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Regulation of Cytokine Production by Treating with Chinese Tonic Herbs in Human Peripheral Blood Mononuclear and Human Acute Monocytic Leukemia Cells

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ABSTRACT

Chinese tonic herbs are often used as remedies for the treatment of cancer or immunity related diseases. Cytokine regulation is one of the pathways that modulates the immune system. In the present study, we investigated the effects of commonly used Chinese herbs on cytokine regulation in human peripheral blood mononuclear cells (PBMC) and human acute monocytic leukemia cell line, THP-1. Among the 15 selected Chinese herbs, *Paeonia lactiflora* exhibited inhibitory effect on phytohemagglutinin (PHA)-activated PBMC proliferation and induced IFN-γ production. We also used a multiplexed flow cytometric bead-based assay to analyze the release of 17 cytokines and reverse transcription polymerase chain reaction (RT-PCR)/quantitative real-time PCR (qRT-PCR) to evaluate gene expression. *Cuscuta chinensis* induced both interleukin-8 and MIP-1β release in THP-1 cells and influenced mRNA transcription. *Dioscorea opposita*, *Cinnamomum osmophloeum*, and *Glycyrrhiza uralensis* enhanced secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) and influenced GM-CSF gene expression. As such, different Chinese tonic herbs may regulate immunomodulatory functions in human cells *via* diverse processes such as the modulation of different cytokines and may be used as adjuvant immunity-boosting drugs. The present study is the first to use high-throughput multiplexed flow cytometric method to analyze cytokine regulatory effects of herbal extracts.

Key words: Chinese tonic herbs, human peripheral blood mononuclear cells, THP-1, multiplexed flow cytometric bead-based assay, RT-PCR, quantitative real-time PCR

INTRODUCTION

Cytokines are messengers of the immune system that modulate immunity and recombinant cytokine proteins have been employed for the treatment of cancer[1,2]. Various cytokines are produced by activated monocytes and macrophages, including tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, IL-6, and IL-8[3,4]. These cytokines share similar functions because redundancy is a characteristic of these proteins. To modulate the secretion of pro-inflammatory cytokines, such as IL-2, IL-6, IL-12, granulocyte-macrophage-CSF (GM-CSF), interferon (INF)-γ, MIP-1β, and TNF-α, the cytokine network balance probably plays critical roles in cancer or immunity related diseases[5-7].

Researchers have used cytokines to enhance immunity, and several reports have suggested that they can have a positive effect on cancer[7]. Some of the cytokines currently under investigation for use in cancer therapy include IL-2, IL-4, IL-12, IFN- γ, GM-CSF, and TNF-α[8,9]. IL-8 and macrophage inflammatory protein-1β (MIP-1β) belong to a small family of cytokines called chemokines that are involved in promoting chemotaxis[10]. IL-8 is known as a pro-inflammatory chemokine induced by stress or pro-inflammatory cytokines. Activation of IL-8 induces angiogenesis in endothelial cells and migration of neutrophils[10]. MIP-1β plays an important role in the regulation of immune responses that enhance the development of humoral, cellular, mucosal, and systemic immunity[11]. MIP-1β has also been shown to induce lymphocyte migration in the nasal mucosa and is highly presented by influenza virus-infected bronchial and nasal epithelial cells[12]. GM-CSF is one of the cytokines that stimulates the

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proliferation of neutrophils and monocyte-lineage cells. Evidence has demonstrated that GM-CSF effectively mobilizes bone marrow-derived progenitor cells into the peripheral circulation[13]. These mobilized progenitor cells can reconstitute bone marrow by transplantation after myeloablative treatment for hematological malignancies. In addition to its hematopoietic effect, GM-CSF improves the immune response by inducing the proliferation, maturation, and recruitment of dendritic cells as well as the expansion and differentiation of B and T lymphocytes[14]. Studies have demonstrated that GM-CSF can exert therapeutically beneficial effects, e.g. a high local GM-CSF concentration enhances dendritic cell presentation of tumor antigens[15].

Traditional Chinese tonic herbs have been known for their tonifying actions in the treatment of Yin, Yang, Qi, and blood deficiencies. These herbs are used for replenishing energy and treating functional deficiencies of the spleen and lungs as well as blood deficiencies in combination with blood tonics. They are also used for nourishing the blood and replenishing the vital essences and body fluids[16]. In previous reports, various herbal medicines were reported to possess immunomodulating properties that may be useful for reducing the risk of various diseases and cancers[17].

The aim of the present study was to investigate the cytokine regulatory actions of Chinese tonic herbs. We examined the effect of selected Chinese herbs on the proliferation of peripheral blood mononuclear cells (PBMCs) as well as on cytokine release from monocytes using THP-1 cells as a model. Analysis was performed using a flow cytometric bead-based assay and reverse transcription polymerase chain reaction (RT-PCR)/quantitative real-time PCR (qRT-PCR).

MATERIALS AND METHODS

I. *Herbal Materials*

Herbal materials were collected from local medicinal markets in Taipei and identified by Dr. H.C. Chang of the Graduate Institute of Pharmacognosy, Taipei Medical University. The specimens were stored at the laboratory of Taipei Medical University.

II. *Preparation of Herbal Medicines*

The dried herbal medicines were pulverized and extracted with a 10-fold 95% ethanol solution at room temperature for 3 days and then filtered three times. The filtrates were combined and concentrated under reduced pressure, freeze-dried, and stored in a closed container. The test samples were dissolved in DMSO. The final concentration of DMSO was less than 0.5% in the medium.

III. *Human Subjects*

Blood was collected from healthy male subjects, 26-37 years of age. The experimental protocol was reviewed and approved by the Institutional Human Experimentation Committee. Written informed consent was obtained from each subject.

IV. *Preparation of PBMCs*

PBMCs were prepared from heparinized, human peripheral blood (50 mL) obtained from healthy volunteers using the Ficoll-Hypaque gradient density method as described previously[18]. Briefly, peripheral blood was centrifuged at 2000 rpm at 4°C for 10 min to remove the plasma. Blood cells were then diluted with phosphate-buffered saline (PBS) and centrifuged in a Ficoll-Hypaque gradient at 1500 rpm for 30 min. The PBMC layers were collected and washed with cold, distilled water and 10 × Hank's buffer saline solution (HBSS) to remove the red blood cells. The cells were then resuspended at 2×10^6 cells/mL in RPMI-1640 medium supplemented with 2% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

V. *Lymphoproliferation Test*

The lymphoproliferation test was modified from that previously described[18]. The density of PBMCs was adjusted to 2×10^6 cells/mL before use. Cell suspensions (100 μL), with or without phytohemagglutinin (PHA; Sigma, St. Louis, MO, USA) (0.25 or 5 μg/mL), were distributed to 96-well plates. Various extracts of the selected Chinese herbs were added to the cells. The plates were incubated in a 5% CO_2-air humidified incubator at 37°C for 3 days. Subsequently, tritiated thymidine (1 μCi/well; New England Nuclear, Boston, MA, USA) was added to each well. After 16 h of incubation, the cells were harvested on glass fiber filters using an automatic harvester (Dynatech, Multimash 2000, Billingshurst, UK). Radioactivity in the filters was measured using a scintillation counter. Inhibition of PBMC proliferation by each tested sample was calculated using the following formula:

Inhibitory activity (%) = (control group [CPM] - experimental group [CPM])/(control group [CPM]) × 100.

VI. *Determination of IFN-γ Production in PBMCs*

PBMCs (2×10^5 cells/well) were cultured with PHA alone or in combination with varying concentrations of the tested samples (6.25, 12.5, 25, 50, and 100 μg/mL) for 3 days. The cell supernatants were then collected and assayed for IFN-γ concentrations using an enzyme immunoassay (R&D Systems, Minneapolis, MN, USA)[19].

VII. *Cells and Cell Cultures*

The human monocytic cell line, THP-1, was obtained from the American Type Culture Collection (Rockville, MD, USA). THP-1 cells were maintained in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma), L-glutamine (200 mmol/L), and 2-mercaptoethanol (5×10^5 mol/L; Sigma). One percent penicillin/streptomycin (Sigma) was incorporated into the growth medium and the cells were grown at 37°C in an environment of 5% CO_2.

VIII. *MTT Assay*

Cell viability was assessed using an MTT assay[20]. THP-1 cells (1.0×10^5) were incubated in a 24 -well plate in RPMI 1640 medium with 10% FBS per well for 24 h. The cells were treated with or without the herbal samples and cultured for an additional 24 h. After centrifuging at 1000 rpm for 10 min, the supernatants were removed. MTT (5 mg/mL; 20 μL) and 180 μL of medium were added and cultured for 24 h, then transferred with an Eppendorf (Hamburg, Germany) pipette. After centrifuging at 1000 rpm for 10 min and removing the supernatant, DMSO (200 μL) was added and mixed completely. The mixture was transferred to an ELISA plate and measured at 570 nm using a model μQuant microplate reader (BioTek, Winooski, VT, USA).

IX. *Cytokine Analyses Using Multiplexed Flow Cytometric Bead-Based Assays*

We investigated the production of inflammatory cytokines in the culture media of untreated or herbal medicine-treated (100 μg/mL for 24 h) THP-1 cells using different flow cytometric bead-based assays[21]. The multiplexed analyses of cytokines with the Bio-Plex system used a liquid suspension array of 17 sets of 5.5 μm beads (Bio-Plex Human Cytokine 17-plex panel; Bio-Rad, Hercules, CA, USA) internally dyed with different ratios of two spectrally-distinct fluorochromes. Eight point standard curves, ranging from 0.2 to 3200 pg/mL, were obtained by serial dilution of the reconstituted lyophilized standards. Data were analyzed using the Bio-Plex manager software (Bio-Rad). The supernatant was frozen at -80°C until simultaneously analyzed for the following 17 cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1 (MCAF), MIP-1β, and TNF-α. This was achieved using a custom 17-plex fluid-phase immunoassay (Bio-Rad) run on a Bio-Plex Suspension Array System (Bio-Plex 100 System; Bio-Rad). A Luminex-100 cytometer (Luminex Corporation, Austin, TX, USA) was used and equipped with StarStation software (Applied Cytometry Systems, Dinnington, UK). All samples were analyzed in triplicate. Cytokine levels were expressed as picograms per milliliter.

X. *RNA Isolation and Reverse Transcription*

Total RNA was isolated using a High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The quality of the total RNA was evaluated based on the A260/A280 ratio. To prepare a cDNA pool from each RNA sample, total RNA (1 μg) was reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Each cDNA pool was stored at -20°C until qRT-PCR analysis was performed.

XI. *PCR Primers*

Specific oligonucleotide primer pairs for qRT-PCR were selected from the Roche Universal ProbeLibrary. The ProbeFinder software (www.universalprobelibrary.com) was used to design an optimal assay composed of the respective labeled probe from the Universal ProbeLibrary Set as well as human and gene-specific primers (Table 1).

Table 1. Primer sequences and reaction conditions for GM-CSF, MIP-1*β*, IL-8, and GAPDH.

	Forward primer	Reverse primer	PCR condition
GM-CSF (for qRT-PCR)	TCTCAGAAATGTTTGACCTCCA	GCCCTTGAGCTTGGTGAG	55 cycles of 10 s at 95°C, 30 s at 60°C, and 1 s at 72°C
MIP-1*β* (for RT-PCR)	CCAAACCAACCGAAGCAAGC	ACAGTGGACCATCCCCATAG	33 cycles of 15 s at 94°C, 30 s at 49°C, and 40 s at 68°C
IL-8 (for RT-PCR)	ATGACTTCCAAGCTGGCCGTG	TTATTGAATTCTCAGCCCTCTTCAAAAACTTCTC	37 cycles of 15 s at 94°C, 30 s at 55°C, and 40 s at 68°C
GAPDH (for RT-PCR)	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	29 cycles of 15 s at 94°C, 30 s at 59°C, and 40 s at 68°C
GAPDH (for qRT-PCR)	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	55 cycles of 10 s at 95°C, 30 s at 60°C, and 1 s at 72°C

Table 2. Traditional uses of the selected Chinese tonic herbs.

No.	Voucher specimen	Species	Family	Yield (%)	Used Part	Indication [22]	Page
1	M215	*Atractylodes macrocephala* Koidz	Compositae	4.7	Rhizoma	Anorexia, abdominal distension, and weakness	352
2	M118	*Cuscuta chinensis* Lam.	Convolvulaceac	1.7	Semen	Weakness in lower body and legs and poor vision	406
3	M197	*Cynomorium songaricum* Rupr.	Cynomoriaceae	7.6	Stem	Impotence, emission, and weakness and paralysis of extremities	164
4	M11	*Dioscorea opposita* Thunb.	Dioscoreaceae	16.7	Rhizoma	Protracted diarrhea, chronic enteritis, cough, and dyspnoea	367
5	M82	*Cinnamomum osmophloeum* Kaneh	Lauraceae	10.3	Herba	Stomach pain, diarrhea, cough, shock, pain, and cold feeling in the lower part of the body	31
6	M25	*Astragalus membranaceus* (Fisch.) Bge.	Leguminosae	17.1	Radix	Perspiration caused by weakness, general fatigue, and chronic diarrhea	259
7	M21	*Glycyrrhiza uralensis* Fisch.	Leguminosae	24.0	Radix	Sore throat, cough, hysteria, dermatitis, and boils	286
8	M5	*Lilium lancifolium* Thunb.	Liliaceae	12.3	Bulbus	Cough, hemoptysis, palpitation, forgetfulness, and convalescence of febrile diseases	8
9	M9	*Ophiopogon japonicus* (Thunb.) Ker.-Gawl.	Liliaceae	33.5	Radix	Cough, anxiety, pharyngitis, and hemoptysis	298
10	M195	*Polygonatum odoratum* (Mill.) Druce	Liliaceae	2.4	Rhizoma	Hyperlipidemia, fever, cough, dizziness, and muscular spasm	386
11	M28	*Polygonatum sibiricum* Red.	Liliaceae	45.8	Rhizoma	General weakness and dry cough	385
12	M19	*Paeonia lactiflora* Pall.	Ranunculaceae	19.4	Radix	Menstrual disorders and dysfunctional uterine bleeding	299
13	M-222	*Morinda officinalis* How	Rubiaceae	10.4	Radix	Impotence, seminal emission, menstrual disorders, pain in the lower abdomen, rheumatic arthralgia, and weakness of the extremities	296
14	M-219	*Rehmannia glutinosa* Libosch.	Scrophulariaceae	30.7	Radix	Anemia, weakness, and low-grade afternoon fever	314
15	M18	*Angelica sinensis* (Oliv.) Diels	Umbelliferae	32.7	Radix	Anemia and menstrual disorders	251

XII. *RT-PCR*

The obtained cDNAs were amplified using the primers shown in Table 1. The reaction conditions are shown in Table 1. The resulting products were analyzed by electrophoresis on 1.5% agarose gels and staining with ethidium bromide. Specific primers for GAPDH were used as controls.

XIII. *qRT-PCR*

qRT-PCR reactions were performed on the Roche LightCycler 2.0 instrument using LightCycler TaqMan Master Mix (Roche Cat. 04 535 286 001). The ProbeFinder software (www.universalprobelibrary.com) was used to design the optimal assay comprised of the respective labeled probe from the Universal ProbeLibrary Set and human and gene-specific primers. Briefly, 20 μL of reactions contained 5 μL of cDNA template, 4 μL of Master Mix, 0.2 μL of 10 μM probe, 0.4 μL of 10 μM forward primer, 0.4 μL of 10 μM reverse primer, and 10 μL of water. The RT-PCR program was 95°C for 10 min, 45 cycles of 95°C for 10 sec, 72°C for 1 sec, and 40°C for 30 sec. At the end of the program, a melt curve analysis was performed. At the end of each RT-PCR run, the data were automatically analyzed and an amplification plot was generated for each cDNA sample. From each of these plots, the LightCycler4 data analysis software automatically calculated the CP value (crossing point: the turning point corresponds to the first maximum of the second derivative curve), which indicated the beginning of exponential amplification. The mRNA level was normalized with reference to the amount of the GAPDH mRNA.

XIV. *Statistical Analysis*

Differences between the data sets were tested for significance using one-way ANOVA followed by Scheffe test. $p < 0.05$ indicated significantly different data sets.

RESULTS

Chinese herbs have been used in alternative medicine for centuries. They are often used to maintain human health and treat diseases. In the present study, we selected Chinese tonic herbs based on compiled ethnobotanical data, which revealed that they have tonifying actions for the treatment of deficiencies in the body (Table 2)[22]. We then evaluated their effects on the regulation of cytokines in PBMCs and THP-1 cells. Many active components from Chinese herbs were extracted by ethanol-water solution[23-25]. Thus, Chinese herbs were extracted with 95% EtOH in the present study. The ethanolic extracts were derived from 11 families, and yields of the 95% EtOH extracts were between 1.7 and 45.8% (Table 2).

I. *Inhibitory Effect of Chinese Herbs on PBMC Proliferation Induced by PHA*

To evaluate the inhibitory effects of the selected Chinese herbs on PBMC proliferation, unstimulated cells or cells activated with PHA were treated with 100 μg/mL of individual herbal extract. Cell proliferation then was determined based on uptake of tritiated thymidine. Among the 15 selected extracts, *P. lactiflora* significantly inhibited PBMC proliferation stimulated with PHA (5 μg/mL), although it had little effect on tritiated thymidine uptake in resting PBMCs (Table 3). The inhibitory effect of *P. lactiflora* on PBMCs was not related to direct cytotoxicity as the viabilities of resting and activated PBMC.

Table 3. Effects of the selected Chinese tonic herbs on PBMC proliferation induced by PHA

Sample	Inhibitory Activity (%)	
	Unstimulated	PHA (5.0 μg/mL)
M215	77.5 ± 13.2	91.3 ± 4.3
M118	59.6 ± 3.6	80.3 ± 2.9
M197	23.7 ± 9.8	46.8 ± 4.0
M11	-19.1 ± 1.4	32.3 ± 4.1
M82	86.4 ± 2.7	95.4 ± 1.0
M25	20.3 ± 1.0	8.5 ± 0.2
M21	47.2 ± 6.8	62.7 ± 3.7
M5	4.6 ± 6.1	35.3 ± 6.4
M9	-3.8 ± 8.2	2.3 ± 7.9
M195	17.3 ± 4.3	38.2 ± 4.3
M28	-5.1 ± 1.0	9.1 ± 2.6
M19	18.5 ± 2.8	71.9 ± 2.8*
M222	32.4 ± 8.6	5.1 ± 4.3
M219	33.4 ± 8.1	38.7 ± 5.5
M18	-23.4 ± 6.3	18.2 ± 10.6

PBMCs (2×10^5/well) were treated with 100 μg/mL of the selected Chinese herbal extracts and PHA (5 μg/mL) for 3 days. The proliferation of cells was estimated based on uptake of tritiated thymidine. After incubation for 16 hr, the cells were harvested using an automatic harvester, after which radioactivity was measured using a scintillation counter. The inhibitory percentage was calculated using the formula described in the Materials and Methods section. Data were analyzed for statistical significance using the nonparametric Mann-Whitney *U*-test. *: $p < 0.05$ *versus* unstimulated group.

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II. *Effects of P. lactiflora on IFN-γ Production in PBMC Cultures*

To elucidate whether the impairment of activated-PBMC proliferation was related to IFN-γ production, we incubated cells with or without *P. lactiflora* for 3 days. Supernatants were then collected and the production of IFN-γ was determined using an enzyme immunoassay. As shown in Figure 1, the level of IFN-γ produced in *P. lactiflora*-treated PBMCs increased in comparison with resting cells.

III. *Effects of the Selected Chinese Herbs on Viability of THP-1 Cells*

An MTT assay for cytotoxicity was employed before further *in vitro* testing of THP-1 cells. Among the 15 extracts tested, a relatively low cytotoxicity was observed (Figure 2), with cell viability above 80% at concentrations of 100 g/mL. After low cytotoxicity was demonstrated as a first test for possible usefulness, the appropriate extracts were further examined for their effects on regulation of cytokines in human monocytes.

IV. *Effects of the Selected Chinese Herbs and LPS on Cytokine Release from THP-1 Cells*

In the present study, we examined the effects of LPS (10 ng/mL) on the release of 17 cytokines from THP-1 cells using the Bio-Plex system (BioRad). Among these cytokines, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, and IL-13 were not detected in the culture supernatants of cells incubated with or without LPS (10 ng/mL) for 24 hours (Table 4). Incubation of THP-1 cells with LPS resulted in a significant increase in the release of IL-8, IFN-γ, MCP-1 (MCAF), MIP-1β, and TNF-α. We also investigated the effects of the selected Chinese herbs on the secretion of these 17 cytokines. THP-1 cells were cultured with or without the selected Chinese herbs at 100 μg/mL. The results showed increased secretion of both IL-8 and MIP-1β in the presence of *C. chinensis* (M-118). We found the GM-CSF secretion increased significantly when cultured with *D. opposite* (M-11), *C. osmophloeum* (M-82), or *G. uralensis* (M-21). An increase in the secretion of MIP-1β was also observed in THP-1 cells cultured with *C. songaricum* (M-197), *D. opposita* (M-11), *C. osmophloeum* (M-82), *A. membranaceus* (M-25), *L. lancifolium* (M-5), or *P. sibiricum* (M-28).

V. *Effects on GM-CSF, MIP-1β, and IL-8 mRNA Expression in THP-1 Cells*

To evaluate whether the induction of IL-8, MIP-1β, and GM-CSF following treatment with the tested Chinese herbs was due to increased gene expression, total cellular RNA was extracted from THP-1 cells in the presence or absence of 100 μg/mL of select Chinese herbs and used for RT-PCR or qRT-PCR analysis. The results were normalized to the levels of the GAPDH mRNA. The MIP-1β and GAPDH mRNAs were detectable in each sample-treated THP-1 cell culture. Compared with controls, the levels of the MIP-1β

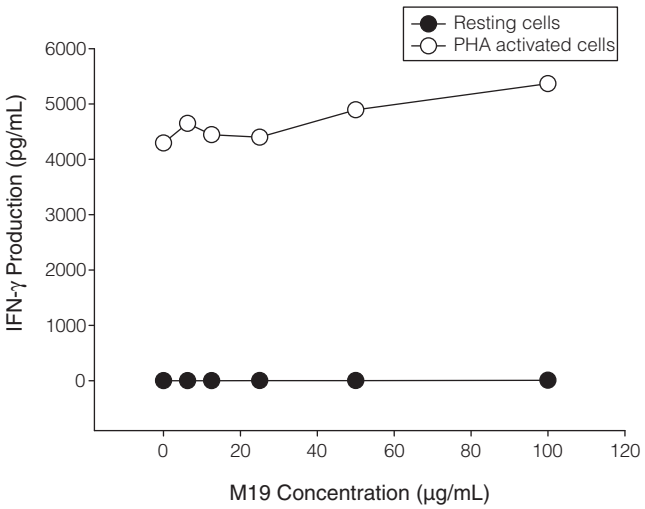

Figure 1. IFN-γ production in PBMCs treated with *P. lactiflora* (M-19). PBMCs (2×10^5 /well) were treated with various concentrations of M-19 with or without PHA (5 μg/mL) for 3 days. Cell supernatants were collected and IFN-γ concentrations were determined using an enzyme immunoassay. Each point represents mean of three independent experiments.

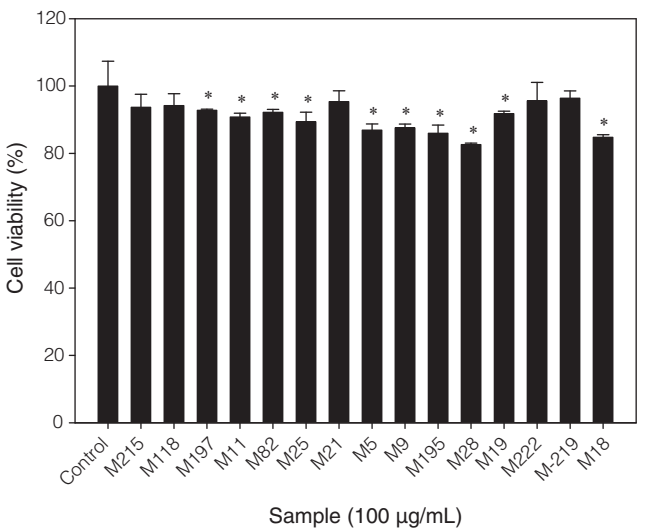

Figure 2. Viability of THP-1 cells following treatment with Chinese herbal medicines. Cells (1.0×10^5) were incubated in a 24-well plate in RPMI 1640 medium with 10% FBS for 24 h. The cells were treated with or without various concentrations of Chinese herbal medicines and cultured for an additional 24 h. Cell viability was assessed using an MTT assay. The values were measured at 570 nm using a model μQuant microplate reader (BioTek, Winooski, VT, U.S.A.). Data were analyzed for statistical significance using the nonparametric Mann-Whitney *U*-test. *: $p < 0.05$ *versus* control group.

Table 4. Cytokine analyses following treatment with selected Chinese tonic herbs (100 mg/mL)

	Med [a]	LPS [a] (10 ng/mL)	M 215	M 118	M 197	M 11	M 82	M 25
(pg/mL)								
IL-1β	ND	8.44 ± 1.91	ND	ND	ND	ND	ND	ND
IL-6	ND	14.02 ± 6.73	ND	ND	ND	ND	ND	ND
IL-8	16.28 ± 6.55	5844.48 ± 1307.35*	20.91 ± 2.80	52.45 ± 16.94*	11.69 ± 1.17	18.39 ± 1.66	17.61 ± 1.47	14.33 ± 0.86
IL-17	ND	6.20 ± 3.07	ND	ND	ND	ND	ND	ND
G-CSF	1.61 ± 0.41	6.67 ± 2.14	ND	ND	ND	ND	ND	ND
GM-CSF	2.04 ± 0.55	2.77 ± 2.13	5.22 ± 1.13	6.52 ± 0.00	14.07 ± 0.16	25.33 ± 7.36*	23.35 ± 3.01*	16.6 ± 5.00
IFN-γ	2.19 ± 1.61	36.03 ± 14.53*	5.44 ± 0.70	ND	7.37 ± 2.04	5.93 ± 0.00	4.42 ± 2.13	7.38 ± 0.68
MCP-1 (MCAF)	2.38 ± 0.25	2012.26 ± 276.47*	5.31 ± 1.24	9.12 ± 1.60	5.69 ± 1.10	6.47 ± 1.55	5.26 ± 1.03	5.43 ± 1.68
MIP-1β	17.64 ± 6.65	356181.53 ± 855.89*	24.66 ± 4.20	42.00 ± 6.18*	24.49 ± 2.40*	25.37 ± 4.37*	25.95 ± 3.99*	23.94 ± 2.60
TNF-α	ND	268.2 ± 98.64*	ND	ND	ND	ND	ND	ND

	M 21	M 5	M 9	M 195	M 28	M 19	M 222	M 219	M 18
(pg/mL)									
IL-1β	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-6	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-8	18.95 ± 7.32	20.11 ± 3.16	11.50 ± 3.61	20.12 ± 9.68	20.76 ± 13.30	13.52 ± 2.48	19.55 ± 15.67	12.42 ± 2.91	11.84 ± 2.87
IL-17	ND	ND	ND	ND	ND	ND	ND	ND	ND
G-CSF	ND	ND	ND	1.09 ± 0.91	ND	ND	ND	ND	ND
GM-CSF	20.22 ± 2.80*	17.38 ± 4.64	15.19 ± 3.90	10.02 ± 4.72	14.44 ± 1.20	13.27 ± 2.34	5.20 ± 3.80	9.86 ± 6.39	17.00 ± 5.02
IFN-γ	6.89 ± 1.36	9.44 ± 1.44	9.74 ± 2.66	6.90 ± 1.36	4.94 ± 1.41	3.90 ± 2.87	ND	ND	5.82 ± 5.58
MCP-1 (MCAF)	5.84 ± 0.92	7.58 ± 0.96	6.20 ± 1.57	6.41 ± 0.42	5.66 ± 0.29	6.50 ± 0.74	5.73 ± 1.38	4.48 ± 0.61	6.48 ± 1.46
MIP-1β	20.76 ± 1.07*	24.51 ± 1.47*	21.21 ± 2.97	39.50 ± 21.28	24.33 ± 1.66*	19.21 ± 0.91	27.59 ± 15.74	21.51 ± 3.00	21.50 ± 2.99
TNF-α	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Abbreviations: Med, medium only; LPS, lipopolysaccharide.
* Statistical significance ($p < 0.05$) between medium and tested groups using one-way ANOVA followed by the Scheffe Test.
ND: Data was beyond the standard curve of the assay. The standard curves ranged from 1.95–32000 pg/mL by serial dilution of the reconstituted lyophilized standards. Cytokine levels were analyzed using a nonlinear regression routine, four-parameter logistic (4PL). IL-2, IL-4, IL-5, IL-7, IL-10, IL-12 (p70), and IL-13 were not detected in the experiment.

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mRNA in cells treated with *C. chinensis*, *D. opposita*, or *P. sibiricum* were higher. Laser densitometry analysis demonstrated that the fold increase for cells treated with *C. chinensis*, *D. opposita*, or *P. sibiricum* were 2.15, 2.10, and 1.16, respectively (Figure 3A). The levels of the IL-8 mRNA also increased by 1.63 fold after treatment with *C. chinensis* in THP-1 cells (Figure 3B). qRT-PCR was used to measure the degree of GM-CSF mRNA expression (relative to GAPDH expression). *D. opposita*, *C. osmophloeum*, and *G. uralensis* upregulated the levels of the GM-CSF mRNA. Compared to the untreated control values, *D. opposita*, *C. osmophloeum*, and *G. uralensis* treatment increased expression of the GM-CSF mRNA by 6.14, 14.38, and 1.19 fold, respectively (Figure 4).

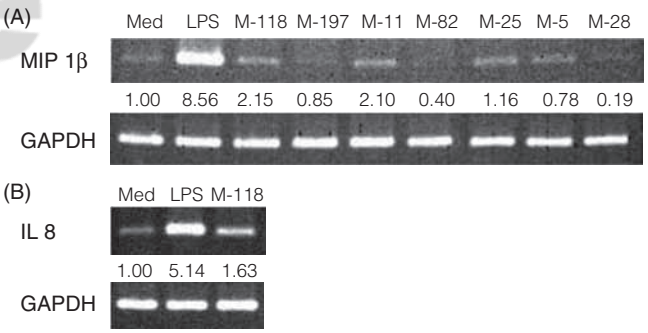

Figure 3. Expression of **(A)** MIP-1β and **(B)** IL-8 mRNAs following treatment of THP-1 cells with LPS or various Chinese herbal medicines, based on RT-PCR analysis. The findings were normalized to the expression of the GAPDH mRNA. Measurements were conducted in triplicate. Med: medium; LPS: 10 ng/mL; Chinese herbal medicines (100 μg/mL).

DISCUSSION

Chinese tonic herbs have traditionally been used to treat various asthenia syndromes. In previous papers, Chinese tonic herbs were found to enhance mitochondrial oxidative processes and exhibited antioxidant[26,27] and antitumor potentials[28], primarily as a result of the enhancement of mitochondrial ATP generation[27]. They have also been shown to affect the respiratory, renal, hepatic, cardiovascular, immunologic, and nervous systems as well as impact cancer, glucose metabolism, and inflammatory conditions[29,30]. In the present study, we evaluated 15 Chinese herbal medicines that are typically used as folk remedies in the treatment of invigoration. We used PBMCs as the target model and multiplexed flow cytometric bead-based assays to measure cytokine release from human monocytes. We demonstrated that *P. lactiflora* exhibited an inhibitory effect on PHA-activated PBMC proliferation. PHA is a mitogen for T lymphocytes[31] that binds *N*-acetylgalactosamine glycoproteins expressed on the cell surface, thus promotes cell proliferation. In this study, T cells were the major proliferating cells in PBMC cultures activated with PHA. The inhibitory effect of *P. lactiflora* on PHA-activated PBMC proliferation could therefore be suggested to suppress T-cell proliferation. As one of the presumed mechanisms of *P. lactiflora's* action on PBMCs, IFN-γ induction may occur through autologous binding to the PBMC receptor and subsequent induction of secondary immune responses to suppress cell proliferation[32].

In this study, *C. chinensis* induced IL-8 and MIP-1β expression, both of which may modulate the immune system. IL-8 serves as a chemical signal that attracts neutrophils to the site of inflammation and, therefore, it is also known as a neutrophil chemotactic factor. When the body encounters an antigen, IL-8 is released to signal other immune cells to migrate to the site of inflammation[33]. Therefore, IL-8 is often associated with inflammation. MIP-1β, a member of the cysteine-cysteine (C–C) chemokine family, is a chemotactic factor in the inflammatory processes[34]. MIP-1β is able to

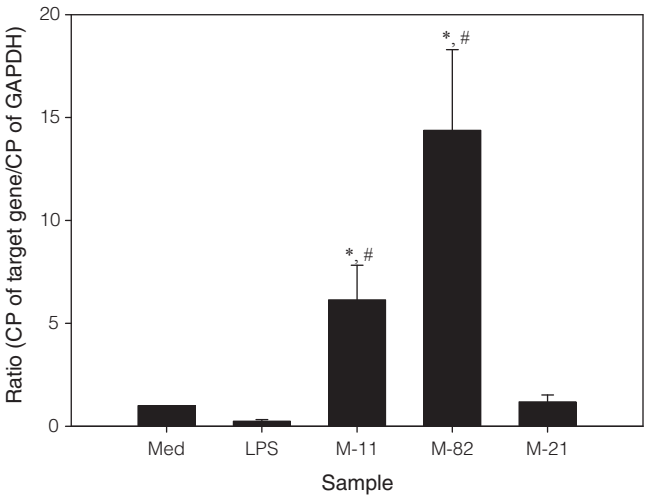

Figure 4. Expression of the GM-CSF mRNA following treatment with *Dioscorea opposita* (M-11), *Cinnamomum osmophloeum* (M-82), or *Glycyrrhiza uralensis* (M-21) based on qRT-PCR analysis. Findings were normalized to the expression of the GAPDH mRNA. Med: medium only; LPS: 10 ng/mL; M-11, M-82, and M-21 (100 μg/mL). Data were analyzed for statistical significance using the nonparametric Mann-Whitney *U*-test. *: $p < 0.05$ *versus* medium only; #: $p < 0.05$ *versus* LPS group.

enhance the development of humoral, cellular, mucosal, and systemic immunity. MIP-1β-mediated immunity is fostered by different regulation of costimulatory molecule expression for support of humoral and cell-mediated immune responses.

In our flow cytometric bead-based assays, we found that *D. opposita*, *C. osmophloeum*, and *G. uralensis* enhanced GM-CSF release compared to controls. Additionally, we analyzed the effects at the mRNA level and found an induction of GM-CSF transcription. GM-CSF has been used to assist patients with cancer undergoing chemotherapy, in patients with AIDS during therapy, and in patients after bone marrow transplantation[35].

Furthermore, GM-CSF is widely used as an adjuvant in clinical trials involving vaccination with autologous tumor cells, peptides, and/or dendritic cells in the treatment of various human neoplasms[36]. In some studies, GM-CSF has appeared to facilitate immune response, but in other studies no effect or even a suppressive effect has been reported. Therefore, these three Chinese herbal medicines may be used as adjuvants for regulating the immune system.

Chemical analysis of *C. chinensis* reported that it contained abundant phenolic compounds such as flavonoids, lignans, and quinic acid derivatives[37]. On the other hand, acidic polysaccharide from *C. chinensis* showed potent stimulating effects on lymphocyte proliferation[38] and exhibited immune enhancement activities, most notably promoting the proliferation of T and B cells *in vitro*[39]. In other previous investigations, phenolic compounds isolated from *D. opposita* exhibited cyclooxygenase inhibitory activity[40]. Cinnamaldehyde and some flavonol glycosides from *C. osmophloeum* showed anti-inflammatory effects via inhibition of cytokine production in murine macrophages[41,42]. Licorice flavonoids of *G. uralensis* were reported to reduce the levels of LPS-induced inflammatory cells in bronchoalveolar lavage fluids[43In the present study, we selected human PBMCs and monocyte-derived macrophage THP-1 cells as target cells to elucidate the immunomodulatory activities of Chinese herbal extracts. PHA is a mitogen for T lymphocytes that binds to *N*-acetylgalactosamine glycoproteins on the cell surface and activates proliferation and cytokine production, including IL-2 and IFN-γ[44]. In this study, T cells were the major proliferating cells in PBMC cultures activated with PHA. Thus, effects of *P. lactiflora* on PHA-activated PBMC proliferation or IFN-γ production could suggest immunomodulatory activities on human T lymphocytes. Although the THP-1 cell line is derived from human acute monocytic leukemia cells, it exhibits macrophage-like characteristics and secretes macrophage-derived cytokines such as GM-CSF and MIP-1β upon stimulation with LPS. Thus, THP-1 cells are particularly useful for studying the effects of Chinese herbs on macrophage-specific cytokines[45]. In the present investigation, we demonstrated that *D. opposita*, *C. osmophloeum*, and *G. uralensis* enhanced GM-CSF secretion in THP-1 cells by modulation of GM-CSF expression and these herbs were potential immunomodulators for macrophages.

CONCLUSIONS

In this study, *P. lactiflora* was shown to exhibit inhibitory effects on PHA-activated PBMC proliferation and induce IFN-γ production. *C. chinensis* induced both IL-8 and MIP-1β release through upregulation of mRNA transcription in human monocytes (THP-1). *D. opposita*, *C. osmophloeum*, and *G. uralensis* were shown to increase GM-CSF expression through enhanced transcription in THP-1 cells. Thus, Chinese tonic herbs may modulate cytokine production in human cells in diverse ways and demonstrate significant potential as adjuvant immune-boosting drugs. The regulatory mechanisms underlying these observations remain to be elucidated.

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