γ and TNF-α production was further enhanced in cells co-incubated with PSP at most concentrations. At 100 and 1000 ng mL^{-1} ciclosporin, treatment with PSP at 500 μg mL^{-1} further suppressed IL-2 ($P < 0.05$), IFN-γ ($P < 0.001$) and TNF-α ($P < 0.001$) production by

T cells. The enhancing effect of PSP was also found at 4000 ng mL^{-1} ciclosporin on IFN-γ ($P < 0.001$) and TNF-α ($P < 0.001$).

For Th2 cytokines, levels of IL-4 were decreased by ciclosporin used alone at 100 ng mL^{-1} ($P < 0.05$), 1000 ng mL^{-1} ($P < 0.01$) and 4000 ng mL^{-1} ($P < 0.01$), as were levels of IL-10 ($P < 0.05$, $P < 0.001$ and $P < 0.001$ at 100, 1000 and 4000 ng mL^{-1} respectively). PSP alone (500 μg mL^{-1}) significantly increased IL-10 levels by 39.76% ($P < 0.01$) but had no effect on IL-4. PSP at 500 μg mL^{-1} antagonized the reduction of IL-10 induced by ciclosporin at 100 ng mL^{-1} ($P < 0.001$) and 1000 ng mL^{-1} ($P < 0.01$). In addition, correlation analysis (Pearson test) indicated a positive correlation between Th1 cytokines IL-2 and IFN-γ ($P < 0.01$) and Th2 cytokines IL-4 and IL-10 ($P < 0.01$).

Immunophenotyping of IL-2 receptor alpha chain ($CD3^+/CD25^+$) expression by flow cytometry

The expression of $CD3^+/CD25^+$ in the resting human T lymphocytes (non-PHA treated) was only about 2.5% (Figures 2A and B). Upon PHA stimulation, the percentage of $CD25^+$ T cells was increased to 67.5% ($P < 0.001$). The elevation of $CD25^+$ in the PHA-stimulated T cells was significantly reduced to 49.5% by 500 μg mL^{-1} PSP ($P < 0.05$) and to 37.9% by 1000 ng mL^{-1} ciclosporin ($P < 0.001$).

Effect of PSP and ciclosporin on IL-2 and IFN-γ gene expression

The effect of PSP on the Th1 cytokines IL-2 and IFN-γ at the gene expression level is shown in Figure 3. The data indicate that the expression of IL-2 and IFN-γ genes was increased upon PHA activation, and treatment with PSP or ciclosporin antagonized the effect of PHA. The reduction in gene expression was further enhanced when the cells were treated with both agents.

Involvement of MAPKp38, STAT1 and STAT5 pathways in the inhibition of T cell proliferation by PSP and ciclosporin

To examine the effect of PSP on phosphorylation of MAPKp38, STAT1 and STAT5, T lymphocytes were pre-incubated with PSP for 24 h before stimulation with PHA. As shown in Figure 4, the phosphorylation of p38 and STAT1 peaked at 40 min, while STAT5 peaked between 40 and 80 min. Down-regulation of phospho-STAT5 or phospho-MAPKp38 levels was observed when T lymphocytes were pre-incubated with PSP before stimulation. As early as 5 min after PHA treatment, densitometry measurement shows that cells pre-incubated with PSP (500 μg mL^{-1}) reduced MAPKp38 phosphorylation when compared with controls, from 12.5 to 4.5 DU. Although both PSP-treated and non-treated T lymphocytes expressed maximum MAPKp38 phosphorylation at 40 min after PHA stimulation, the intensity of the expression was weaker in the control group (40.4 DU of control vs 24.1 DU of PSP-treated group). PSP also affected phosphorylation of STAT5; cells pretreated with PSP had maximum STAT5 phosphorylation when compared with the control, with peak STAT5 phosphorylation at 40 min (67.8 DU of control vs 29.7 DU of PSP-treated group) and 80 min (72.3 DU of control vs 13.4 DU of PSP-treated group). In contrast, STAT1 phosphorylation was unaffected by PSP.

Discussion

PSP from *C. versicolor* is currently used as an immunomodulatory agent and an anticancer adjuvant, with successful clinical outcomes (Ng 1998; Kidd 2000). We have recently reported that PSP exhibited ciclosporin-like immunomodulatory properties in activated human T lymphocytes using a proteomic approach (Lee et al 2007). To further characterize this immunomodulatory property of PSP, this study investigated the time- and dose-dependent effect of PSP on the production of Th1 and Th2 cytokines of PHA-activated human T cells, both alone and in combination with ciclosporin.

The data show that PSP suppressed activated T cell proliferation, as did ciclosporin (Figure 1). Compared with the effect of ciclosporin, PSP exhibited similar suppressive effects on the Th1 cytokines (IL-2, IFN-γ and TNF-α) but stimulated the production of the Th2 cytokine IL-10 differently to inhibit T cell proliferation (Tables 1 and 2). The different responses between resting and activated T cells to PSP is not surprising because the resting cells are in the G0/G1 phase whereas activated cells are in the S and G2/M phase of the cell cycle. We have previously shown that PSP induces S-phase arrest and apoptosis in cancerous HL-60 cells (Hui et al 2005; Yang et al 2005). Whether the same mechanism of action occurred in the current study has yet to be determined.

Further experiments revealed that the Janus kinase (JAK)-STAT and MAPKp38 signalling pathways were involved in the inhibitory activity of PSP. IL-2 is produced in abnormally high quantities during autoimmune responses and is a central Th1 cytokine involved in the proliferation of antigen-stimulated T cells (Wagner et al 1980). IFN-γ also plays a major role in inflammation and regulates several aspects of the autoimmune responses, such as stimulating the activity of phagocytes and antigen presentation (Boehm et al 1997). TNF-α is a major cytokine that acts as an inflammatory mediator and as a growth factor for inflammatory effectors (Tracey & Cerami 1993). Thus, the selective reduction of IL-2, IFN-γ and TNF-α by PSP suggests that PSP may play important roles in the pathogenesis of human allergy and inflammatory diseases. In contrast, the Th2 cytokine IL-10 is known to promote humoral immune responses and is a potent immunosuppressive cytokine that down-regulates synthesis of Th1 cytokines (Pestka et al 2004). Human IL-10 was able to prevent acute liver rejection with the same efficacy as ciclosporin (Hong et al 2003). By promoting the expression of IL-10 and inhibiting the expression of Th1 cytokines, PSP may regulate the cytokine network and influence the progression of the inflammatory response.

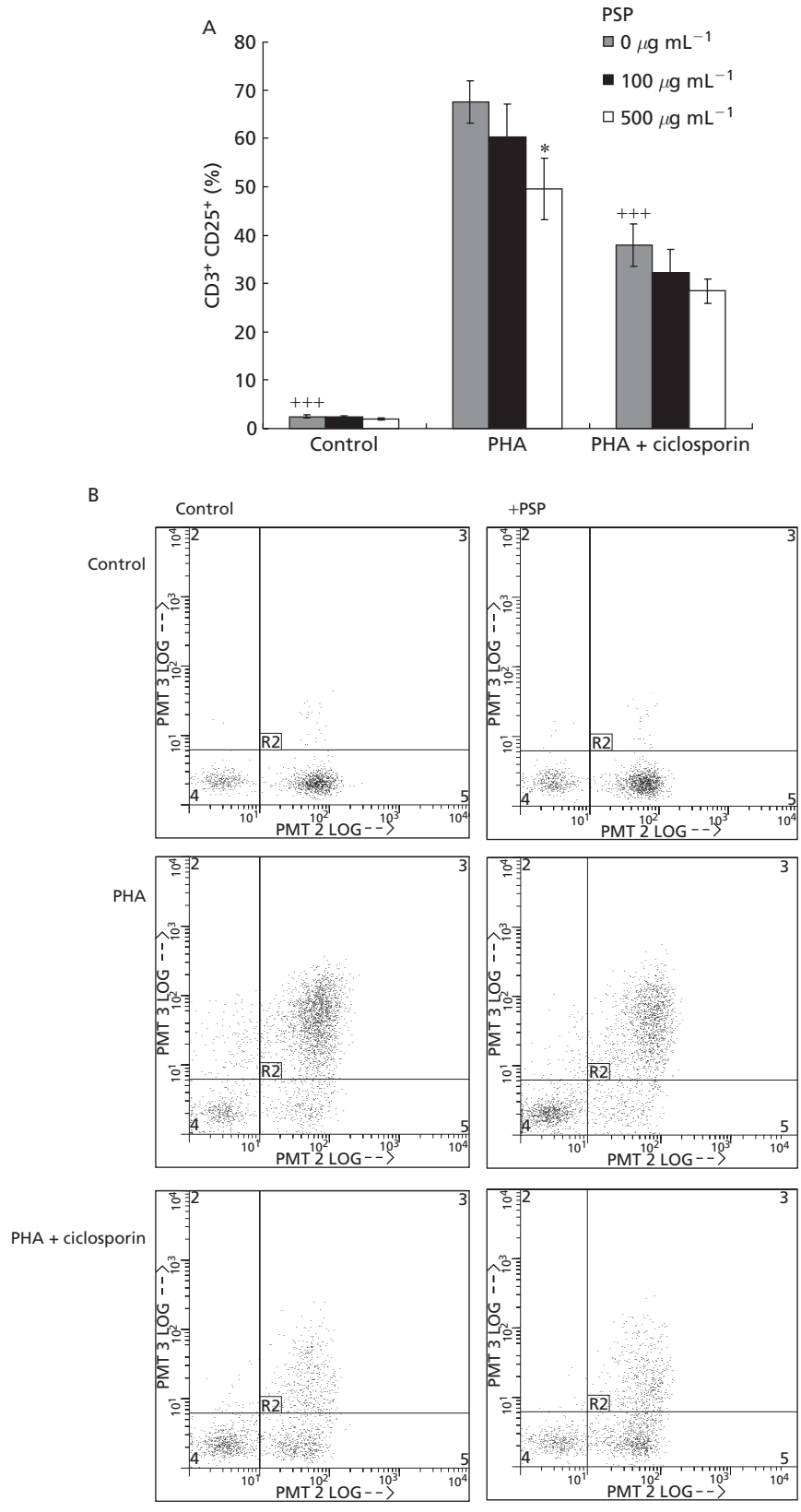

Figure 2 (A) Effect of PSP on IL-2α receptor ($CD3^+/CD25^+$) expression; (B) Dot-plot of IL-2α receptor ($CD3^+/CD25^+$) expression. Normal or PHA (5 μg mL^{-1})-stimulated T lymphocytes were treated with ciclosporin (1000 ng mL^{-1}) and/or PSP (100 and 500 μg mL^{-1}) for 72 h by bivariate flow cytometry. Cells labelled with both fluorescence-labelled anti-CD3 and anti-CD25 antibodies were counted as IL-2α-receptor positive lymphocytes. Data are mean ± s.e.m. (n = 6). $^*P < 0.05$ vs. without PSP; $^{+++}P < 0.001$ for control and PHA + ciclosporin (without PSP).

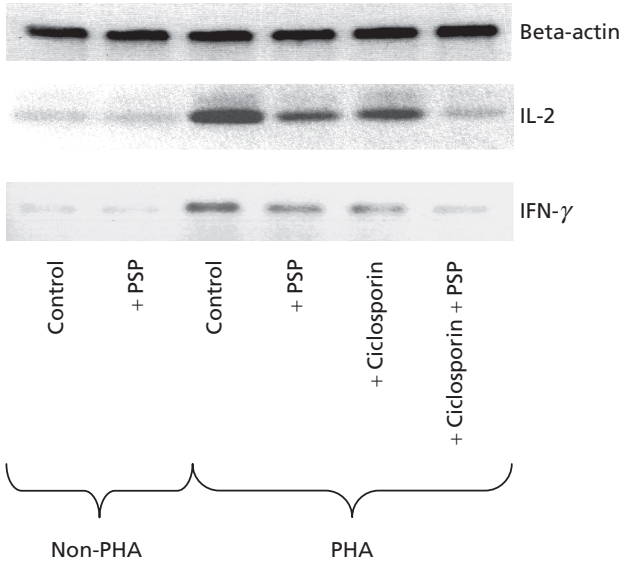

Figure 3 The combined effect of ciclosporin and PSP on IL-4, IL-2 and IFN-γ expression. PHA (5 μg mL^{-1})-stimulated human T lymphocytes were incubated with ciclosporin (1000 ng mL^{-1}) and PSP (500 μg mL^{-1}). Cytokine expression was determined from the reverse-transcripted cDNA by RT-PCR with the corresponding primers. The result is one example from four individual experiments.

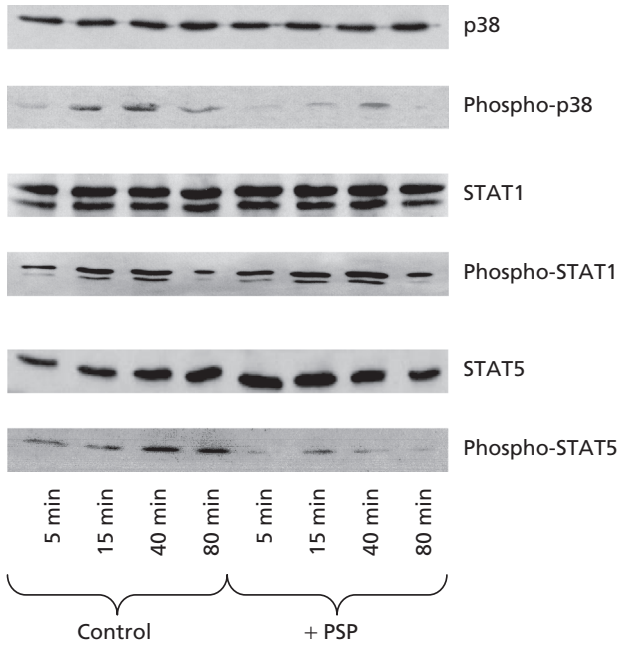

Figure 4 The combined effect of ciclosporin and PSP on MAPKp38, STAT 1 and STAT 5 activation. T lymphocytes were pretreated with/without 500 μg mL^{-1} PSP for 24 h before stimulation by PHA (5 μg mL^{-1}) for 5, 15, 40 and 80 min. Cell lysates were examined by Western blot analysis with antibodies to MAPKp38, STAT1 and STAT5 and their corresponding phosphorylated form. The protein bands were measured and quantified by densitometry. The result is one example from four individual experiments.

Stimulation of T cells induced the subsequent expression of IL-2 and its receptor. Most T cells in the peripheral blood are CD25 negative. The expression of CD25 in activated T lymphocytes has been shown to be associated with cell activation and IL-2 synthesis (Kelly & Siebenlist 1995). Binding of IL-2 with high-affinity IL-2 receptors stimulates an autocrine effect, leading to clonal expansion of T cells. Similar to ciclosporin, PSP significantly reduced the stimulation of T cell proliferation by inhibiting CD25 expression (Figures 2A and B).

Binding of IL-2 and IFN-γ to their receptors leads to phosphorylation of their corresponding receptor-associated JAK, which is crucial to STAT phosphorylation (Shuai et al 1993; Silva et al 1994; Frank et al 1995; Ihle 1996). The activated STAT proteins are translocated into the nucleus, where they regulate transcription of the target genes (Imada & Leonard 2000). On the other hand, phosphorylation of JAK activates MAPKp38 (Lee et al 1994). To further explain the mechanism for the functional change of Th1/Th2 by PSP, we measured the signalling proteins for Th1 (MAPKp38, STAT1 and STAT5) function, and found that PSP suppressed PHA-induced T cell production of cytokines through a similar pathway, by inhibition of tyrosine phosphorylation of STAT5 and MAPKp38 (Figure 4). MAPKp38 but not ERK or JNK is being studied because it clearly plays a critical role in the Th1 inflammatory cytokine network in CD4$^+$ and CD8$^+$ T cells, whereas ERK and JNK are related to diverse downstream targets (Rincón et al 2000; Berenson 2004). MAPKp38 also plays an important role in T cell homeostasis and inflammation regulation (Lee et al 1994; Lavoie et al 1996; Rincón et al 2000). On the other hand, STAT1 and STAT5 are essential signalling molecules in Th1 cytokine-producing cells under stimulation (Gollob et al 1998). They convert IFN-γ and IL-2 signals into gene expression level and they have major roles in cell growth, survival and differentiation through transcriptional regulation (Ihle 1996; Darnell 1997; Ramana et al 2000). However, the present data are preliminary, as MAPKp38 and STAT5 pathways are only part of the pathways involved in ciclosporin-mediated Th1/Th2 cytokine balance. More detailed experimental studies to investigate the involvement of other pathways are still required to get a full picture of how PSP modulates cytokine balance and the immune system.

Unlike the non-selective suppressive activities of ciclosporin on T cells, the immunomodulatory role of PSP is selective. The suppressive effect of PSP on Th1 cytokines has been demonstrated on stimulated T cells in this study, but in other studies has been shown to have the opposite enhancing effect in resting and depressed immunity (Ooi & Liu 2000; Hsieh et al 2002; Ho et al 2004). We speculate that the mechanisms of action involves the unique proteoglycan structure of PSP with its immuno-flexibility and that it shares receptors with some inflammatory mediators. In the resting T cell, the immuno-stimulator is absent and PSP acts as an agonist to give a stimulatory effect, whereas in stimulated T cells PSP acts as an antagonist to the inflammatory mediators to compete with their binding. Structurally complex oligosaccharides appear to have affinities for these receptors and generate immune responses. As the affinity and biological potency of PSP is likely to be weaker than the original immunostimulant for that receptor because of structural difference, the

competition reduces the stimulatory response. Studies with *Astragalus membranaceus*, *Phellinus linteus* and *Ganoderma lucidium* show that their proteoglycan and polysaccharides exhibit binding activity for the toll-like receptor, which is the receptor of bacterial cell surface lipopolysaccharide and other immunogenic ligands, indicating that fungal proteoglycan and polysaccharide are capable of controlling immune function through specific receptor binding (Kim et al 2004; Shao et al 2004a, b). The modulation of the immune system thus appears as a cycle of suppression and activation around a homeostatic attractor, and finally creates a sustained normalization to the immune response (Shinkai et al 2008).

Conclusions

This study shows that PSP alone suppresses the proliferation of activated T cells. PSP also exhibits similar and additive inhibitory effects to ciclosporin to suppress activated T cell proliferation, Th1 cytokines and reduce $CD3^+/CD25^+$ cells expression, but antagonizes secretion of the Th2 cytokine IL-10, which helps the cytokine balance shift towards Th2 dominance. These suppressive actions of PSP involve the MAPKp38 and STAT5 pathways. For clinical consequences, these data suggest a potential role of PSP as an immunomodulatory adjuvant of ciclosporin, based on the following observations: PSP promotes the immunosuppressive effect of ciclosporin on activated T cells and shifts the cytokine balance towards Th2; PSP has no suppressive effect under normal conditions; PSP is non-toxic and does not have side-effects at high clinical doses (Ng 1998); thus, the dosage of ciclosporin could be reduced in order to avoid its side-effects. This preliminary ex-vivo study indicates the promising therapeutic potential of PSP as a single agent or adjuvant with ciclosporin for the treatment of Th1-related disorders such as rheumatoid arthritis and graft rejection. Further in-vivo studies and clinical trials are required to test this.

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