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Immunomodulatory Activities of HERBSnSENSES™ *Cordyceps* — *in Vitro* and *in Vivo* Studies

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The commercially available HERBSnSENSES™ *Cordyceps* (HSCS) belongs to a cultivated strain of *Cordyceps sinensis* whose immunomodulatory activities has been renowned in traditional Chinese medicine (TCM) for centuries. The present report is the first that describes its immunomodulatory features through a series of *in vitro* and *in vivo* experiments. We measured, in peripheral blood mononuclear cells the *in vitro* effects of HSCS on the gene expression of cytokines and cytokine receptors, cytokine release, and surface expression of cytokine receptors using cDNA expression array, cytometric bead array (CBA), and immunoflorescence staining, respectively, as well as macrophage phagocytosis and monocyte production of H_2O_2 using flow cytometry. Sixty female BALB/c mice were fed with either HSCS (40 mg/kg/day) or water consecutively for 14 days. Proliferation, cytokine liberation, and CD3/4/8 expression of splenic cells were measured using 5-bromo-2′-deoxyuridine proliferation ELISA, CBA, and cytometry immunoflorescence staining, respectively. *In vitro* results demonstrated that HSCS induced the production of interleukin(IL)-1β, IL-6, IL-10 and tumor necrosis factor alphaα from PBMC, augmented surface expression of CD25 on lymphocytes, and elevated macrophage phagocytosis and monocyte production of H_2O_2. *In vivo* results showed that HSCS did not induce splenomegaly and cytokine overliberation. Our results possibly provide the biochemical basis for future clinical trials.

Keywords *Cordyceps Sinensis*, Cytokines, Immunomodulatory Agent, Lymphocytes, Monocytes, Traditional Chinese Medicine.

INTRODUCTION

The heavy reliance on antimicrobial agents in the past decades has prompted the emergence of resistant pathogenic strains, requiring the recent re-appraisal

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of natural immunomodulatory agents.[1] Traditional Chinese medicine (TCM) has been renowned as a herbal holistic remedy in China for centuries and may facilitate current studies. *Cordyceps sinensis*, or winter-worm summer-plant, is the TCM possessing both suppressive (*yin*) and enhancing (*yang*) modulatory properties in the human body.[2] Numerous reports have evinced this dual modulation from the immunological perspective. A cellular study has shown that *C. sinensis* possesses immunosuppression by attenuating blastogenesis, natural killer (NK) cell activity, and interleukin(IL)-2 productions in the activated peripheral blood mononuclear cells (PBMC).[3] Animal trials also have suggested that extracts of *C. sinensis* might alleviate autoimmune disease by significantly inhibiting the anti double strand DNA production,[4] mitigating proteinuria and serum creatinin levels and renal mesangial proliferation.[5–6] *In vivo* studies also have depicted the immunoactivating activities of *C. sinensis*, which possibly acts *via* the enhancement of bone marrow cell proliferation, macrophage functions, and synthesis of hematopoietic growth factor such as IL-6[7] and phagocyte and T helper (Th) cell counts in murine peripheral blood and spleen.[8] The active ingredients that contribute to the above pharmacological activities may be cordycepin, a nucleoside derivative, and polysaccharides, macromolecules shown as possible immunomodulators from rigorous studies during the past 40 years.[9–11]

C. sinensis grows naturally at high altitude such as the *Qinghai-Tibetan* plateau in China and thus there is difficulty in assessing values of *C. sinensis* as a very precious TCM. With the recent advancement of plant technology, cultivation of TCM including *C. sinensis* has become feasible. Yet there has been scarce systematic and scientific reports depicting the pharmacological activities of *C. sinensis* in its cultivated strains. Therefore, we selected a commercially available cultivated *C. sinensis*, HERBSnSENSES™ *Cordyceps* (HSCS), as our subject. Based on the evidence that *C. sinensis* is an immunomodulatory agent and the hypothesis that cultivated strains retain pharmacological activities of the wild form, we speculated that HSCS also could produce immunomodulatory effects. In this investigation, we conducted both cellular and animal studies for the investigation of our hypothesis.

MATERIALS AND METHODS

HSCS in capsules was manufactured by the GreaterChina Technology Group Ltd., Hong Kong conforming to the Good Manufacturing Practice standard. Each capsule was composed of microcrystalline cellulose (12 %) and cultivated fruiting bodies of *C. sinensis* (88 %). Active ingredients including cordycepin and adenosine were verified by CUCAMed Company Limited, Hong Kong. In accordance with traditional protocol of TCM preparation, the water-soluble active ingredients of the HCSC were isolated by heating at 80°C for 4 hr in autoclaved deionized water. The extracted content was subsequently lyophilized,

reconstituted by phosphate buffered saline (PBS, Gibco™ Invitrogen Corp, CA, USA) and finally stored at –20°C. The preparation was filtered through 0.2 μm polyethersufone filter before use.

Endotoxin Assay

The level of Gram-negative bacterial endotoxin contaminated in HSCS was measured by colorimetric method using QCL-1000® Chromogenic *Limulus* Amebocyte Lysate kit (Bio-Whittaker, Walkersville, MD, USA), that detects as low as 0.1 endotoxin unit (EU)/mL in a given sample by comparing with the endotoxin standard curve[12].

Animals

Female BALB/c mice aged 4–6 weeks and weighed between 20–25 g were grown under normal laboratory conditions (21 ± 2°C, 12 hr light-dark cycle) with proper feeding of standard rodent chow and water *ad libitum*. The mice were either treated with distilled water or HSCS at 40 mg/kg/day consecutively for 14 days and were sacrificed for blood collection and spleen isolation.

Cell Isolation of Monocytes and Lymphocytes

Peripheral blood mononuclear cells (PBMC) were purified from human buffy coat (Blood Transfusion Center, Hong Kong Red Cross, Hong Kong) by density gradient centrifugation using Ficoll-Pague™ Plus solution (Amersham Pharmacia Biotech Ltd., Uppsala, Sweden). The PBMC were incubated in 75 cm^2 culture flask for 1 hr to separate the suspending lymphocytes and the adhering monocytes. The monocytes were further detached by incubating with enzyme-free cell dissociation buffer (Gibco) for 20 min at 4°C. Both lymphocytes and monocytes were resuspended at 1×10^6 cells/mL in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone Co., UT, USA) for further assays.

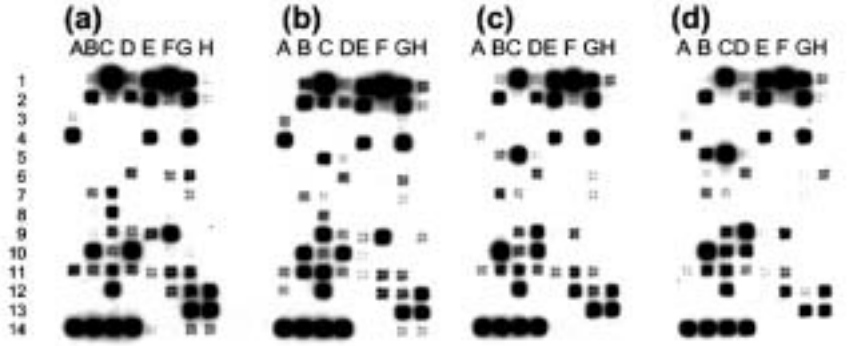
Cell Isolation of Mononuclear Splenic Cells

Murine spleen cells were extracted from the isolated spleen by grounding on a 70 μm nylon mesh immersing in RPMI 1640 medium supplemented with FBS (10 %). Mononuclear splenic cells were further purified by density gradient separation using Ficoll-Pague Plus solution and were resuspended in RPMI 1640 medium supplemented with 10% FBS for the subsequent experiments.

cDNA Expression Array

Total RNA was extracted from the untreated control or *Cordyceps*-treated human PBMC using RNeasy Mini Kit (Qiagen, Hilden, Germany). Nonradioactive

GEArray™ Q-series reagent kits (Superarray Bioscience Corp., MD, USA) were used in conjunction with the GEArray Probe Synthesis (Superarray Bioscience Corp) to quantify the gene expression profiles of the treated cells. One aliquot of total RNA (3 μg) was used as the template to produce biotinylated cDNA probes. The GEArray Q-series membranes dotted with tetra-spot cDNA fragment of 96 human inflammatory cytokine and receptor genes (Figure 1e) were hybridized for 18 hr with the cDNA probes. The labeled biotin on the membrane was detected by chemiluminescent method and the luminescence intensities of hybridized cDNA probes were analyzed using BioRad Quantity One™ software (Bio-Rad Laboratories, CA, USA). The relative amount of a given gene transcript was estimated by comparing its signal intensity with that derived from the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH)[12] (Figures 1 and 2).



(e)

	A	B	C	D	E	F	G	H
1	CXCR5	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7
2	CCR8	CCR9	CCXCR1	CX3CR1	CXCR4	IFNG	IL-10	IL-10Rα
3	IL-10Rβ	IL-11	IL-11Rα	IL-12α	IL-12β	IL-12Rβ$_1$	IL-12Rβ$_2$	IL-13
4	IL-13Rα$_1$	IL-13Rα$_2$	IL15	IL-15Rα	IL-16	IL-17	IL-17R	IL-18
5	IL-18R1	**IL-1α**	**IL-1β**	IL-1R$_1$	IL-1R$_2$	IL-2	IL-20	IL-21
6	IL-25	IL-2Rα	IL-2Rβ	IL-2RG	IL-4	IL-5	IL-5Rα	**IL-6**
7	IL-6R	Gp130	IL-9	IL-9R	Leptin	TNFβ	LT-b	LTbR
8	MIF	I-309	Eotaxin	MCP-4	HCC-1	MIP-1α	HCC-4	TARC
9	PARC	SCYA19	MCP-1	MIP-3a	MIP-2	MDC	MPIF-1	MPIF-2
10	TECK	MIP-1a	MIP-1b	RANTES	MCP-3	MCP-2	IP-10	IP-9
11	SCYB13	ENA-78	GCP-2	Lymphotactin	SCYC2	Fractalkine	SCYE1	SDF1
12	SDF2	TGFα	TGF-β$_1$	TGF-β$_2$	TGF-β$_3$	**TNFα**	TNFR1	TNFR2
13	pUC18 Plasmid DNA			BLANK			GAPD	
14	Cyclophilin A				Ribosomal protein IL13a		β-actin	

Figure 1: Gene expression of PBMC after stimulation with HSCS. Human PBMC (1×10^6 cells/mL) were (a) left untreated or stimulated with HSCS at (b) 0.5 mg/mL, (c) 1.0 mg/mL, and (d) 1.5 mg/mL for 24 hr, with (e) the location of individual genes on the membrane denoted in the table. Data shown in this image are from the human inflammatory cytokines and receptors GEArray and are representatives from four separate experiments.

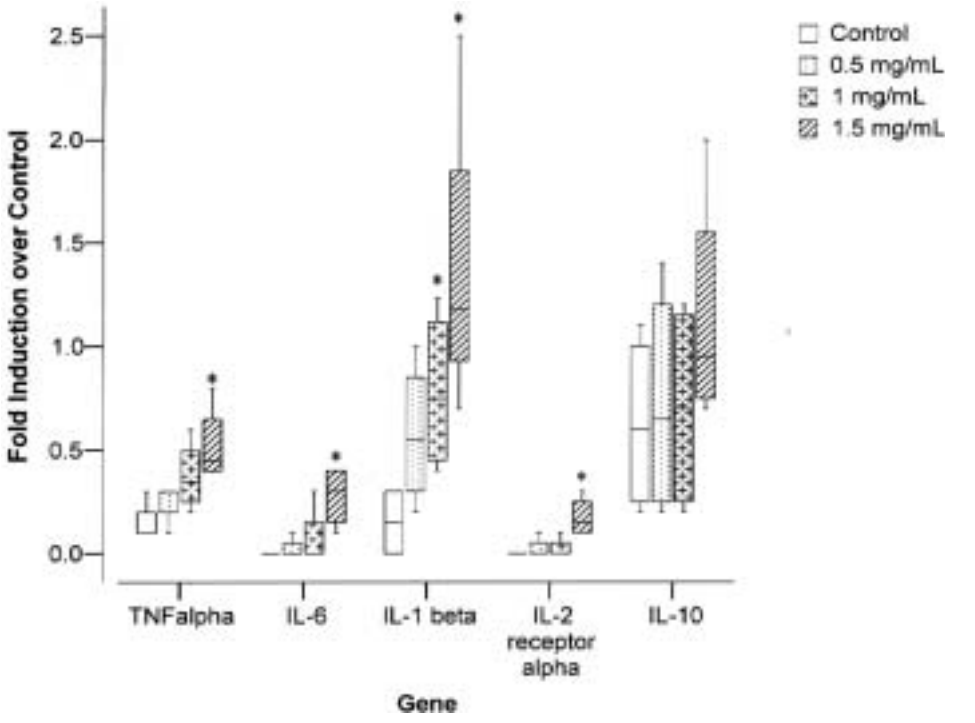


Figure 2: Upregulation of gene expression of cytokines and cytokine receptor by HSCS. The data were normalized to the housekeeping gene GAPD. Values shown in the box-plots are from four separate experiments. Statistical analysis was performed using Kruskal-Wallis test Test. *$p < 0.05$, significantly different from unstimulated cells.

Cytometric Bead Array

Interleukin (IL)-1β, IL-12p70, IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)α in the culture supernatant of treated human monocytes and lymphocytes were measured by the human inflammation cytometric bead array (CBA) kit (BD Pharmingen, CA, USA) using flow cytometer (FASCalibur, Becton Dickinson) (Figures 3 and 4). IL-6, IL-10, IL-12p70, TNFα, monocyte chemoattractant proteins (MCP)-1, interferon (IFN)γ in the murine serum, and supernatant of murine splenic mononuclear cells were similarly assayed by mouse inflammation CBA kit (BD Pharmingen)[12] (data not shown).

Immunophenotyping of T Cell Markers

Fresh murine splenic cells or treated human lymphocytes (5×10^5 cells) were harvested, washed, and re-suspended in PBS supplemented with 0.1 % bovine serum albumin (Gibco). Pooled serum (3 %) was further loaded to block for nonspecific binding sites. The cells were either incubated with FITC-conjugated

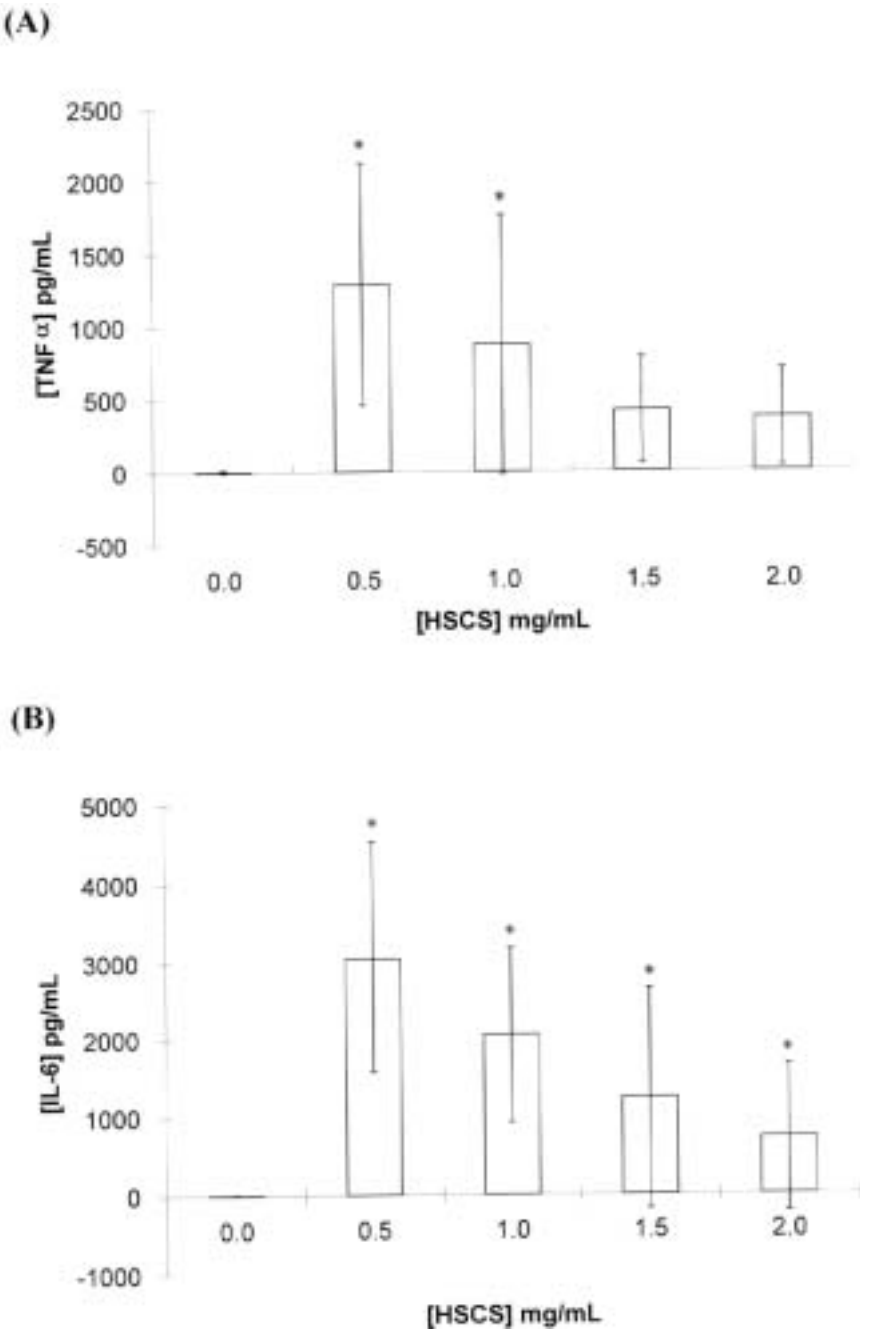


Figure 3: *In vitro* effects of HSCS on the induction of (A) TNFα, (B) IL-6, and (C) IL-10 from lymphocytes. Human lymphocytes (1×10^6 cells/mL) were left untreated or stimulated with HSCS at 0.5, 1.0, and 1.5 mg/mL for 20 hr. Culture supernatant was collected for cytokine assay using CBA. Values shown in the box-plots are from five separate experiments. Statistical analysis was performed by comparing the untreated control to stimulated cells using Kruskal-Wallis test. *$p < 0.05$, significantly different from unstimulated cells.

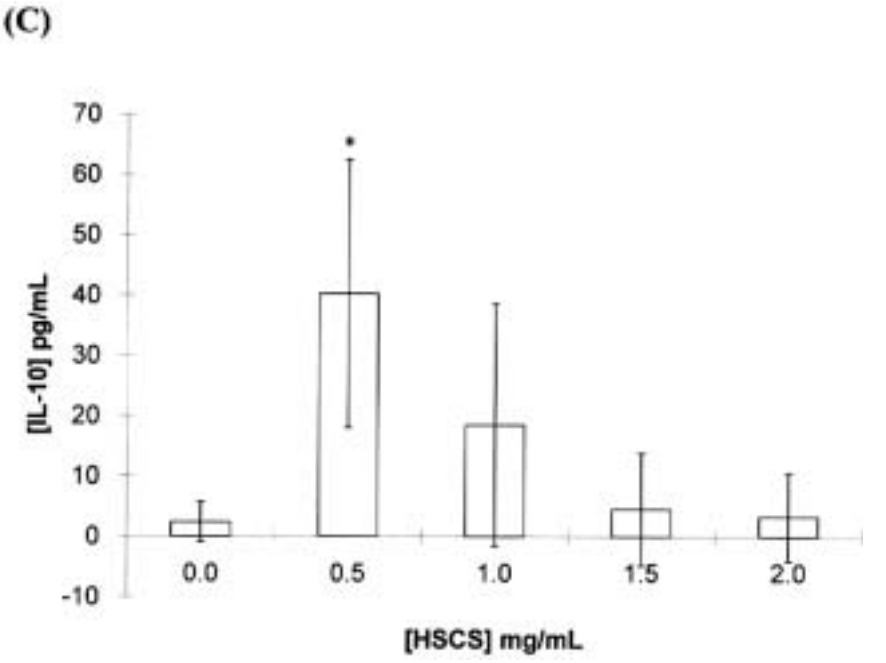


Figure 3: Continued.

monoclonal antibody or the corresponding fluorescein-conjugated mouse isotype at 4°C in dark for 30 min. After washing away the unbound antibodies, the cells were resuspended in paraformaldehyde (1%) (Sigmal Chemical Co. St. Louis, MO, USA). A total of 10,000 events (gated to exclude nonviable cells) were collected in the log mode using flow cytometry. Results were expressed as histograms of relative florescence intensity (Figures 5 and 8).

Assessment of Phagocytosis

Human monocytes (1×10^6 cells/mL) were primed with lipopolysaccharides (LPS) isolated from strain *Escherichia coli* 0127:B8 (Sigma) at 20 μg/mL for 2 hr and then incubated with HSCS for 18 hr. The treated cells were loaded with FITC-conjugated zymosan A from *Saccharomyces cerevisiae* (15 μg/mL; Sigma) for 45 min at 37°C in a CO_2 incubator. The intracellular florescence intensity was measured by flow cytometry using BD CellQuest software. Percentage of macrophage activation was assessed by comparing the mean fluorescence intensity (MFI) of the test sample with that of the macrophage control (Figure 7).

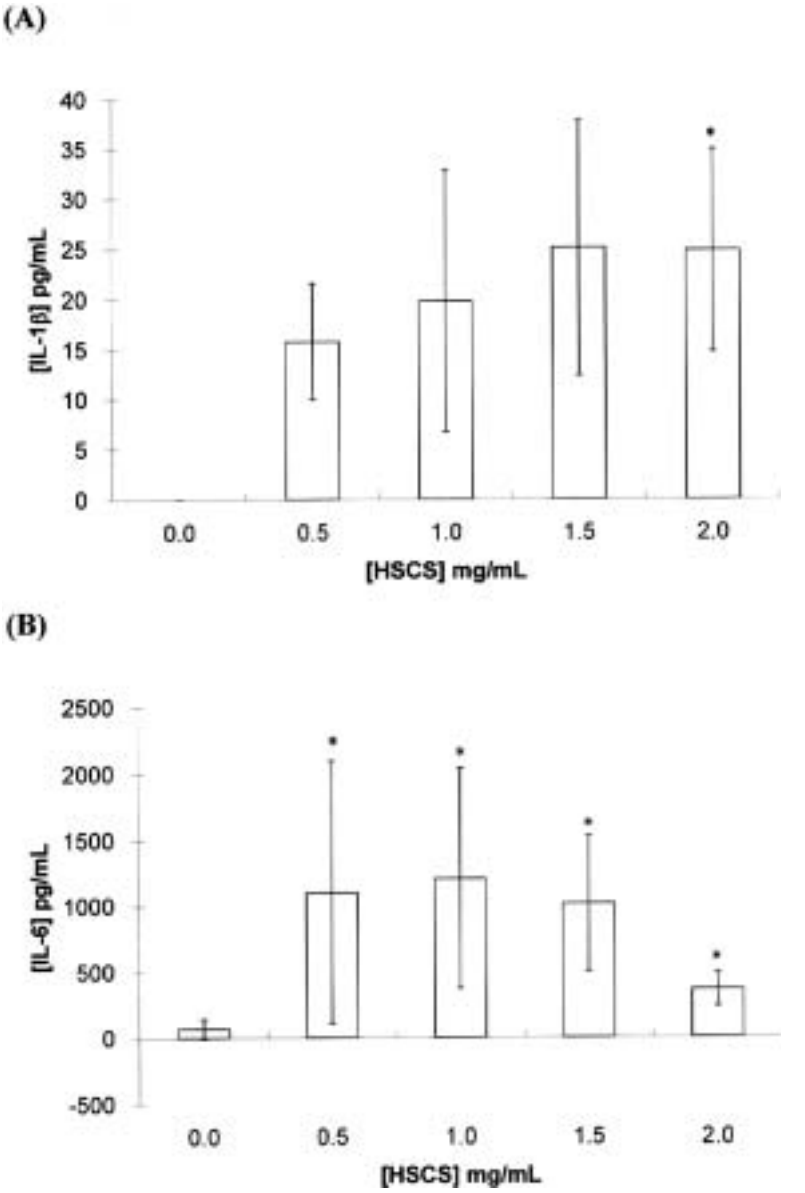


Figure 4: *In vitro* effects of HSCS on the induction of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNFα from monocytes. Human monocytes (1×10^6 cells/mL) were left untreated or stimulated with HSCS at 0.5, 1.0, and 1.5 mg/mL for 20 hr. Culture supernatant was collected for cytokine assay using CBA. Values shown in the box-plots are from five separate experiments. Statistical analysis was performed by comparing the untreated control to stimulated cells using Kruskal-Wallis test. *$p < 0.05$, significantly different from unstimulated cells.

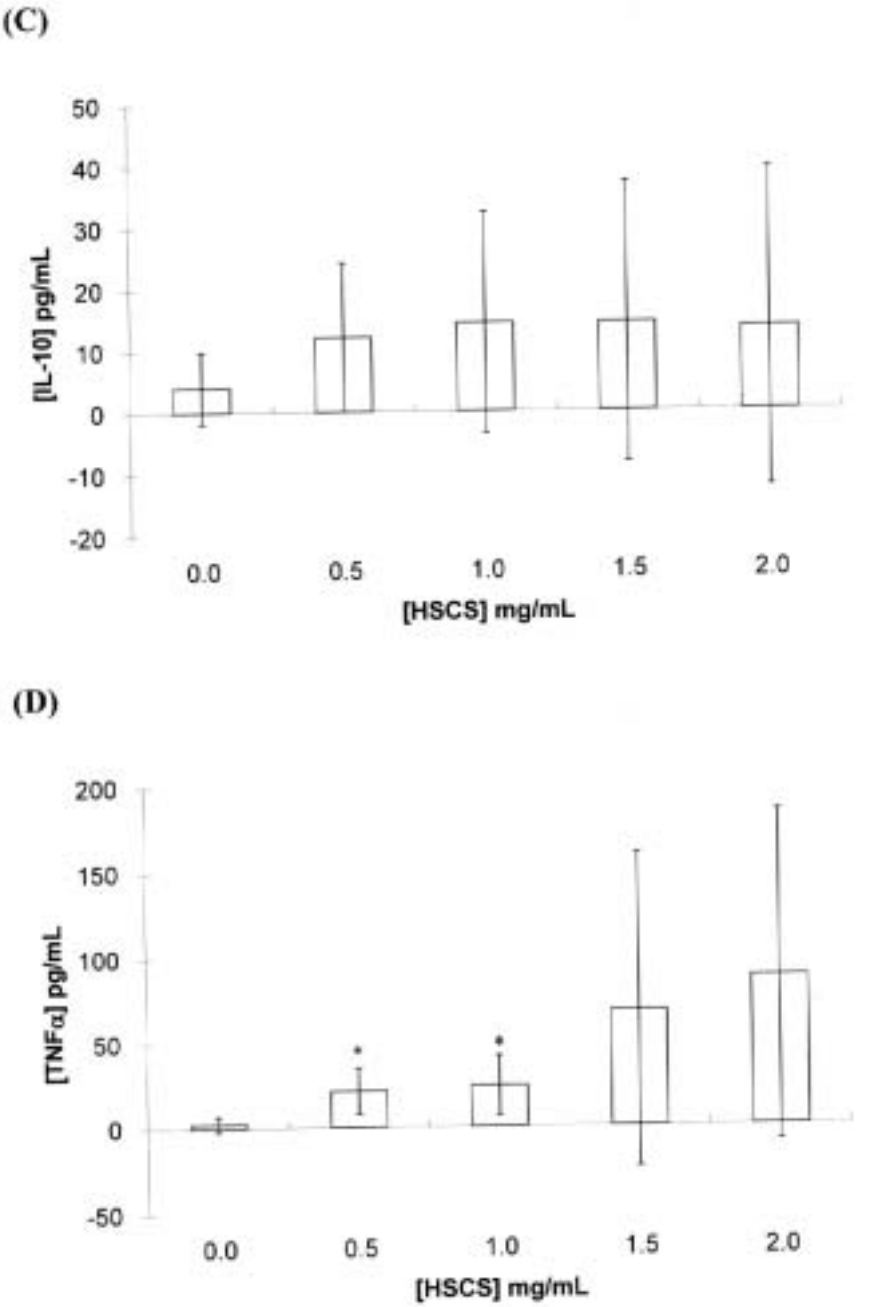


Figure 4: Continued.

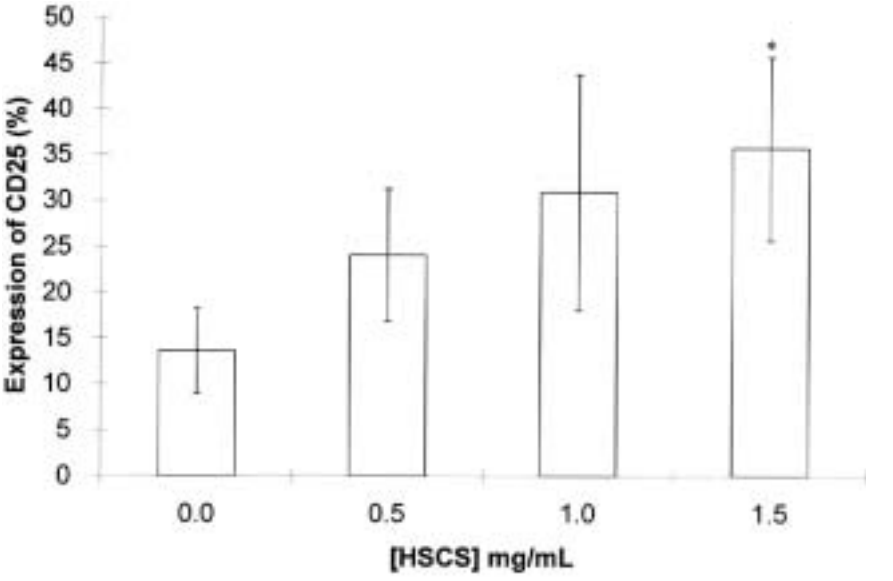


Figure 5: *In vitro* effects of HSCS on the cell surface expression of IL-2 receptor chain α (CD25). Human lymphocytes (1×10^6 cells/mL) were left untreated or stimulated with various concentrations of HSCS for 20 hr. The expression of CD25 was measured using immunoflorescence staining by flow cytometry. Percentage expression of CD25 was normalized with the isotypic control. Values shown in the histograms are from five separate experiments. Statistical analysis was performed by comparing the untreated control to stimulated cells using Kruskal-Wallis test. $*p < 0.05$, significantly different from unstimulated cells.

Flow Cytometric Analysis of Hydrogen Peroxide (H_2O_2)

Human monocytes (1×10^6 cells/mL), after incubating with various concentrations of HSCS for 3 hr at 37°C in CO_2 incubator, were loaded with 1 μg/mL dihydrorhodamine-123 (DHR; Molecular Probe Europe BV, Leiden, The Netherlands) for 15 min at 37°C. The nonfluorescent DHR was readily taken up by the phagocytes during respiratory burst and was converted to a green florescent compound in the presence of H_2O_2. Percentage of H_2O_2 release was estimated by comparing the percentage of fluoresced cells to the nonfluoresced cells[13] (Figure 6).

Mitogenic Assay

Splenic mononuclear cells were incubated with LPS at 20 μg/mL or phytohemagglutinin (PHA) at 5 μg/mL for 24 hr in a 96-well microtiter plate. The mitogenic activity of HSCS on splenic mononuclear cells was quantified by colorimetric 5-bromo-2′-deoxyuridine (BrdU) cell proliferation ELISA kit (Roche Diagnostic GmbH, Roche Applied Science, Penzberg, Germany). The absorbance

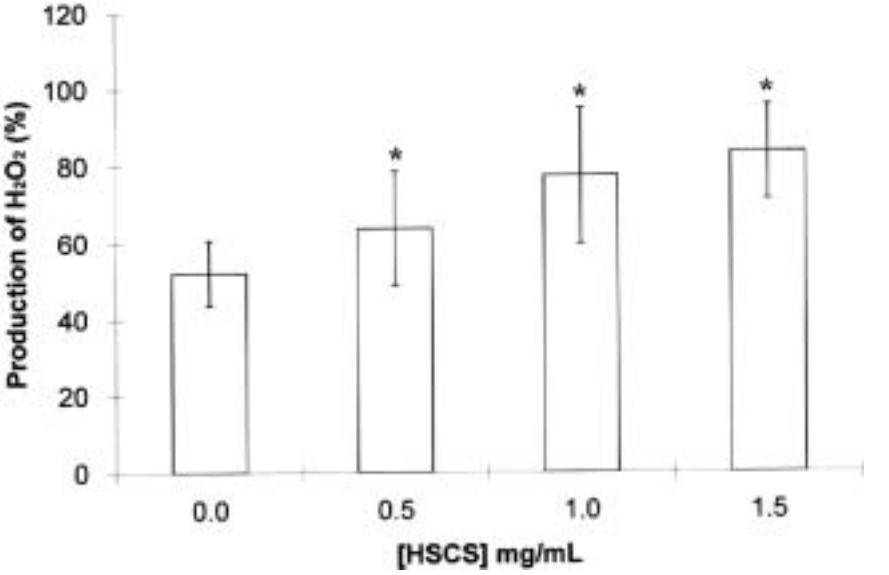


Figure 6: *In vitro* effects of HSCS on the production of hydrogen peroxide (H_2O_2). Monocytes (1×10^6 cells/mL) were left untreated or stimulated with various concentrations of HSCS for 3 hr. The H_2O_2 synthesis was quantified by florescence staining of DHR using flow cytometry. Percentage production of H_2O_2 was calculated by comparing the percentage of DHR fluoresced cells to the nonfluoresced cells and expressed as mean ± SD. Values shown are from five separate experiments. Statistical analysis was performed by comparing the untreated control to stimulated cells using Kruskal-Wallis test. *$p < 0.05$, significantly different from unstimulated cells.

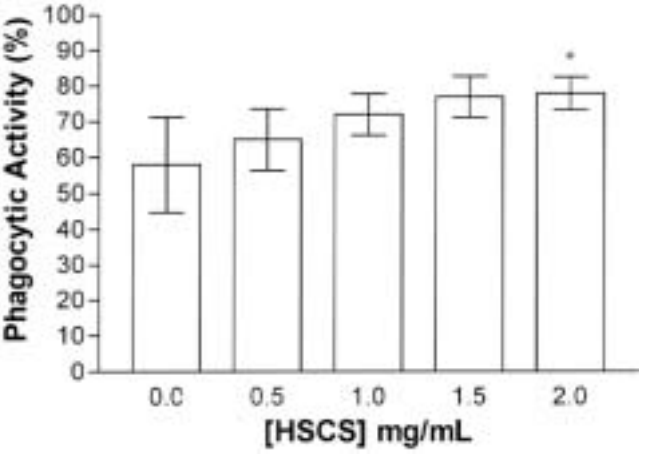


Figure 7: *In vitro* effects of HSCS on the activation of macrophage phagocytosis. Monocytes (1×10^6 cells/mL) were prestimulated by LPS (20 μg/mL) for 2 hr and were then left untreated or incubated with various concentrations of HSCS for another 18 hr. The phagocytic activity of macrophage was assessed by flow cytometry and the percentage of zymosan$^+$ cells relative to the zymosan$^-$ cells was presented. The bar chart expresses mean ± SD from five separate experiments. Statistical analysis was performed by comparing the untreated control to stimulated cells using Kruskal-Wallis test. *$p < 0.05$, significantly different from unstimulated cells.

was recorded at 405 nm with a reference wavelength of 470 nm in an EL340 plate reader (Bio-Tek Instruments, VT, USA) (data not shown).

Statistical Analysis

Nonparametric Mann-Whitney U test was used to compare the effectiveness of HSCS in the present studies. All statistical analyses were performed using Statistical Package for the Social Science (SPSS) statistical software for Windows, version 12 (SPSS Inc. IL, USA). ($p < 0.05$ was considered as significantly different.

RESULTS

Endotoxin Level in HSCS

Undetectable endotoxin level was found in the HSCS solution at concentration < 5 mg/mL. Since the lowest detection limit of the assay kit was 0.1 EU/mg, the amount of endotoxin present in the experimenting drug was considered negligible.

In vitro Effect of HSCS

As shown by cDNA array analysis by comparing the signal intensities with densitometry (Figure 1), inflammatory cytokines including IL-1α (coordination: 5B), IL-1β (coordination: 5C), IL-6 (coordination: 6H), and TNFα (coordination: 12F) (bold and shaded in Figure 1e) were upregulated upon 24-hr induction with HSCS at 0, 0.5, 1.0, and 1.5 mg/mL while the expression of anti-inflammatory cytokines IL-10 remained unchanged among the 96 genes being screened. Figure 2 shows that gene expression of these cytokines was dose-dependently upregulated by HSCS, with statistical difference usually reached at a concentration of 1.5 mg/mL ($p < 0.05$). The gene expression of IL-2 receptor α also was significantly upregulated by HSCS at 1.5 mg/mL ($p < 0.05$), although the induction was rather modest with less than 0.3-fold.

The CBA results indicate that low dosages of HSCS (0.5 and 1 mg/mL) stimulated in vitro production of TNFα and IL-6 from human lymphocytes (Figure 3A and 3B) and TNFα and IL-6 from human monocytes (Figure 4B and 4D) ($p < 0.05$). However, HSCS at 0.5 mg/mL could only induce IL-10 synthesis from lymphocytes (Figure 3C, $p < 0.05$) but not monocytes ($p > 0.05$) (Figure 4C). Low dosages (0.5 and 1.0 mg/mL) and all tested dosages (0.5, 1.0, 1.5, and 2.0 mg/mL) of HSCS induced *in vitro* production of TNFα from lymphocytes (Figure 3A) and IL-6 from monocytes (Figure 4B).

Although the gene expression of IL-1β was induced at 1.0 and 1.5 mg/mL of HSCS, we could only observe similar induction of IL-1β at 2.0 mg/mL (Figure 4A). Figure 5 illustrates that HSCS upregulated the cell surface

expression of IL-2 receptor α at 1.5 mg/mL ($p < 0.05$) but not at dosages less than 1.0 mg/mL ($p > 0.05$), which was consistent with the cDNA array results.

In vitro effects of HSCS on Hydrogen Peroxide

At 1.0 and 1.5 mg/mL, HSCS induced the production of H_2O_2 by 25% and 33%, respectively ($p < 0.05$) (Figure 6). HSCS stimulated peripheral blood monocytes to engulf the FITC-conjugated yeast in a dose-dependent manner. When compared with the control, HSCS significantly enhanced phagocytosis at 2.0 mg/mL ($p < 0.05$). HSCS at lower concentrations (< 2.0 mg/mL) also elevated the phagocytic response, although statistical significance could not be reached ($p > 0.05$).

Effect of HSCS on Spleenomagaly

In vivo safety of HSCS was assessed by whether spleenomagaly was triggered in the BALB/c mice in the whole course of treatment. Results indicate no significant changes between the spleen weights of the two groups of mice ($p > 0.05$). In addition, no obvious differences in the sizes, shapes, and color of spleens isolated from the two treatment groups were observed.

Mitogenic Activity of HSCS

After 14 days treatment with the HSCS (40 mg/kg/day), median proliferation of the spleen cells were elevated when compared with that of the water-treated (control) mice, although statistical differences could not be reached ($p > 0.05$). When compared with the unstimulated spleen cells, HSCS elevated the median mitogenic activity of the LPS and PHA-treated spleen cells to a greater extent than that of the control ($p > 0.05$).

Effect of HSCS on Induction of Cytokines

There were no statistical differences in the serum concentrations of cytokines IL-12p70, TNFα, IFNγ, MCP-1, IL-10, and IL-6 in mice after the 14-day treatment with HSCS ($p > 0.05$). Similarly, in the culture supernatant of spleen cells after the *Cordyceps* treatment, we could not detect any significant differences in the level of these cytokines ($p > 0.05$). However, observable elevation was noted in the mean level of the release of certain cytokines between the *Cordyceps* and water treatment groups, including IL-12p70; TNFα; MCP-1 in serum; TNFα, MCP-1, IL-10, and IL-6 in the culture supernatant of unstimulated spleen cells; TNFα, IFNγ, MCP-1, IL-10, and IL-6 in the culture supernatant of LPS-stimulated spleen cells; and IL-12p70, TNFα, IFNγ, MCP-1, IL-10, and IL-6 in the culture supernatant of PHA-stimulated spleen cells.

Effect of HSCS on Activation of Murine T lymphocytes

Cytofluorogram in Figure 8 illustrates that the 14-day treatment with HSCS enormously upregulated the expression of cell surface antigen CD3 and CD4 on spleen cells by 209% and 120 %, respectively. However, we could only detect a 14 % elevation in the expression of CD8 on spleen cells (data not shown).

DISCUSSION

Despite the fact that HSCS is a dietary supplement frequently consumed in Hong Kong, relatively little is known about its immunomodulatory effects. Here we described cytokine induction, T cell activation, and enhancement of macrophage functions that may contribute to the *in vitro* immunomodulatory activities of HSCS. Animal studies showed that HSCS may strengthen the *in vivo* cell-mediated immunity *via* the activation of T helper cells.

Cytokines control immunological functions by regulating cell proliferation, survival, differentiation, antigen presentation and trafficking and are therefore important parameter in the present study. Our *in vitro* results showed that both the gene expression and protein synthesis of cytokine IL-1β, TNFα, and IL-6 were upregulated by HSCS, with statistical significance usually observed at a dosage of 1.5 mg/mL (Figures 2–4). IL-1β, TNFα, and IL-6 are proinflammatory cytokines that contribute to the elimination of infectious agents and to the promotion of wound healing for the restoration of tissue integrity.[14] Specifically, IL-1β may activate the innate immunity by sensitizing dendritic cells.[15] IL-6 is involved in the cell-mediated immunity by inducing the expression of IL-2 receptor α chain (CD25),[16–17] by functioning as a secondary signal for the IL-2 production,[18] and by participating in the activation, growth, and differentiation of T cells.[19–21] TNFα may regulate the immune response, hematopoiesis, and inflammation and usually acts synergistically with IL-1β and IL-6.[22] Production of IL-1β, TNFα, and IL-6 by HSCS may enhance immunity *via* both innate and cell-mediated immunity.

Also our *in vitro* results concur with a previous study performed by Chen *et al*.[23] that also demonstrated the induction of TNFα and IL-1 from PBMC by polysaccharides extracted from natural *C. sinensis*.[23] This may indicate that HSCS retains pharmacological activities of the natural form. Apart from the induction of proinflammatory cytokines, HSCS also stimulated liberation of the anti-inflammatory cytokines IL-10 *in vitro*. IL-10 may modulate inflammatory cytokines IL-1β, IL-6, TNFα, and IL-10 itself by inhibiting their production.[24] Besides, IL-6 also may act pleiotropically by inhibiting IL-1β and TNFα at the transcriptional level.[25]

The negative regulation induced by IL-6 and IL-10 may thereby modulate the overactivated immune system that is potentially detrimental to the host.

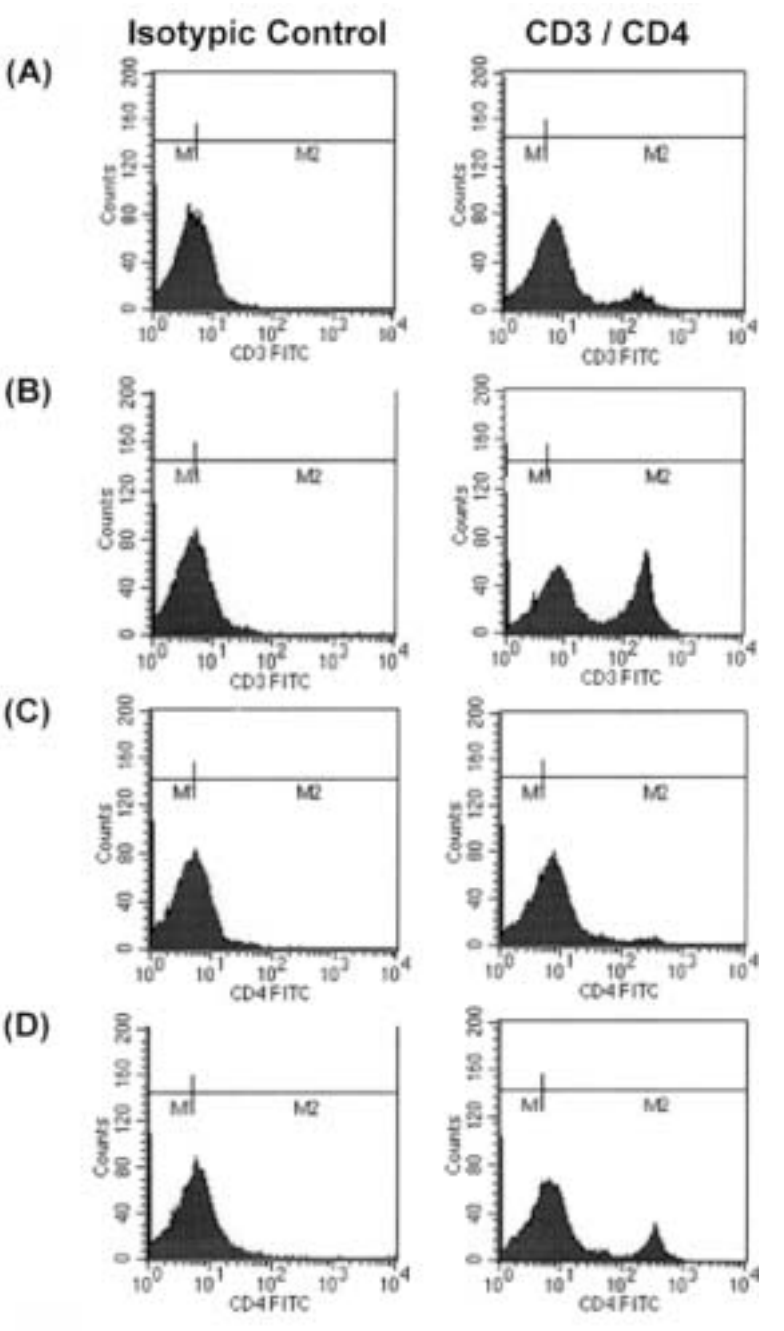


Figure 8: *In vivo* effects of HSCS on the surface expression of (A & B) CD3 and (C & D) CD4 antigen on splenic mononuclear cells. (A, C) BALB/c mice treated with water. (B, D) BALB/c mice treated with HSCS. The cytoflurograms represents a total count of 10,000 cells pooled from 15 homogenous BALB/c mice. Region M2 shown in the cytoflurogram indicates the activated T cells that express CD3 or CD4 antigen. Results were a representative from triplicate experiments.

However, high dosages of HSCS may accumulate inhibitory substances for suppressing the IL-6, TNFα, and IL-10 release—even the gene expression was elevated at these dosages. Further attempts to optimize the dosage of HSCS in humans are necessary.

Activated Th cells express the low affinity IL-2 receptor chain α (CD25) as the marker on cell surface. Our i*n vitro* results showed that both the gene and cell surface expression of CD25 were elevated ($p < 0.05$ at 1.5 and 2.0 mg/mL). This suggests that HSCS modulates T cells to respond at low concentration of IL-2 (Figure 5). The binding of IL-2 cytokine to IL-2 receptor subsequently triggers rapid proliferation and differentiation of T cells,[26] thereby enhancing the cell-mediated immunity. In addition, this upregulation of IL-2 receptor expression may be mediated by the co-activating effects of IL-1β, IL-6, and TNFα[27,28] that are induced simultaneously by HSCS from the PBMC.

Macrophages are the primary effector cells of innate immunity. A previous study suggested that ethanol extracts of natural *C. sinensis* may enhance macrophage functions.[29] The present study using hot water extract of *C. sinensis* demonstrated that the *in vitro* macrophage functions were promoted by HSCS, possibly through the upregulation of phagocytosis and H_2O_2 synthesis (Figures 6 and 7). Apart from the elimination of pathogenic bodies, phagocytosis may promote engulfment of apoptotic cells[30] for the ultimate remodeling of tissues.[31] The production of H_2O_2, on the other hand, exerted potential antitumor and microbicidal activities.[32,33] More recent evidence suggests that H_2O_2 is a secondary messenger for regulating the intracellular signaling of the mammalian immune system.[34–38] HSCS can potentially facilitate the upregulation of the host innate immunity via the activation of macrophages. In addition, the *in vitro* pharmacological activities possessed by HSCS were considered a contribution from the active ingredients that it contains rather than by bacterial contamination, as suggested by the negligible endotoxin amount in the drug powder.

Our *in vitro* findings positively suggest that HSCS possesses immunomodulatory features (Figures 1–8). We further validated our *in vitro* observation by performing pre clinical animal trials that focused on the effects of HSCS on the cell-mediated immunity. We found that normal mice orally fed with HSCS or control vehicle (H_2O) did not possess remarkable differences in cytokine production. This *in vivo* result may assure the safety of HSCS by suggesting that consecutive use over 14 days did not raise inflammation in normal individuals *via* the overliberation of cytokines.[39] Yet mean serum levels of cytokines IL-12p70, TNFα, and MCP-1 were detected at an elevating trend after the consecutive 14-day treatment of HSCS.

IL-12p70 may represent a growth factor for the activated $CD4^+$ T cells, $CD8^+$ T cells, natural killer (NK) cells,[40] and lymphokine-activated killer (LAK) cells.[41–42] IL-12p70 may also promote Th1-specific immune response by inducing IFNγ and IL-18 for the cell-mediated immunity.[43] TNFα may

activate dentritic cells for the ultimate induction of tumor-specific T cells, [44–45] where as MCP-1 may induce granule release from NK and $CD8^+$ cells.[46] Therefore, HSCS may be potentially antitumorigenic and antiinfectious. Further investigation using tumor-bearing mice as the study model is pending.

Mitogens were added to the murine spleen cells to mimic the pathogen stimulation. Our *in vivo* results demonstrated that HSCS elevated the proliferation of mitogen-stimulated splenic cells when compared with the control. This may suggest a beneficial role of HSCS in enhancing the immunity for combating infectious diseases. Also, HSCS may aid in health maintenance as because it increased proliferation of unstimulated spleen cells. These proliferative spleen cells were probably the T helper cells, as suggested from the heightened cell surface expression of CD3 and CD4 spleen cells (Figure 8). Spleen weight of normal mice fed with HSCS, however, did not significantly increase when compared with their placebo-treated control. This further indicates that HSCS did not unnecessarily overexaggerate spleen size.

From the *in vivo* results, we speculate that HSCS exerts its immunomodulatory effects directly on the T cell surface receptors, probably through the polysaccharides and cordycepin contained in HSCS, rather than by cytokine signaling. The activated T cells contribute to the enhanced cell-mediated immunity in a normal individual, which ultimately enhances the body's immune system against invading pathogens.

Our *in vitro* results indicated that HSCS possesses immunomodulatory activities. The *in vivo* animal study further strengths the *in vitro* observation by demonstrating that HSCS enhances the host cell-mediated immunity. Therefore, HSCS may be a useful daily dietary supplement to benefit the human immune system.

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REFERENCES

1. Masihi, K.N. Immunomodulatory agents for prophylaxis and therapy of infections. Int. J. Antimicrob. Agents. **2000**, 14(3), 181–191.
2. Siu, K.M.; Mak, D.H.; Chiu, P.Y.; Poon, M.K.; Du, Y.; Ko, K.M. Pharmacological basis of 'Yin-nourishing' and 'Yang-invigorating' actions of *Cordyceps*, a Chinese tonifying herb. Life. Sci. **2004**, 76(4) 385–395.
3. Kuo, Y.C.; Tsai, W.J.; Shiao, M.S.; Chen, C.F.; Lin, C.Y. *Cordyceps sinensis* as an immunomodulatory agent. Am. J. Chin. Med. **1996**, 24(2), 111–125.
4. Chen, J.R.; Yen, J.H.; Lin, C.C.; Tsai, W.J.; Liu, W.J.; Tsai, J.J.; Lin, S.F.; Liu, H.W. The effects of Chinese herbs on improving survival and inhibiting anti-ds DNA antibody production in lupus mice. Am. J. Chin. Med. **1993**, 21(3–4), 257–262.

5. Yang, L.Y.; Chen, A.; Kuo, Y.C.; Lin, C.Y. Efficacy of a pure compound H1-A extracted from *Cordyceps sinensis* on autoimmune disease of MRL lpr/lpr mice. J. Lab. Clin. Med. **1999**, 134(5), 492–500.

6. Lin, C.Y.; Ku, F.M.; Kuo, Y.C.; Chen, C.F.; Chen, W.P.; Chen, A.; Shiao, M.S. Inhibition of activated human mesangial cell proliferation by the natural product of *Cordyceps sinensis* (H1-A): an implication for treatment of IgA mesangial nephropathy. J. Lab. Clin. Med. **1999**, 133(1), 55–63.

7. Nakamura, K.; Yamaguchi, Y.; Kagota, S.; Shinozuka, K.; Kunitomo, M. Activation of *in vivo* Kupffer cell function by oral administration of *Cordyceps sinensis* in rats. Jpn. J. Pharmacol. **1999**, 79(4), 505–508.

8. Chen, G.Z.; Chen, G.L.; Sun, T.; Hsieh, G.C.; Henshall, J.M. Effects of *Cordyceps sinensis* on murine T lymphocyte subsets. Chin. Med. J. **1991**, 104(1), 4–8.

9. Cunningham, K.G.; Manson, W.; Spring, F.S.; Hutchinson, S.A. Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn.) Link. Nature **1950**, 166(4231), 949.

10. Kuo, Y.C.; Lin, C.Y.; Tsai, W.J.; Wu, C.L.; Chen, C.F.; Shiao, M.S. Growth inhibitors against tumor cells in *Cordyceps sinensis* other than cordycepin and polysaccharides. Cancer Invest. **1994**, 12(6), 611–615.

11. Tzianabos, A.O. Polysaccharide immunomodulators as therapeutic agents: structural aspects and biologic function. Clin. Microbiol. Rev. **2000**, 13(4), 523–533.

12. Wong, C.K.; Tse, P.S.; Wong, E.L.; Leung, P.C.; Fung, K.P.; Lam, C.W. Immunomodulatory effects of yun zhi and danshen capsules in health subjects—a randomized, double-blind, placebo-controlled, crossover study. Int. Immunopharmacol. **2004**, 4(2), 201–211.

13. Emmendorffer, A.; Hecht, M.; Lohmann-Matthes, ML.; Roesler, J. A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. J. Immunol. Methods. **1990**, 131(2), 269–275.

14. Durum, S.K. Proinflammatory cytokines. In *Cytokines Reference*; Oppenheim, J.J., Feldmann, M., Eds.; Academic Press: London, San Diego, 2001, 95–97.

15. Luft, T.; Jefford, M.; Luetjens, P.; Hochrein, H.; Masterman, K.A.; Maliszewski, C.; Shortman, K.; Cebon, J.; Maraskovsky, E. IL-1 beta enhances CD40 ligand-mediated cytokine secretion by human dendritic cells (DC): a mechanism for T cell-independent DC activation. J. Immunol. **2002**, 168(2), 713–722.

16. Noma, T.; Mizuta, T.; Rosen, A.; Hirano, T.; Kishimoto, T.; Honjo, T. Enhancement of the interleukin 2 receptor expression on T cells by multiple B-lymphotropic lymphokines. Immunol. Lett. **1987**, 15(3), 249–253.

17. Le, J.M.; Fredrickson, G.; Reis, L.F.; Diamantstein T.; Hirano T.; Kishimoto T.; Vilcek J. Interleukin 2-dependent and interleukin 2-independent pathways of regulation of thymocyte function by interleukin 6. Proc. Natl. Acad. Sci. USA **1988**, 85(22), 8643–8647.

18. Garman, R.D.; Jacobs, K.A.; Clark, S.C.; Raulet, D.H. B-cell-stimulatory factor 2 (beta 2 interferon) functions as a second signal for interleukin 2 production by mature murine T cells. Proc. Natl. Acad. Sci. USA **1987**, 84(21), 7629–7633.

19. Van Snick, J. Interleukin-6: an overview. Annu Rev Immunol. **1990**, 8, 253–278.

20. Houssiau, F.; Van Snick, J. IL-6 and the T-cell response. Res. Immunol. **1992**, 143(7), 740–743.

21. Kopf, M.; Ramsay, A.; Brombacher, F.; Baumann, H.; Freer, G.; Galanos, C.; Gutierrez-Ramos, J.C.; Kohler, G. Pleiotropic defects of IL-6-deficient mice including early hematopoiesis, T and B cell function, and acute phase responses. Ann. NY Acad. Sci. **1995**, 762, 308–318.

22. Mizutani, H.; May, L.T.; Sehgal, P.B.; Kupper, T.S. Synergistic interactions of IL-1 and IL-6 in T cell activation. Mitogen but not antigen receptor-induced proliferation of a cloned T helper cell line is enhanced by exogenous IL-6. J. Immunol. **1989**, 143(3), 896–901.

23. Chen, Y.J.; Shiao, M.S.; Lee, S.S.; Wang, S.Y. Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. Life Sci. **1997**, 60(25), 2349–2359.

24. Rene de Waal Malefyt. IL-10. In *Cytokines Reference*; Oppenheim, J.J., Feldmann, M., Eds.; Academic Press: London, San Diego, 2001, 165–185.

25. Schindler, R.; Mancilla, J.; Endres, S.; Ghorbani, R.; Clark, S.C.; Dinarello, C.A. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood **1990**, 75(1), 40–47.

26. Taniguchi, T.; Minami, Y. The IL-2/IL-2 receptor system: a current overview. Cell **1993**, 73(1): 5–8.

27. Holsti, M.A.; Raulet, D.H. IL-6 and IL-1 synergize to stimulate IL-2 production and proliferation of peripheral T cells. J. Immunol. **1989**, 143(8), 2514–2519.

28. Panzer, S.; Madden, M.; Matsuki, K. Interaction of IL-1 beta, IL-6 and tumour necrosis factor-alpha (TNF-alpha) in human T cells activated by murineantigens. Clin. Exp. Immunol. **1993**, 93(3), 471–478.

29. Shin, K.H.; Lim, S.S.; Lee, S.; Lee, Y.S.; Jung, S.H.; Cho, S.Y. Anti-tumour and immuno-stimulating activities of the fruiting bodies of Paecilomyces japonica, a new type of Cordyceps spp. Phytother. Res. **2003**, 17(7), 830–833.

30. Roos, A.; Xu, W.; Castellano, G.; Nauta, A.J.; Garred, P.; Daha, M.R.; van Kooten, C. Mini-review: a pivotal role for innate immunity in the clearance of apoptotic cells. Eur. J. Immunol. **2004**, 34(4), 921–929.

31. Fadeel, B. Programmed cell clearance. Cell Mol Life Sci. **2003**, 60(12), 2575–2585.

32. Nathan, C.F.; Arrick, B.A.; Murray, H.W.; DeSantis, N.M.; Cohn, Z.A. Tumor cell antioxidant defenses: inhibition of the glutathione redox cycle enhances macrophage-mediated cytolysis. J. Exp. Med. **1981**, 153(4), 766–782.

33. Farias-Eisner, R.; Chaudhuri, G.; Aeberhard, E.; Fukuto, J.M. The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. J. Biol. Chem. **1996**, 271(11), 6144–6151.

34. Remick, D. G.; Villarete, L. Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. J. Leukoc. Biol. **1996**, 59(4), 471–475.

35. Forman, H. J.; Torres, M.; Fukuto, J. Redox signaling. Mol. Cell. Biochem. **2002**, 235(1–2), 49–62.

36. Foley, E.; O'Farrell, P.H. Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in Drosophila. Genes Dev. **2003**, 17(1), 115–125.

37. Asehnoune, K.; Strassheim, D.; Mitra, S.; Kim, J.Y.; Abraham E. Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. J. Immunol. **2004**, 172(4), 2522–2529.

38. Ryan, K.A.; Smith, M.F. Jr.; Sanders, M.K.; Ernst, P.B. Reactive oxygen and nitrogen species differentially regulate Toll-like receptor 4-mediated activation of NF-kappa B and interleukin-8 expression. Infect. Immun. **2004**, 72(4), 2123–2130.

39. Miossec, P. Acting on the cytokine balance to control auto-immunity and chronic inflammation. Eur. Cytokine Netw. **1993**, 4(4), 245–251.

40. Gately, M.K.; Desai, B.B.; Wolitzky, A.G.; Quinn, P.M.; Dwyer, C.M.; Podlaski, F.J.; Familletti, P.C.; Sinigaglia, F.; Chizonnite, R.; Gubler, U. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). J. Immunol. **1991**, 147(3), 874–882.

41. Gately, M.K.; Wolitzky, A.G.; Quinn P.M.; Chizzonite, R. Regulation of human cytolytic lymphocyte responses by interleukin-12. Cell Immunol. **1992**, 143(1), 127–142.

42. Kobayashi, M.; Fitz, L.; Ryan, M.; Hewick, R.M.; Clark, S.C.; Chan, S.; Loudon, R.; Sherman, F.; Perussia, B.; Trinchieri, G. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J. Exp. Med. **1989**, 170(3), 827–845.

43. Wong, C.K.; Lam, C.W. Clinical applications of cytokine assays. Adv. Clin. Chem. **2003**, 37: 1–46.

44. Jonuleit, H.; Knop, J.; Enk, A.H. Cytokines and their effects on maturation, differentiation and migration of dendritic cells. Arch. Dermatol. Res. **1996**, 289(1), 1–8.

45. Gerosa, F.; Baldani-Guerra, B.; Nisii, C.; Marchesini, V.; Carra, G.; Trinchieri, G. Reciprocal activating interaction between natural killer cells and dendritic cells. J. Exp. Med. **2002**, 195(3), 327–333.

46. Rollins, B.J. Chemokines. Blood **1997**, 90(3), 909–928.