PSP activates monocytes in resting human peripheral blood mononuclear cells: Immunomodulatory implications for cancer treatment

Bhagwant Kaur Sekhon, Daniel Man-Yuen Sze [1,*], Wing Keung Chan [2], Kei Fan, George Qian Li, Douglas Edwin Moore, Rebecca Heidi Roubin

Faculty of Pharmacy, The University of Sydney, NSW 2006, Australia

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ABSTRACT

Polysaccharopeptide (PSP), from *Coriolus versicolor,* has been used as an adjuvant to chemotherapy, and has demonstrated anti-tumor and immunomodulating effects. However its mechanism remains unknown. To elucidate how PSP affects immune populations, we compared PSP treatments both with and without prior incubation in phytohaemagglutinin (PHA) – a process commonly used in immune population experimentation. We first standardised a capillary electrophoresis fingerprinting technique for PSP identification and characterisation. We then established the proliferative capability of PSP on various immune populations in peripheral blood mononuclear cells, using flow cytometry, without prior PHA treatment. It was found that PSP significantly increased the number of monocytes (CD14$^+$/CD16$^-$) compared to controls without PHA. This increase in monocytes was confirmed using another antibody panel of CD14 and MHCII. In contrast, proliferations of T-cells, NK, and B-cells were not significantly changed by PSP. Thus, stimulating monocyte/macrophage function with PSP could be an effective therapeutic intervention in targeting tumors.

1. Introduction

Polysaccharopeptides PSP and PSK are chemically-related microbial metabolites isolated by hot-water extraction from deep-layer cultivated mycelia of the *COV-1* and *CM-101* strains, respectively, of the edible fungus *Coriolus versicolor* (also known as *Trametes versicolor* or *Polystictus versicolor*, with the common name in China of Yunzhi) (Cui & Chisti, 2003). Both PSP and PSK are proteoglycans of approximately 100 kDa with a bioactive β-d-glucan region bound to a protein core. The polysaccharide moiety of PSP includes glucose, mannose, xylose, galactose, arabinose and small amounts of rhamnose (Cui & Chisti, 2003) residues. Analysis of the polysaccharide moiety of PSP, using gas chromatography-mass spectrometry (GC–MS), reveals a predominance of $1 \rightarrow 4$, $1 \rightarrow 2$ and $1 \rightarrow 3$ glucose linkages (molar ratio 3:1:2), together with small amounts of $1 \rightarrow 3$, $1 \rightarrow 4$ and $1 \rightarrow 6$ galactose,

$1 \rightarrow 3$ and $1 \rightarrow 6$ mannose, and $1 \rightarrow 3$ and $1 \rightarrow 4$ arabinose linkages (Ng, 1998). The peptide moiety of PSP contains 18 different amino acids, with aspartic and glutamic acid residues being most predominant (Ng, 1998).

PSP and PSK have been generally characterised in terms of their total protein and polysaccharide contents by separate non-specific colorimetric assays. Such characterisation is inadequate because of the extensive isomerism which may occur within polysaccharopeptides, due to their geometric diversity, variable sequence and linkage forms. A "fingerprint" technique is required to provide a profile of the variable nature of the constituents in order to standardise the extracts. In the Chinese State Drug Administration's specification for Yunzhi powder or PSP (WSi-XG-021–2002), the identity of the substance is determined by comparing the profile obtained by size-exclusion chromatography with that of a reference standard. Size-exclusion gel chromatography is a time-consuming technique for the separation of macromolecules, based on their molecular weight. For PSP, the chromatogram obtained shows a broad band indicating an average molecular weight of 100 kDa. In principle, any polysaccharide of similar average molecular weight would produce a similar profile with insufficient detail to allow satisfactory discrimination between samples. GC–MS is used for the analysis of volatile substances of molecular weight less than 1000 Da, many of which may require prior derivatisation to enhance stability and volatility at operating temperatures that

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* Corresponding author. Tel.: +852 6811 9213; fax: +852 3904 7867.
E-mail addresses: bhagwant.sekhon@sydney.edu.au (B.K. Sekhon), daniel.sze@sydney.edu.au (D.Man-Yuen Sze), wing.chan@sydney.edu.au (W.K. Chan), kei.fan@sydney.edu.au (K. Fan), george.li@sydney.edu.au (G.Q. Li), douglas.moore@sydney.edu.au (D.E. Moore), rebecca.roubin@sydney.edu.au (R.H. Roubin).
[1] Present address: Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hong Kong Special Administration Region, PR China.
[2] Present address: Department of Paediatrics and Adolescent Medicine, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administration Region, PR China.

range up to 300 °C. The method is not applicable to the direct quality analysis of polysaccharopeptides, such as PSP, because of these limitations. However, structural information has been obtained using GC–MS after acid hydrolysis of PSP and chemical derivatisation of the resulting low molecular weight saccharide fragments. On the other hand, capillary electrophoresis (CE) is a relatively straightforward technique that can be used for the separation and characterisation of glycoproteins and polysaccharides, based on their size and electro-osmotic characteristics (Kakehi & Honda, 1996). Recently, CE fingerprint analysis has been applied to herbal medicines in cases where species identification or potential for adulteration are issues in quality control (Rabanes, Guidote, & Quirino, 2012). In particular, one cannot confirm a CE peak based only on retention time, but the CE fingerprint analysis of multi-component mixtures is well recognised as a practical solution for natural product extracts, such as medicinal mushrooms *Antrodia, Ganoderma* and *Cordyceps* (Cheung, Ng, & Hood, 2001; Li, Yang, & Tsim, 2006; Rabanes et al., 2012). The detailed nature of the glucan linkages can be determined after chemical digestion of the macromolecules, whereby GC/MS can then be employed on the resulting lower molecular weight fragments.

The Therapeutic Goods Administration (TGA) in Australia has designated the official name of PSP and PSK as "*T. versicolor* Proteoglycan Concentrate" and the Compositional Guidelines for this substance specify the use of CE as the principal identification method in addition to protein and polysaccharide content (TGA, 2012). In the present study, CE has been used to compare the fingerprint profiles of commercial PSP and PSK extracts obtained from different sources, prior to testing their biological activity.

Both PSP and PSK have been reported to be potent immunomodulators against cancers and infections (Cui & Chisti, 2003; Ng, 1998). Immunomodulatory effects of PSP include elevation of pro-inflammatory cytokines, such IFN-γ, interleukins, such as IL-2; NK cell activity, activation of complement-3, and T-cell proliferation (Yang, 1999). However, the related roles of PSP in immunity remain unclear. Clinical studies on PSP in cancer patients have shown conflicting results in T-cell immunity. For instance, one randomised controlled study of 50 gastric cancer patients receiving surgery and chemotherapy reported an increase in CD4^{+} and in the CD4^{+}/CD8^{+} ratio (Shi, Chen, & Lian, 1993); while a phase II randomised study in 151 inpatients with nonparvicellar lung cancer, receiving chemotherapy, did not find any significant differences in the proportion of CD4^{+}, CD8^{+} or in the CD4^{+}/CD8^{+} ratio (Liao & Zhao, 1993). However, both studies reported an increase in NK cell activity after PSP treatment.

Several *in vitro* studies have reported that PSP stimulates T-cell immunity (Lee, Lee, & Wan, 1999; Liang, Sheng, & Wang, 1999). One study found that PSP significantly stimulated an increase in the CD4^{+}/CD8^{+} ratio of T-cell subsets in PBMCs in a phytohaemagglutinin (PHA) *in vitro* model (Liang et al., 1999). However, it is known that PHA, being a strong mitogen of both T-cell subsets, T-helper (CD4) and T-suppressor cells (CD8), may directly affect the CD4/8 ratio (Jason & Inge, 2000). Therefore, in order to examine the direct immunomodulatory effects of PSP on unstimulated PBMCs, it is vital to conduct the study in the absence of PHA.

In this study we examined the immunomodulatory effects of PSP on PBMCs, without prior PHA stimulation. We evaluated the potential immunomodulatory effects of PSP on PBMCs, using our previously established protocol (Sekhon, Roubin, Tan, Chan, & Sze, 2008). The changes of the relative proportion of various immune cell subsets in PBMCs were delineated using flow analysis with antibodies targeting specific cell surface markers.

2. Materials and methods

2.1. PSPs and chemicals

PSP from the Chinese *Cov-1* strain of *Coriolus versicolor* was obtained from two sources: Essence of Mushroom Yunzhi® capsules from Winsor Health Products Ltd (Hong Kong); and Yun Zhi Tang Tai Jiao Nang® capsules from Shanghai Xin Kang Pharmaceutical Factory (Shanghai, China). The two PSP products were referred to as Winsor (W) and Xin Kang (XK). PSK Trammune® capsules from Vertos Healthcare (Australia) were used as a reference sample for CE comparison. PSK (polysaccharopeptide-Krestin) was derived from the Japanese *P7806* strain of *Coriolus versicolor* and typically contains 30–35% polysaccharides. PHA (Lectin L4144) and GM-CSF were obtained from Sigma (St. Louis, MO, USA). Endotoxin levels of PSP products (W and XK) were measured, using a Lonza Limulus Amoebocyte Lysate QCL-1000 assay kit (Walkersville, MD, USA) according to manufacturer's instructions. The endotoxin concentrations of the PSP samples were comparable to those of other commercial mushroom extracts.

2.2. Preparation of PSP stock solutions

Stock solutions of XK or W in Milli-Q water (5 mg/ml) were gently vortexed for 1 min, followed by inversion, and vortexed again for 1 min. The samples were allowed to settle for 5 min at room temperature. Aliquots were taken from the clear solution for analysis, and referred to as the non-filtered samples. The filtrate obtained after passing the non-filtered solutions through a 0.2 μm filter were referred to as the filtered samples.

2.3. CE fingerprinting of PSP

CE fingerprinting was carried out on a Beckman P/ACE system 5500 (Beckman-Coulter, Fullerton, CA, USA) equipped with a fixed wavelength UV-detector. The separation steps were controlled by Beckman P/ACE Station Version 1.21 software (Beckman-Coulter). Capillary tubing (57 cm total length, 50 cm effective length, 75 μm ID, 375 μm OD) was mounted in a standard Beckman cartridge. The capillary temperature during electrophoresis was maintained at 25 °C. The capillary was prepared by rinsing in succession with 1 M HCl for 2 min, distilled water for 2 min, 1 M NaOH for 2 min and then distilled water for 5 min. Before each injection, the capillary was rinsed with 0.1 M NaOH for 2 min, distilled water for 2 min and then rinsed with run buffer for 2 min. Samples were diluted in the running buffer and then were injected with pressure for 5 s with the following optimal standard separation conditions: voltage at 20 kV, capillary temperature 25 °C, UV detector wavelength 214 nm and the running buffer 20 mM di-sodium tetraborate, pH 9.73.

2.4. Isolation and culture of human PBMCs

PBMCs were obtained from 5 healthy donors' buffy coat preparations with prior consent from the Australian Red Cross Blood Service (Sydney, Australia). PBMCs were isolated by standard Ficoll-Hypaque gradient separation (GE Health Science, Uppsala, Sweden). The PBMCs were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) with 10% foetal bovine serum (Invitrogen, Life Technologies, CA, USA), 1% penicillin–streptomycin (Invitrogen), 2 μM l-glutamine (Invitrogen).

2.5. Cell count and viability assays

Before culturing in 96 well flat-bottom plates, PBMCs cell count was determined by 0.5% (w/v) crystal violet (Sigma) and viability by 0.5% (w/v) trypan blue (Sigma, St. Louis, MO, USA), using a Neubauer haemocytometer.

2.6. ATP cell proliferation assays

PBMC proliferation after PSP stimulation was performed using the ATP-based assay, as previously described (Sekhon et al., 2008). Briefly, PBMCs were suspended in supplemented RPMI-1640 media and cultured on 96 well plates at 2×10^4 cells/well and incubated overnight at 37 °C in a humidified, 5% CO_2 atmosphere. The next day, XK and W (non-filtered or filtered, 1 mg/ml) were serially diluted 10-fold (0.1, 1, 10, and 100 μg/ml) immediately prior to use. PHA (2 μg/ml), added to PBMCs without any PSP, was used as a positive control. The cells were incubated at various time points (0, 24, 48, and 72 h). In the last 40 min, the plate was left to equilibrate at room temperature, and 50 μl of ATP reagent (Promega, Australia) were added. The plate was then placed on an orbital shaker for 2 min and then equilibrated at room temperature for 10 min. The luminescence was read in a POLARstar plate reader (BMG Labtech, Australia). Assay was performed in quadruplicate. The data are represented as the means ± standard error of the mean (SEM) of five independent experiments.

2.7. Flow analysis of W-treated PBMCs

PBMCs, at 2×10^6 cells/well, were cultured in supplemented RPMI-1640 media on 24 well plates overnight at 37 °C in a humidified, 5% CO_2 atmosphere. W was filtered and added at the following doses: 1, 3, 10 and 100 μg/ml, followed by incubation for 48 h. PHA (2 μg/ml) was added to PBMCs without W as a positive control. The cells were then harvested and stained with various fluorochrome conjugate combinations. Phycoerythrin (PE) –conjugated and fluorescein isothiocyanate-conjugated (FITC) monoclonal antibodies (MAbs) and mouse IgG1 isotype controls were purchased from BD Biosciences (Palo Alto, CA) and Dako (Glostrup, Denmark). B cells ($CD19^+$), T cells ($CD3^{+-}CD4^+$, $CD3^+CD8^+$), NK cells ($CD16^+CD56^+$, CD56+, CD3+) and monocytes ($CD14^+CD19^-CD3^-$ and $CD14^+MHCII^+$) were incubated with MAbs for 20 min at 4 °C in the dark at the volume recommended by the manufacturer (20 μl/10^6 cells). The stained cells were washed and fixed in 1% paraformaldehyde (Sigma). Samples were analysed on a FACSCalibur Sort flow cytometer (BD Biosciences, Palo Alto, CA, USA) at 0 and 48 h. Typically, 20,000 events were collected and gated according to the relevant isotype control. The data were analysed with FlowJo software (Tree Star, Inc. Ashland, USA).

2.8. Statistical analyses

Results are expressed as either means ± standard deviation of the mean (SD), or means ± standard error of the mean (SEM). Statistical analyses between groups were performed using a one-way ANOVA, followed by post hoc tests when appropriate. Statistical significance was achieved when $p < 0.05$ and $p < 0.01$ was considered to be highly significant when compared to the untreated PBMCs control.

3. Results

3.1. Standardisation of CE fingerprinting method for PSP

PSP commercial products were examined using the Australian TGA method specified for the PSK active ingredient, T. versicolor proteoglycan concentrate (TvPC). According to the TGA compositional guidelines, TvPC should contain 30–35% polysaccharides and 10–40% protein. A reference standard is not currently available for TvPC, requiring the following approach to be used in establishing a working standard.

Samples from six different batches of TvPC prepared by the TGA method were obtained with certificates of analysis that showed an average polysaccharide content of 32.3% ± 1.8 and average protein content of 22.3% ± 2.4. The six samples were tested by CE in order to establish a standard for fingerprint identification of PSP. Under the experimental conditions used, all samples gave reproducible electropherograms containing the characteristic profile, shown as the Reference Standard in Fig. 1. The four major peaks at about 4.8, 5.2, 5.3, and 6.7 min were compared between samples in terms of the migration time relative to the unretained components, represented by the small broad peak at about 3.2 min, and the relative peak area, expressed as a percentage of the total area under all peaks between 3 and 10 min. All samples were measured at 5 mg/ml in water.

The variability between samples of the relative migration times of the major peaks did not exceed 1.2%, while the relative peak areas varied by no more than 3.9% in intra-day experiments. The inter- day variabilities, on the basis of analysing three replicate samples on separate days, were below 2.7% and 8% for relative migration time and peak area, respectively. Because of the wider variability between days due to slight variations in buffer composition, temperature and detector response, the importance of using a reference standard in parallel, under the same conditions, was established, as is the case in chromatographic analysis. The electropherogram for batch number 050408, shown in Fig. 1, was nearest to the average from the 6 batches, so this batch was taken as the reference standard for TvPC.

The stability of the TvPC sample solutions was tested by storage for 24 h at ambient temperature in comparison with aliquots of the same solutions kept at 4 °C for the same time. The electrophoretic profiles showed no significant variation (within the above limits) for the samples stored at 4 °C for 24 h. However, slight variations in the profile (peak height changes and the appearance of some small additional peaks) were observed for the sample solutions stored at ambient temperature. This indicated that TvPC in aqueous solution was liable to undergo degradation if not stored at a low temperature.

3.2. Fingerprint profiles of PSP and PSK products

The three commercial products, XK, W, and PSK, were dissolved in water to a concentration of 5 mg of capsule contents/ml. Dissolution was assisted by sonication for 10 min in a water bath at 80 °C. Some insoluble material was removed by centrifugation before analysis by CE. Reference standard TvPC (5 mg/ml) was analysed before and after the product sample solutions, under the same conditions. Solutions were also tested after filtering through a 0.45 μm membrane filter, but no difference was observed. The electropherograms for the 3 products are compared with the Reference Standard in Fig. 1.

The CE profile for PSK was identical in appearance to that of the TvPC reference standard, but the peak areas were slightly smaller than that of the standard. This difference is attributed to the fact that each capsule contains 500 mg of the active ingredient with
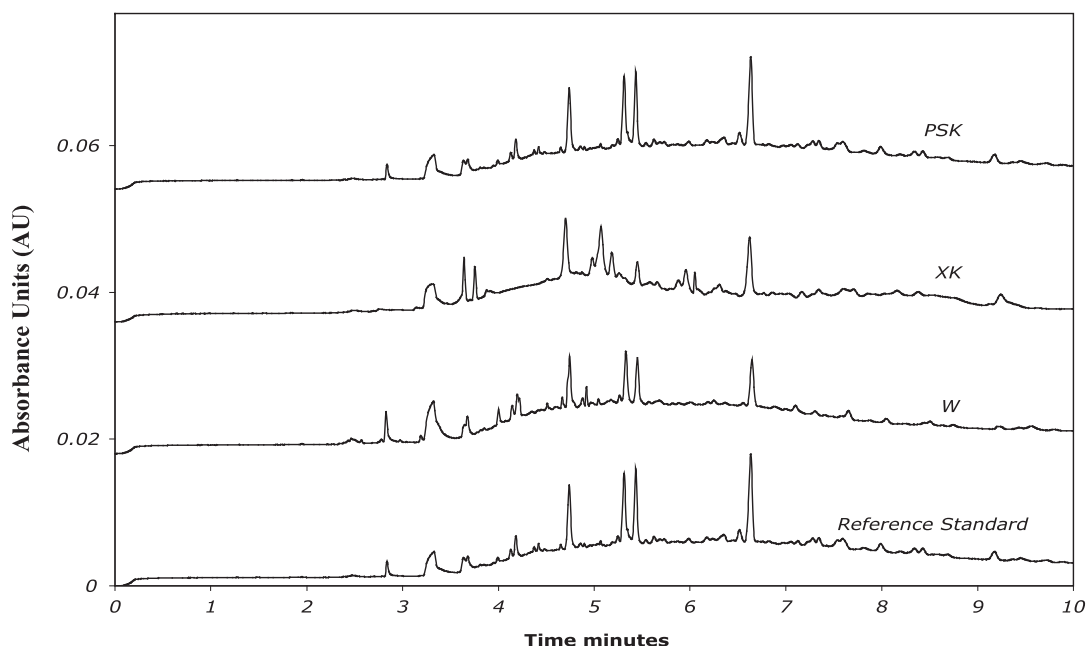
Fig. 1. The electropherograms for the PSP products (W, and XK) compared with PSK and TvPC reference standard. The CE profile for PSK was identical in appearance to the TvPC reference standard although the peak areas were slightly smaller than that of the standard. The CE profile for W showed a close correlation with the standard in relation to the appearance of the major peaks. The CE profile for XK had similarities to the standard in regard to the peaks at 4.8 and 6.8 min, but the patterns between these peaks were quite dissimilar, suggesting that the composition of XK was somewhat different from that of the standard TvPC. Each sample was injected at least three times within each day and on separate days.

about 44 mg of excipients (information provided by the manufacturer).

The CE profile for W showed a close correlation with the standard, particularly in relation to the appearance of the major peaks. However, the peak areas were lower, by approximately 30% compared to the standard. The label statement of product W is that each 400 mg capsule contains "100% essence of Yunzhi". This was verified by weighing, indicating that no excipients were involved. If the area under the peaks in the CE profile is taken as representing the amounts of the active ingredients, the possibility arises that W would not be as concentrated as PSK.

The CE profile for XK had similarities to the standard, particularly in regard to the peaks at 4.8 and 6.8 min, but the patterns between these peaks were quite dissimilar, suggesting that the composition of XK was somewhat different from the standard TvPC.

3.3. Proliferative effect on human PBMCs after filtered PSP treatment

To ensure that filtering the PSP samples did not affect their bioactivity, we determined whether non-filtered versus filtered XK or W had different proliferative effects on human PBMCs. We tested a 10-fold concentration range of XK and W incubated with 2×10^4-PBMCs/well for 48 h and the luminescence was measured using the ATP cell viability assay (Fig. 2a–d). As shown in Fig. 2a, non-filtered W, at 10 µg/ml, showed a significant increase in PBMCs cell viability when compared to the untreated PBMCs control ($p < 0.05$). At the highest concentration (100 µg/ml), the non-filtered W was found to be cytotoxic to PBMCs, causing a significant decrease in cell viability ($p < 0.05$) when compared to the untreated PBMCs control (Fig. 2a). Non-filtered XK, at 0.1 and 1 µg/ml, showed proliferative effects on PBMC though the effect was not statistically significant (Fig. 2c). The higher concentrations of filtered XK, including 10 and 100 µg/ml, on PBMCs appear to be consistent with those of filtered W. However, both filtered W

and XK did not show any significant proliferative or cytotoxic effect at any of the concentrations tested (Fig. 2b and d).

3.4. PSP product W activates monocytes, but not T, B, and NK cells

In our ATP studies, we found that 10 µg/ml of non-filtered W stimulated the proliferation of PBMCs. We hypothesised that this proliferation might be due to the expansion of specific subsets of PBMCs rather than non-specific expansion. Since the ATP assay is limited to measuring relative proliferation percentages of PBMCs and not the subsets responsible, we decided to investigate the effects of PSP on PBMC subsets, using flow cytometry. To test our hypothesis, filtered W was chosen as the material for the subsequent study because of its clear separation of peaks obtained by CE, as seen in Fig. 1. Filtered W was also chosen to eliminate any stimulation caused by particulate matter, consistent with other reports on preparation of PSP for in vitro studies. From the trypan blue exclusion assay, we confirmed that there was no significant decrease in viability for any of the concentrations of W tested compared to untreated counterparts (Fig. 3).

To examine the specific subsets of PBMC expansion, we used the following monoclonal antibody combinations to identify the following major immunological cell subsets: B cells ($CD19^+$), T cells ($CD3^+CD4^+$ or $CD3^+CD8^+$), NK cells ($CD16^+CD56^+$), and monocytes ($CD14^+CD19^-CD3^-$ or $CD14^+MHCII^+$). For the lymphoid cells, W (1, 3, 10 and 100 µg/ml) did not increase the expression of the $CD56^-/CD3^+$ T cells, nor the $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells (Fig. 4a–c). There was no significant change in the percentage of $CD19^+$ B and $CD56^+/CD3^-$ NK cells (Figs. 4d and 5e). PHA, a well-known mitogen for T cells was used as a positive control and it stimulated the proliferation of $CD4^+CD8^+$ T cells from 1.6% ± 0.3 to 12.4% ± 4.6 ($p < 0.05$) when compared to the untreated PBMCs control (Fig. 4c). PHA also increased the expression of $CD56^+/CD3^-$ NK cells from 7.5% ± 2.3 to 13.6% ± 3.5 ($p < 0.05$) and $CD19^+$ B cells from 4.2% ± 2.0 to 14.9% ± 1.0 ($p < 0.05$) when compared to

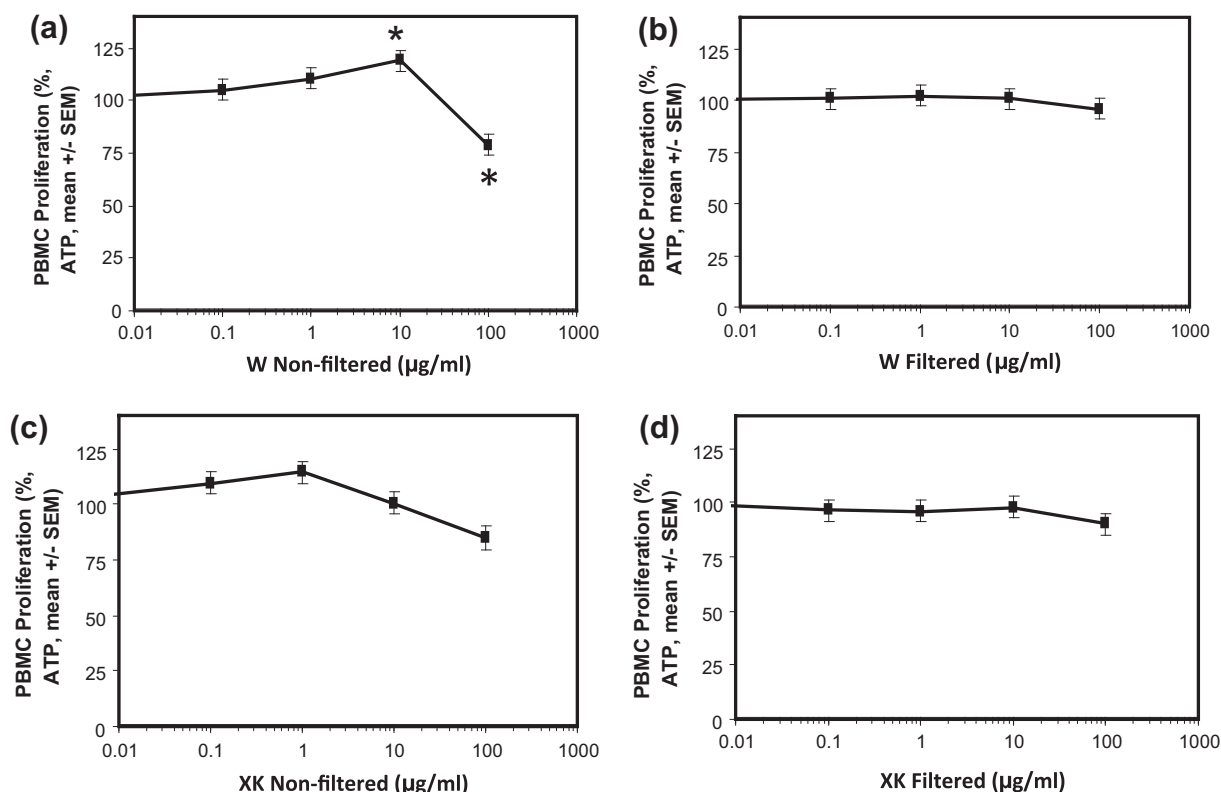**Fig. 2.** Absence of PBMC stimulation after PSP treatment. PBMCs were incubated with PSP (W/XK) at the doses 0, 1, 3, 10, and 100 µg/ml for 48 h. (a) XK (non-filtered) stimulated PBMC proliferation at 10 µg/ml and was cytotoxic at 100 µg/ml. The results represent the means ± SEM of four replicates of five independent experiments performed. (b) XK (filtered) did not have stimulatory or cytotoxic effects on PBMCs. The results represent the means ± SEM of four replicates of five independent experiments performed. (c–d) W (filtered and non-filtered) had no stimulatory or cytotoxic effects on PBMCs. The results represent the means ± SEM of four replicates of five independent experiments performed. $^*p < 0.05$, $^{**}p < 0.01$ compared to the untreated PBMCs control.
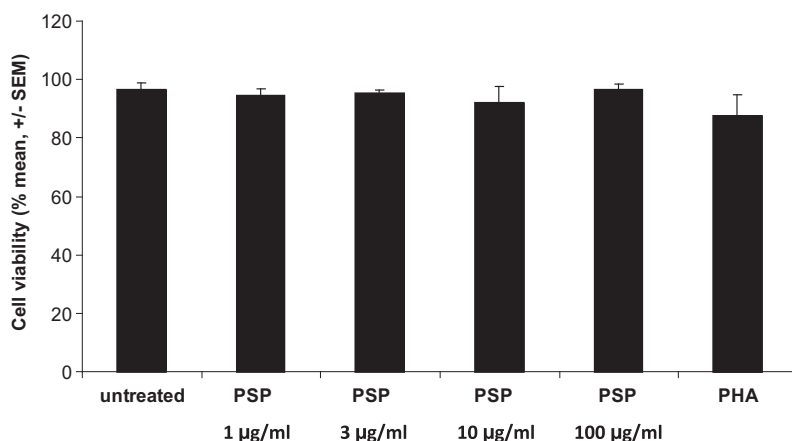

Fig. 3. Cytotoxicity testing of PSP. There was no significant decrease in viability for any of the concentrations of W tested compared to untreated counterparts, using the trypan blue exclusion assay.

the untreated PBMCs control (Fig. 4d and e). This increased expression may be the result of indirect stimulation via T cells.

For filtered W treatment at 100 µg/ml, monocytes were the only immune subset that was found to increase. This was shown by the increase in the percentage of CD14$^+$/CD16$^-$ subset from 3.45% ± 0.8 to 7.9% ± 0.5 ($p < 0.01$), when compared to the untreated PBMCs control (Fig. 5a and b). This result was confirmed by measuring the same subset using another antibody combination, MHCII and CD14 (Fig. 5c). This increased expression in the monocyte subset was dose-dependent at 10 µg/ml (from 6.4% ± 1.0 to 12.1% ± 1.3,

$p < 0.01$) and 100 µg/ml (from 6.4% ± 1.0 to 13.0% ± 1.2, $p < 0.01$) when compared to the untreated PBMCs control.

4. Discussion

PSK, or Krestin, is a polysaccharopeptide used in Japan as an adjuvant to conventional cancer treatment since 1977 (Yang et al., 1992). PSP has been used in China as an immune-enhancer and has been the subject of research for its anti-cancer and immune-enhancing effects for more than 25 years with many
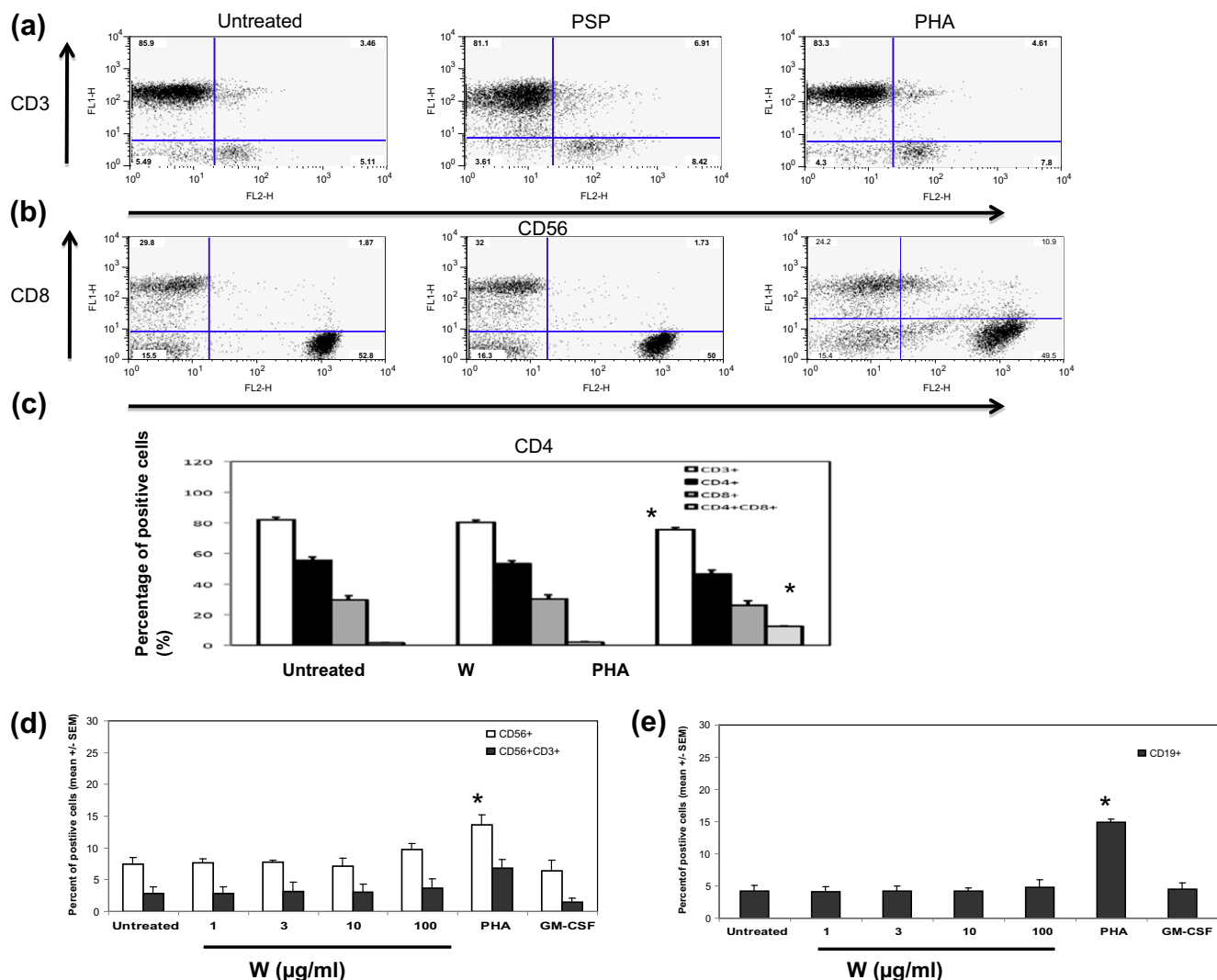
Fig. 4. Absence of T cell, NK cell and B cell activation after W treatment. (a) The distribution of CD3⁺CD56⁻ T cells after incubation with 100 μg/ml of W with PHA as positive control. W, at 100 μg/ml, was incubated with PBMCs for 48 h and then stained for CD3⁺CD56⁻ flow cytometry analysis. (b) The percentages of helper and cytotoxic T cells were analysed by staining CD4 and CD8, respectively. The results were one representative of five independent experiments. (c) The inter-donor variations in effects of W on T cell subsets percentages were summarised as means ± SD from five independent experiments. (d) The distribution of NK cells after incubation with different doses of W (1, 3, 10, and 100 μg/ml). The percentage of NK cells were analysed by staining for CD56⁺ and CD3⁻. The results were one representative of five independent experiments. (e) The percentages of B cells were analysed by staining for CD19 expression after incubation with different doses of W (1, 3, 10, and 100 μg/ml). The results were one representative of five independent experiments.*$p < 0.05$, $_{**}p < 0.01$ compared to the untreated PBMC control.

in vitro, in vivo and clinical studies aiming to demonstrate corresponding immunological changes, but the immunological mechanism of action has yet to be elucidated. Despite its wide usage and lack of clear mechanism of action, there is currently no simple chemical fingerprinting method that can be used for the identification and characterisation of PSPs. We report the development of a CE method for fingerprint analysis of PSP derived from *Coriolus versicolor*. The fingerprints of 6 batches of PSK were obtained with a standardised procedure. The fingerprint of PSK showed "4" common peaks as PSP W, representing the characteristics of the herb's constituents. The intra-day and inter-day precisions for relative migration times and peak areas were (1.2–2.7%, and 3.9–6.2%), respectively. The primary application in qualitative identification and consistency assessment of PSP was investigated with a limited number of samples. PSK can therefore be used as a reference sample for the identification of PSPs. Currently, CE is not used as a routine analytical method for PSPs, though it is clearly a versatile technique with additional benefits compared with other techniques, such as high separation efficiency, high speed, low cost of analysis, low solvent consumption and rapid method development,

that can be easily applied to PSP. The PSP and PSK extracts contain the non-cellulosic structural components that comprise about half the mass of the mycelial cell walls. Gel chromatographic methods have only been able to define the material as a broad peak of average molecular weight 100 kDa, whereas the CE technique can resolve it into several peaks. It is likely that these peaks represent polysaccharopeptides of discrete molecular weight contributing to the cell wall structure. As yet, a suitable supporting electrolyte has not been found to enable the application of a mass spectrometric detector to the CE separation of these macromolecules.

We report that non-filtered W (10 μg/ml) at 48 h showed a significant increase in the proliferation of PBMCs; conversely non-filtered W (100 μg/ml) was cytotoxic compared to the untreated PBMCs control. Interestingly, non-filtered XK (0.1–1 μg/ml) showed proliferative effects on PBMCs; however, the effect was not statistically significant. In contrast, one study showed that, after co-incubating lymphocytes for 48 h with PHA (100 μg/ml) and sterilised XK (6.25–400 μg/ml), there was a significant increase in proliferation of PHA-activated lymphocytes at all doses and this was most potent at 50–100 μg/ml (Liang et al., 1999).
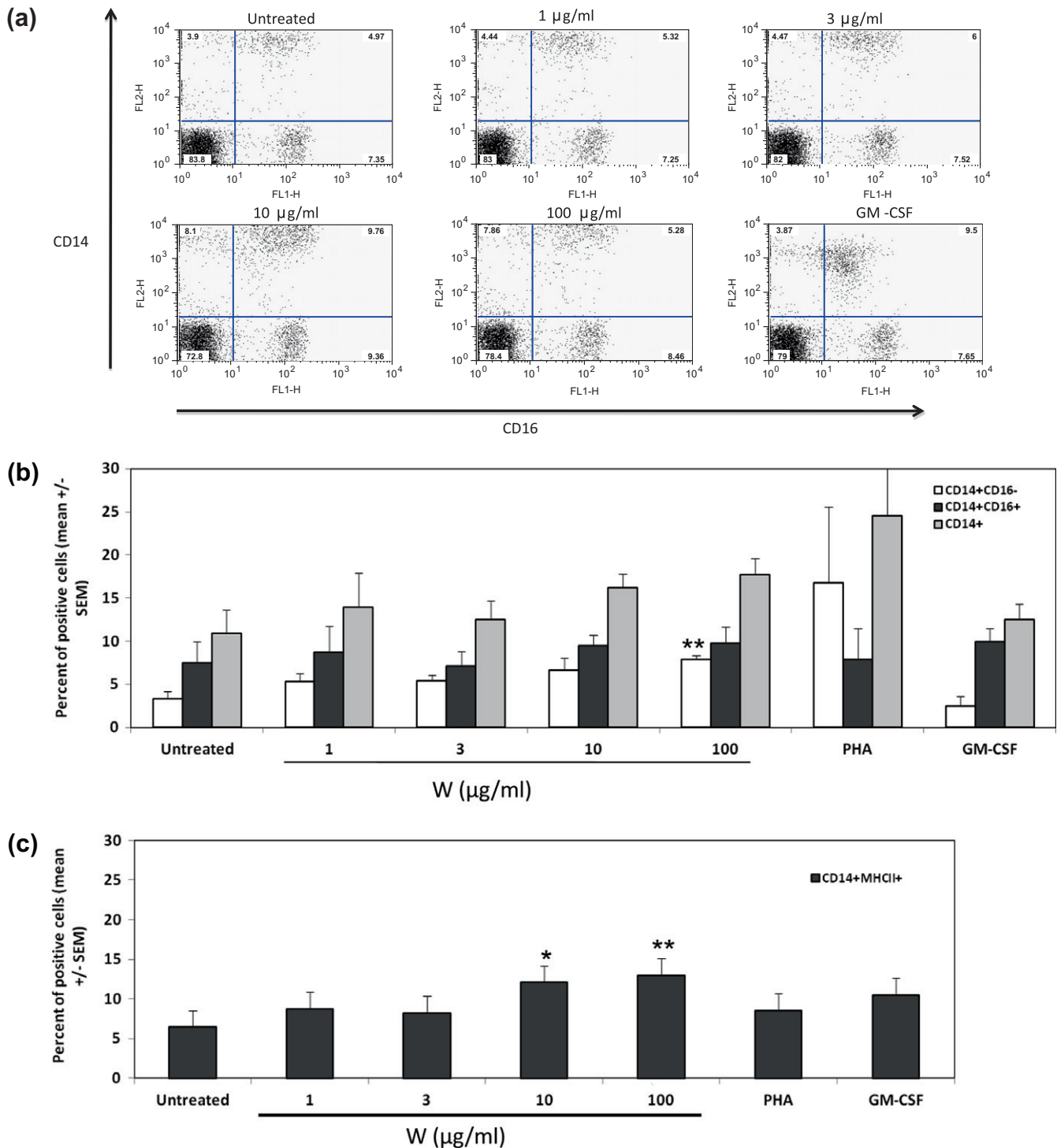
Fig. 5. PSP product W at the doses of 0, 1, 3, 10, and 100 µg/ml stimulated an increase in the expression of CD14⁺CD16⁻ after 48 h. (a) The CD14⁺ cells were up-regulated across the increasing doses while the CD14⁺CD16⁺ monocyte subset remained as untreated control. The results were from one representative experiment of five independent experiments performed. (b) The CD14⁺CD16⁻ monocytes were significantly up-regulated. The results were the means ± SD from five independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared to the untreated PBMCs control. (c) The percentage of MHCII⁺ cells in CD14⁺ populations after treatment with PSP at 1, 3, 10, and 100 µg/ml. The results were the means ± SD from five independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared to the untreated PBMCs control.

Our results demonstrate that filtered PSP is not able to induce proliferation of PBMCs, in contrast to the previous reports that have shown that PSP can induce proliferation in a subset of PBMCs (lymphocytes) after PHA activation.

In this study, we report, for the first time, that, in the absence of PHA, PSP increases the proportion of monocytes, but not T-cells, B-cell or NK-cell subsets of PBMCs *in vitro*. We found that PSP treat-ment of PBMCs at 100 µg/ml significantly increased CD14⁺/CD16⁻ monocytes. Classical monocytes, considered the major subset of monocytes, are strongly positive for the CD14 cell surface molecule (CD14⁺CD16⁻ monocytes). These classical monocytes are more ma-ture than a minor subset of CD14⁺/CD16⁺ monocytes which has been shown to have high human leukocyte antigen-DR expression and high antigen-presenting cell activity (Ziegler-Heitbrock, 2007).

The increase in monocytes we observed was confirmed by positive staining, for both MHCII and CD14. This further supported the role of PSP in antigen presentation.

Clinical trial research and case studies in cancer patients on PSP treatment have demonstrated increases in NK cells' subsets (Shi et al., 1993; Liao & Zhao, 1993; Sun & Zhu, 1999) and in T-cell subsets, CD4/8 ratio (Shi et al., 1993; Sun & Zhu, 1999). In contrast, other researchers did not find significant changes in the CD4/8 ratio after PSP treatment (Liao & Zhao, 1993; Zhang, Zhong, & Zhou, 1999). Our results are consistent with previous studies reporting no significant changes in the CD4/CD8 ratio in PBMCs after PSP treatment. In contrast, we report significant changes in the proportion of monocytes that were not observed in the clinical studies. This could be explained in that monocytes were not investigated in the clinical studies, where a greater emphasis was placed on evaluating shifts in the CD4/8 ratio and NK cells function that cover a large repertoire of immunological responses.

Since cancer patients vary between having localised and widespread metastases, stimulating monocyte/macrophage function with agents such as PSP can be an effective therapeutic intervention in targeting tumors. Though monocytes can only differentiate into macrophages after infiltrating tissues, they appear to play an important role in tumor eradication. Particularly, most malignant tumors appear to be infiltrated by macrophages, which can comprise more than 50% of the tumor mass (Leek et al., 1996). These tumor-associated macrophages (TAMs) are capable of releasing angiostatic compounds and killing tumor cells, though there are reports that TAMs are also capable of stimulating tumor growth by producing angiogenic factors and metalloproteinases (Leek et al., 1996; Polverini, 1996). However, in prostate (Shimura et al., 2000) and stomach tumors (Migita et al., 1999), TAMs presence has been associated with improved prognosis. PSP's ability to increase monocyte presence and differentiation into macrophages could be an effective strategy for tumor eradication.

During the course of inflammation triggered by external factors, microbial infection or autoimmune responses, monocytes are recruited to the inflammatory foci where they differentiate into macrophages (Gordon & Taylor, 2005). Inflammatory macrophages play a critical role in defence against infection by initiating the innate immune responses to pathogens and also in fulfilling multiple effector and regulatory functions. PSP may also be a potentially effective agent against infection, commonly associated with the side effects of chemotherapy and inflammatory states existing in the tumor environment.

In support of the role of PSP in pathogen defence, one study observed that PSP, administered in drinking water to C57BL/6 mice, resulted in increased production of reactive oxygen species, nitrogen intermediates and tumor-necrosis factor in peritoneal macrophages (Liu, Ng, Sze, & Tsui, 1993). Other studies have also shown macrophage-stimulating activity of β-glucans from PSP, resulting in increased tumoricidal activity, nitric oxide production and phagocytic activity in young but not aged mice (Jang, Namkoong, & Sohn, 2010). In another study investigating the effect of cultivation duration on immunomodulation, the authors demonstrated that in the unstimulated PBMCs, PSP was capable of inducing a significant increase in pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ (Lee, Yang, & Wan, 2006). PSPs ability to alter various pro-inflammatory cytokines and induce cell recruitment presents a powerful strategy for tumor eradication.

Monocyte-derived DCs are known to be controlled by GM-CSF and have been shown to be blocked by pathogen receptors, such as TLR receptors (Palucka, Taquet, Sanchez-Chapuis, & Gluckman, 1999). β-(1,3)-d-Glucan is considered to be the active ingredient responsible for PSP, exerting various stimulating effects on different immune subsets. Dectin-1, which is highly expressed on blood and splenic monocytes, neutrophils and alveolar inflammatory macrophages, is highly specific for β-(1,3)-d-glucan, although it does not recognise all β-(1,3)-d-glucans equally, based on glycan backbone chain length and side branches, which strongly affect its binding affinity (Barsanti, Passarelli, Evangelista, Frassanito, & Gualtieri, 2011). Complement 3 receptor (CR3), which is highly expressed on monocytes and neutrophils and NK cells, has one carbohydrate-binding lectin site for β-(1,3)-d-glucan and a second for the complement cleavage fragment iC3b (Barsanti et al., 2011). CR3 is involved in mediating inflammatory processes and may serve as a receptor for β-(1,3)-d-glucan.

Future studies, investigating the cytokine profiles responsible for stimulating the monocyte/macrophage function and their role in receptor function, by blocking TLR2/6/4 and Dectin-1 to confirm their role in PSP stimulation, are warranted.

The role of PSP in increasing the expression of monocytes without PHA stimulation is important because it illustrates an immunological mechanism of action different from that of PHA itself, which specifically triggers cell division in T-lymphocytes. For instance, Lee et al. (1999) demonstrated that after co-incubating lymphocytes for 48 h with PHA (2.5 µg/ml) and PSP (1.0 mg/ml) in vitro, CD4$^+$ significantly increased compared to PSP control without PHA.

Our results provide new insights into the complex array of mechanisms involved in PSP immunity. In a study investigating the effects of PSK on T-cell activation of Epstein-Barr virus-infected cord blood mononuclear cells, PSK was found to inhibit virus-induced B-cell growth through activation of monocytes mediated by IL-15, phosphatidylinositol 3–(PI-3)–kinase and reactive oxygen species (Liu, Arbiser, & Holmgren, 2005).

In relation to PSP, PSK was found to promote the phenotypic and functional maturation of DC derived from CD14$^+$ PBMCs antigen (monocytes), by increasing the expression of DC specific markers, such as HLA class II antigen, CD40; it increased the number and expression of CD80$^+$CD86$^+$CD83$^+$ cells and induced antigen-specific cytotoxicity (Kanazawa et al., 2004). Mature DCs, which are professional antigen presenting cells, are unique in their ability to present tumor-specific antigens and activate a specific antitumor T cell response in vivo. Similarly, another well investigated PSP derived from Ganoderma lucidum was reported to induce proliferation of PBMCs and monocytes and also monocyte specific maturation of DC by upregulating CD40, CD80 and CD86 (Chan, Lam, & Law, 2005).

In vitro studies, in particular PHA treated PBMCs models report indirect stimulation of T-cell subsets and cytokine secretion but fail to demonstrate the cascade of events involved in the immune responses from antigen presentation to antibody production. There is an absence of models used to investigate the effects of immunomodulators, such as PSP, in demonstrating antigen presentation to DC and the cognate interaction between T-cells and B-cells that follow, leading to antibody production and memory B-cells. Future studies investigating whether PSP has a direct effect, in vivo, in activating T-cell-dependent B-cell responses are needed.

Monocytes not only differentiate into inflammatory DCs, but also represent the precursors for DCs located in antigen capture areas. Future research should investigate, in depth, the potential relevance of monocyte-derived DCs and the mechanisms involved in response to potent immunomodulatory agents, such as PSP. We suggest the convenient use of positive controls, such as PHA and GM-CSF, used separately to compare the direct immune modulating effects rather than co-incubation with PBMCs. The stimulation of monocyte-macrophage function by PSP may have potential clinical value in different clinical situations, including protection against opportunistic infections in patients with cancer; or with inflammatory, immunoregulatory and stimulatory function disorders in response to pathogens and external factors.

5. Authors contribution

B. K. Sekhon: Data collection, data analysis, and manuscript preparation.

D. M. Sze: Conception, design, data analysis; and review of the manuscript.

W. K. Chan: Data collection, data analysis. K. Fan: data analysis.

G. Q. Li: Essential resources and equipment and review of the manuscript.

D. E. Moore: Design and review of the manuscript.

R. H. Roubin: Conception, design, data analysis, and manuscript preparation.

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