JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 927–936 927
DOI:10.1021/jf902952z

Immunostimulatory Bioactivity of Algal Polysaccharides from *Chlorella pyrenoidosa* Activates Macrophages via Toll-Like Receptor 4

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Much research suggests that a dietary supplement of *Chlorella pyrenoidosa* may be helpful to human health, but the molecular mechanism involved remains unclear. The aim of this research was to investigate the effects of certain hot-water-soluble polysaccharides from *Chlorella pyrenoidosa* (CWSP) on cytokine production, human leukocyte antigen (HLA) expression, and costimulatory molecule expression in macrophages. We demonstrated that CWSP induced IL-1β secretion in macrophages via Toll-like receptor 4 (TLR4) mediated protein kinase signaling pathways. In addition, CWSP also stimulated the cell surface expression of HLA-DA, -DB, and -DC, and HLA-DR, -DP, and -DQ as well as the expression of costimulatory family molecules such as CD80 and CD86 in macrophages. Furthermore, we demonstrated that preinjection of C57BL/6J mice with CWSP increased lipopolysaccharide (LPS)-induced tumor necrosis factor-α and IL-1β secretion into serum in vivo. This outcome was consistent with the corresponding outcome for cells treated with CWSP in vitro. Our current results provide support for the possible use of CWSP as a modulation agent of immune responses in humans and certain animal species. Finally, in using GC-MS to analyze the polysaccharides, we found that the major monosaccharides of CWSP were rhamnose (31.8%), glucose (20.42%), galactose (10.28%), mannose (5.23%), and xylose (1.27%). This study is the first to report the molecular mechanism of immune-modulated signal transduction in vitro from the polysaccharides of *Chlorella pyrenoidosa*.

KEYWORDS: *Chlorella*; polysaccharides; Toll-like receptor; protein kinase; cytokine

INTRODUCTION

Chlorella, unicellular green algae, has often been used for health-improvement purposes, including the treatment of hypertension and the modulation of human immune responses (*1*). It has been previously reported that a hot-water extract of *Chlorella* may elicit various beneficial pharmacological effects against cancers (*2*), bacterial infections (*3*), and viral replication (*4*). From an earlier study, *Chlorella vulgaris*-extract-administered mice produced more INFγ and IL-2 levels in serum than did control mice (*5*). It has also been reported that oral administration of hot-water extract of *Chlorella vulgaris* enhances resistance in mice to *Listeria monocytogenes* through augmentation of cytokine production (*6*). Pugh et al. demonstrated the function of polysaccharides isolated from the *Chlorella pyrenoidosa* alga as regards the stimulation of IL-1β mRNA and TNFα mRNA expression and NF-κB activation (*7*), but most of this research rarely focused on the molecular mechanisms involved. Here, we will investigate how polysaccharides of *Chlorella pyrenoidosa* regulate immune signal transduction, especially in IL-1β. It is well known that IL-1β, one of the critical cytokines for modulating immune responses, is secreted mainly from activated macrophages (*8*). Mice pretreated with recombinant IL-1β 2 h prior to infection with *E. coli* reveal a significantly diminished level of mortality compared with that of infected mice that had not been similarly pretreated (*9*). IL-1β is produced as a 31-kDa precursor that must be cleaved into a 17-kDa form in order to become bioactive. The conversion of preIL-1β into bioactive IL-1β can be accomplished by the IL-1β converting enzyme (*10*).

Toll-like receptors (TLRs) are of a family that has been shown to be essential for the recognition of a range of microbial

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Published on Web 11/16/2009

components (*11*). A glycoprotein isolated from a *Chlorella vulgaris* culture medium stimulated TLR-dependent IL-12 production in spleen-adherent cells in mice (*12*). In our previous studies, we demonstrated that mitogen-activated protein kinases (MAPKs) play important roles in cytokine expression in macrophages in response to stimulation by certain polysaccharides (*13*−*16*), but how *Chlorella* polysaccharides mediate MAPK activation in the regulation of cytokine expression is unclear. Therefore, in this research we investigated the role of hot-water-soluble polysaccharides from *Chlorella pyrenoidosa* (CWSP) in immune signal transduction in macrophages.

Macrophages, one of the antigen-presenting cells (APCs), play important roles in host defense mechanisms. In response to certain extracellular signals, macrophages develop a mature antigen-presentation function and stimulate the activation of T helper cells (Th cells), which influence the overall immune responses (*17*). Macrophages in the resting stage are not able to efficiently induce activation of Th cells. One of the reasons that macrophages are not always able to efficiently induce activation of Th cells is their rather low cell-surface expression of human leukocyte antigen (HLA) molecules and costimulatory family molecules such as CD80 and CD86 (*18*−*20*).

The potential beneficial immunological properties of polysaccharides derived from *Chlorella pyrenoidosa* have been less studied. Herein we report on how we have isolated and analyzed the chemical characteristics of CWSP and investigated their immune-modulation functions, including stimulation of IL-1β expression in human and murine macrophages. In addition, we demonstrated that CWSP are able to increase cell surface expression of HLA as well as CD80 and CD86 in human macrophages. Further, we have found that pretreatment with CWSP of macrophages from C57BL/6J mice results in the upregulation of tumor necrosis factor-α (TNFα) and IL-1β secretion (in cultured medium and mouse serum, respectively) after LPS stimulation. Thus, the results of this study may lead to the further development of CWSP as agents capable of contributing to the modulation of cytokine expression.

MATERIALS AND METHODS

Cell Cultures. Murine macrophage J774A.1 cells (J774A.1 cells) were obtained from American Type Culture Collection (Manassas, VA). HeNC2 (with functional TLR4) and GG2EE (lacking functional TLR4) were from D. Radzioch (McGill University, Montreal, Canada). Human primary monocytes were obtained from normal blood donor buffy coats (Taipei Blood Center, Taipei, Taiwan). Buffy coat cells were mixed with an equal volume of PBS, layered on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 400x*g* for 30 min at 20 °C. The interface, containing mononuclear cells, was collected and washed twice with PBS. Thereafter, human primary monocytes (98% $CD14^+$, analyzed by flow cytometry) were isolated from mononuclear cells by Monocyte Isolation Kit II (Miltenyi Biotech, Auburn, CA, USA). Human primary monocytes were cultured in RPMI-1640 supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA). Human primary macrophages were obtained by culturing monocytes for 7 days in RPMI-1640 supplemented with 15% fetal calf serum at a density of $1.5 \times 10^5/cm^2$. During the culturing period, nonadherent cells were removed by washing with PBS. After 7 days of incubation, the adherent cells were used as human blood monocyte-derived macrophages. Human THP-1 monocytes (1×10^6 cells/mL) were differentiated into macrophages in 60 mm dishes by culturing cells in RPMI-1640 supplemented with 10% fetal calf serum and 100 ng/mL phorbol 12-mysistate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA) for 5 days. Nonadherent cells were removed by washing with PBS, and the remaining adherent cells were used as human THP-1 macrophages. Murine J774A.1 macrophages were obtained from ATCC (Rockville, MD, USA) and propagated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine (Life Technologies, Inc., Rockville, MD, USA), and then cultured in a 37 °C, 5% CO_2 incubator.

Materials. *Chlorella pyrenoidosa* is a commercially available product from Taiwan *Chlorella* Manufacturing Co., Ltd. (Taipei, Taiwan). LPS (from *Escherichia coli* 0111: B4), antidiphosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) antibody, antidiphosphorylated c-Jun N-terminal kinase 1/2 (JNK1/2) antibody, antidiphosphorylated p38-MAKP (p38) antibody, antiactin antibody, PD98059, Ro-31-8220, SB203580, curcumin, and LY294002 were purchased from Sigma Co. (St. Louis, MO, USA). Mouse IL-1β and TNFα ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). A human IL-1β ELISA kit was purchased from BioSource International, Inc. (Camarillo, CA, USA). Anti-IL-1β, antirabbit IgG-HRP, and antimouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The CaspACE Assay System, Fluorometric was purchased from Promega (Madison, WI, USA).

Preparation of CWSP. *Chlorella pyrenoidosa* samples were collected from aquaculture ponds at the Taiwan *Chlorella* Manufacturing Co., Ltd. After harvesting from the culture pool, the algae were cleaned with water under a 200 mesh net and then air-dried. Twenty grams of *Chlorella pyrenoidosa* samples were extracted with distilled water (300 mL) for 20 min at 121 °C by an autoclave. The extracts were filtered by a 0.2 μm membrane, followed by vacuum-concentration at 50 °C, giving a final volume of 100 mL to which five volumes of 95% ethanol were added slowly at 4 °C. The mixture was centrifuged to produce the precipitates (CWSP, 1150 mg) and supernatants (polysaccharide-free *Chlorella pyrenoidosa* water-soluble extract, PF-CWE).

Gel Filtration Chromatography and Sugar Composition Analysis of CWSP. One hundred milligrams of CWSP was purified by gel filtration chromatography using a Sephadex G-100 column (100×1.6 cm) (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden), with distilled deionized water as the eluent. The flow rate was set at 0.3 mL/min, and 5.0 mL per tube was collected. Under the same conditions, a Shodex standard P-82 column was used as an internal standard for molecular weight. All chromatography processes were executed at 4 °C. After the chromatography processes, each fraction was subjected to analysis to determine its total carbohydrate content; this was done using the phenol-sulfuric acid colorimetric method (with glucose as the calibration standard) (*21*). For monosaccharide analysis, 0.5 mg of polysaccharide was methanolyzed with 0.2 mL of 0.5 N methanolic HCl (Supelco, Bellefonte, PA, USA) at 80 °C for 16 h; re-N-acetylated with 500 mL of methanol, 10 mL of pyridine, and 50 mL of acetic anhydride; and then treated with Sylon HTP trimethylsilylation reagent (Supelco, Bellefonte, PA, USA) for 20 min at room temperature, after which it was dried by nitrogen blowing and redissolved in hexane. GC-MS analysis of the trimethylsilylated derivatives was carried out using a Hewlett-Packard (HP) Gas Chromatograph 6890 connected to an HP 5973 mass selective detector. Samples were dissolved in hexane prior to splitless injection into an HP-5MS fused silica capillary column ($30 \text{ m} \times 0.25$ mm I.D.). The column head pressure was maintained at around 8.2 psi to give a constant flow rate of 1 mL/min using helium as the carrier gas. Initial oven temperature was held at 60 °C for 1 min; increased to 140 at 25 °C/min; then to 250 at 5 °C/min; and then to 300 at 10 °C/min. The CWSP investigated herein revealed a higher molecular-weight group comprising monosaccharides larger than 1,000 kDa (data not shown).

Measurement of Intracellular H_2O_2 Production. Intracellular H_2O_2 stimulated by CWSP and LPS was measured by detecting the fluorescent intensity of carboxyl-2′,7′-dichlorofluorescein diacetate (CM-DCFH) (Molecular Probes, Inc., Eugene, OR, USA) oxidized product, CM-DCF. Briefly, J774A.1 cells (1×10^6/mL) grown in serum- and phenol red-free RPMI medium for 24 h were then preincubated with CM-DCFH (2 μM) and N-acetyl cysteine (NAC) (10 mM) at 37 °C for 30 min in the dark. This was followed by adding fresh starvation medium containing CWSP (100 μg/mL) or LPS (1 μg/mL) for an additional time, as indicated. The relative fluorescent intensity of the fluorophore CM-DCF, which was formed by peroxide oxidation of the nonfluorescent precursor, was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a Cytofluor 2300 fluorometer (Millipore, Inc., Bedford, MA, USA).

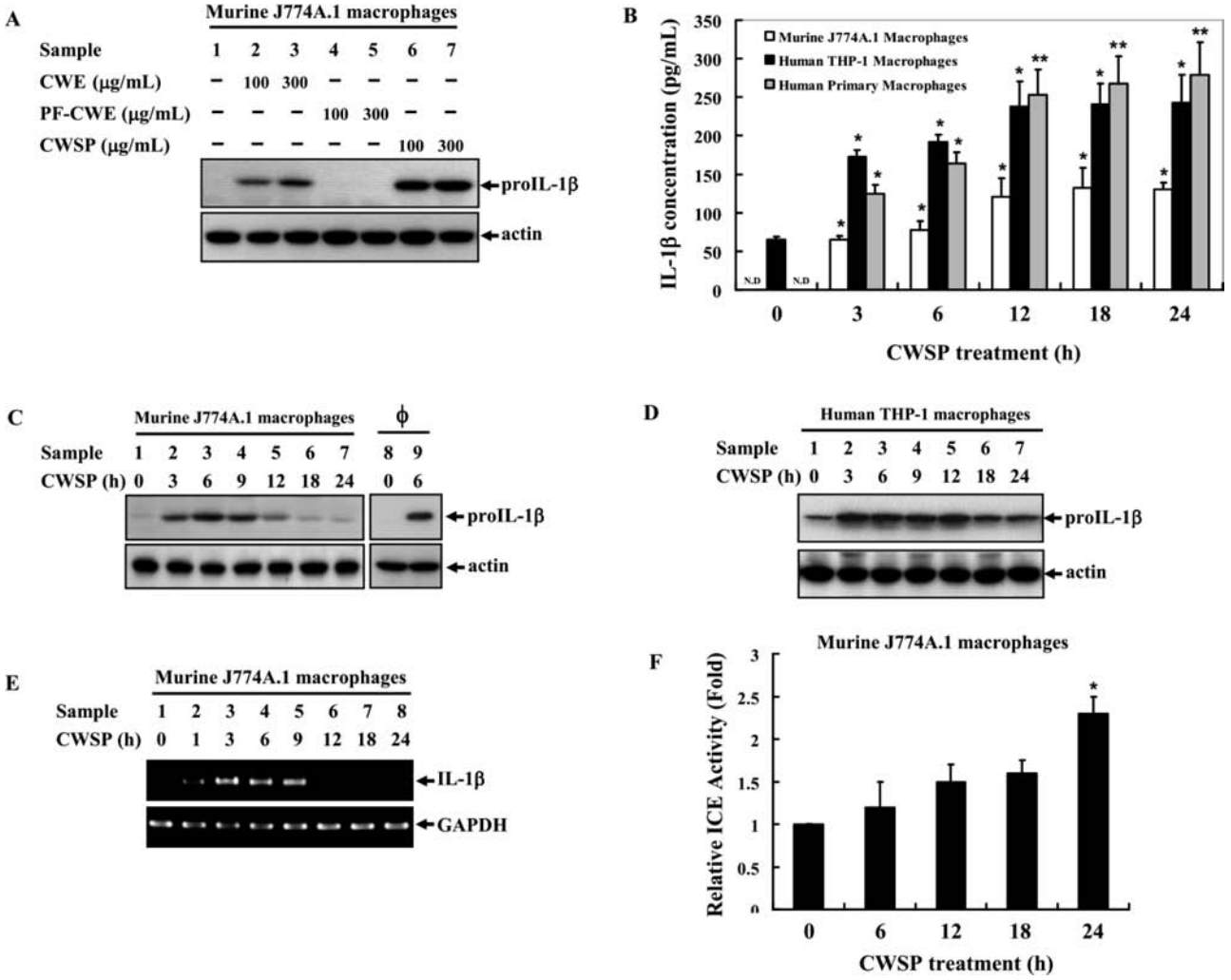

Figure 1. CWSP increase IL-1β expression in macrophages. (**A**) Incubation of J774A.1 cells with hot-water extract of *Chlorella pyrenoidosa* (CWE) and hot-water-soluble polysaccharides of *Chlorella pyrenoidosa* (CWSP), but not polysaccharide-free CWE (PF-CWE), for 6 h induces proIL-1β expression. (**B**) CWSP (100 μg/mL) stimulate IL-1β secretion in J774A.1 macrophages (white bars), human THP-1 macrophages (black bars), and human primary macrophages (gray bars). Shown are the mean values of three experiments $\pm$ SD. N.D means nondetectable; *p < 0.05; **p < 0.01 versus control. (**C**) CWSP (100 μg/mL) increase proIL-1β expression in J774A.1 macrophages (samples 1−7) and human blood monocyte-derived macrophages (samples 8 and 9). One of three experiments is presented. (**D**) CWSP (100 μg/mL) increase proIL-1β expression in human THP-1 macrophages. One of three experiments is presented. (**E**) CWSP (100 μg/mL) increase IL-1β mRNA expression in J774A.1 macrophages. GAPDH was used as an internal control. One of three experiments is presented. (**F**) CWSP (100 μg/mL) increase IL-1β converting enzyme (ICE) activity in J774A.1 macrophages. Shown are the mean values of three experiments $\pm$ SD *p < 0.05 versus control.

Mice Model for Testing CWSP. All studies were approved by the Institutional Animal Care and Use Committee at National Yang-Ming University, Taipei, Taiwan, and met university guidelines for the use of experimental animals in research. Male, 6- to 8-week-old C57BL/6J mice (National Laboratory Animals Center, Taiwan) were used in all studies. Mice were housed in a monitored, light−dark cycled environment and provided standard lab chow and water. Mice were intraperitoneally (IP) injected with CWSP (100 mg/kg of body weight in 0.1 mL of PBS); control mice received PBS (0.1 mL) in the same regimen. After 24 h, all mice received a challenge dose of LPS (10 mg/kg, IP). Serum was collected from test mice after 1.5 h of LPS challenge for further assessment of cytokine expression by ELISA.

Monitoring of LPS Contamination of CWSP in Experiments. Our reagents and utensils for the preparation of CWSP were either of LPS-free grade or were washed with PBS containing 50 μg/mL polymyxin B (PMB), then rinsed with PBS. In order to rule out possible LPS contamination of CWSP samples, J774A.1 cells were preincubated with or without PMB (10 μg/mL) for 30 min, followed by treatment for 6 h with CWSP (10, 100, or 300 μg/mL) or LPS (1 or 2 μg/mL), respectively. ProIL-1β expression was analyzed by Western blotting. In addition, LPS concentration in the CWSP samples was analyzed using a Limulus ameboctye lysate PYROCHROME assay kit (Associates of Cape Cod, Inc., Falmouth, MA, USA).

Flow Cytometry Analysis. For cell surface expression experiments of HLA and costimulatory molecules, THP-1 macrophages were incubated with medium (control), CWE (100 μg/mL), CWSP (100 μg/mL), polysaccharide-free CWE (100 μg/mL), or LPS (1 μg/mL) for 24 h. Cells were fixed, and cell surface expression of HLA and costimulatory molecules were measured by staining cells for 30 min with specific fluorescence-conjugated antibodies on ice. After washing, cells were subjected to flow cytometry analysis on FACSCalibur using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated by the REzol C&T method according to the manufacturer's instructions. For reverse transcription, the reaction was performed at 42 °C for 30 min, at 99 °C for 5 min, and then cooled to 4 °C. For future PCR, the PCR mixture was held at 94 °C for 2 min and then cycled 30 times at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min followed by 10 min at 72 °C at the final cycle. The products from the PCR reaction were examined by 1% agarose gel electrophoresis with ethidium bromide (EtBr) and normalized by comparison to RT-PCR of mRNA of GAPDH, a constitutively expressed gene. Each EtBr-stained band was

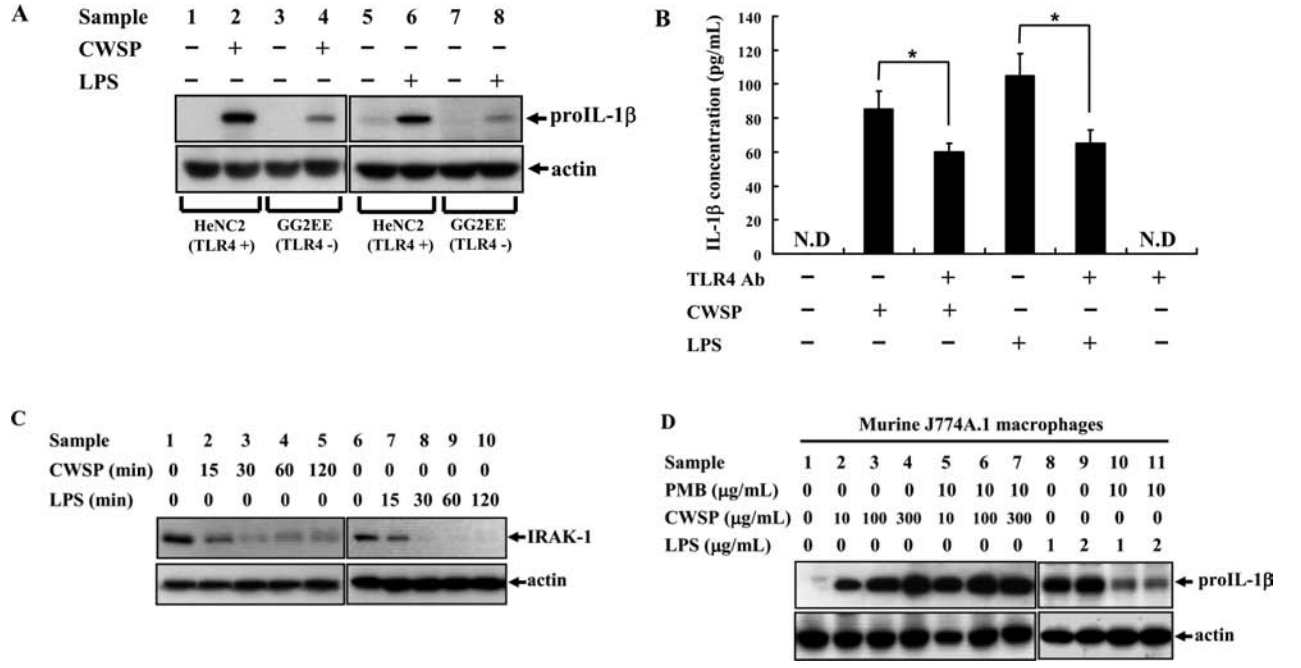

Figure 2. CWSP induce IL-1β expression via TLR4. (**A**) CWSP (100 μg/mL) induce proIL-1β expression in HeNC2 macrophages (functional TLR4 normal) but not in GG2EE macrophages (functional TLR4 deficient). One of three experiments is presented. (**B**) TLR4 neutralizing antibody (HTA125, 10 μg/mL) blocks CWSP (100 μg/mL)-induced IL-1β secretion in human THP-1 macrophages. Shown are the mean values of three experiments ± SD; *$p < 0.05$. (**C**) CWSP (100 μg/mL) and LPS (1 μg/mL) downregulate IL-1β receptor-associated kinase-1 (IRAK-1) expression in J774A.1 macrophages. One of three experiments is presented. (**D**) Polymyxin B sulfate (PMB) inhibited LPS-induced, but not CWSP-induced, proIL-1β expression in J774A.1 macrophages. One of three experiments is presented.

quantified using ImageQuaNT software from a PhosphorImager from Molecular Dynamics (Sunnyvale, CA).

Western Blotting Analysis. Whole cell lysates were separated by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and electrotransferred to a PVDF membrane. The membrane was incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h. The membrane was incubated with anti-IL-1β antibody or anti-MAPKs antibody at room temperature for 2 h. After washing three times in PBS with 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody directed against the primary antibody. The membrane was developed by an enhanced chemiluminescence Western-blotting detection system (DuPont NEN Research Product Co, Boston, MA) according to the manufacturer's instructions.

Enzyme Linked Immunosorbent Assay (ELISA). The J774A.1 cell, THP-1 cells, and human primary cells (1×10^6/mL) were stimulated with CWSP (100 μg/mL) for the indicated time points within 24 h. The IL-1 concentration in the condition medium was analyzed by ELISA according to the protocol from R & D Mouse IL-1beta ELISA System (R & D Systems, Inc.). In brief, 50 μL of biotinylated antibody reagent and 50 μL of supernatant were added to an antimouse IL-1 precoated stripwell plate and incubated at room temperature for 3 h. After washing the plate three times with washing buffer, 100 μL of diluted streptavidin-HRP concentrate was added to each well and incubated at room temperature for 30 min. The washing process was repeated, and 100 μL of a premixed TMB substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition of 100 μL of provided stop solution to each well to stop the reaction, the absorbance of the plate was measured by a MRX microplate reader (Dynex Tech. Inc.) at a 450−550 nm wavelength.

Statistical Analysis. All values are given as the mean ± SD. Data analysis involved one-way ANOVA with a subsequent Scheffé test.

RESULTS

CWSP Induce IL-1β Expression in Macrophages and Sugar Composition Analysis. The immune-modulation activity of *Chlorella pyrenoidosa* was monitored by its proIL-1β (precursor of IL-1β) induction activity in J774A.1 macrophages. The hot-water extract of *Chlorella pyrenoidosa* (CWE) induced proIL-1β expression in a dose-dependent manner (**Figure 1A**, samples 2 and 3). In order to investigate the role of polysaccharides on proIL-1β expression in CWE-stimulated J774A.1 macrophages, the polysaccharides in CWE were removed. We found that polysaccharide-free CWE was unable to induce proIL-1β expression (**Figure 1A**, samples 4 and 5). Furthermore, we isolated CWSP and found that CWSP induced proIL-1β expression to a greater level than CWE did (**Figure 1A**, samples 6 and 7), indicating that polysaccharides play important roles in *Chlorella pyrenoidosa*-induced proIL-1β expression. In addition, in order to test whether CWSP are able to stimulate macrophages to secrete IL-1β, J774A.1 macrophages were stimulated with CWSP during a testing period of 0 to 24 h. IL-1β was detected in conditioned media as early as 3 h after CWSP stimulation of J774A.1 macrophages, and the IL-1β concentration remained elevated for up to 24 h post-CWSP stimulation (**Figure 1B**, white bars). We have tested the effect of dextran and the polysaccharides from *Isochrysis galbana* (M.W. > 1 M Da) on IL-1β expression, and we found that both of them were unable to induce IL-1β expression in murine macrophages (data not shown). In addition, the effect of CWSP on IL-1β expression in human THP-1 macrophages was investigated. IL-1β protein levels of around 170, 190, 210, 240, and 250 pg/mL were detected in the conditioned medium of CWSP-treated THP-1 macrophages at poststimulation times of, respectively, 3, 6, 9, 12, 18, and 24 h (**Figure 1B**, black bars). This IL-1β induction activity of CWSP was also confirmed in human blood monocyte-derived primary macrophages (**Figure 1B**, gray bars). These results indicate that CWSP could induce IL-1β secretion in both murine and human macrophages. We further investigated the molecular mechanism controlling the secretion of mature IL-1β in CWSP-stimulated J774A.1 macrophages. The expression of proIL-1β was detected between 3 and 12 h subsequent to CWSP

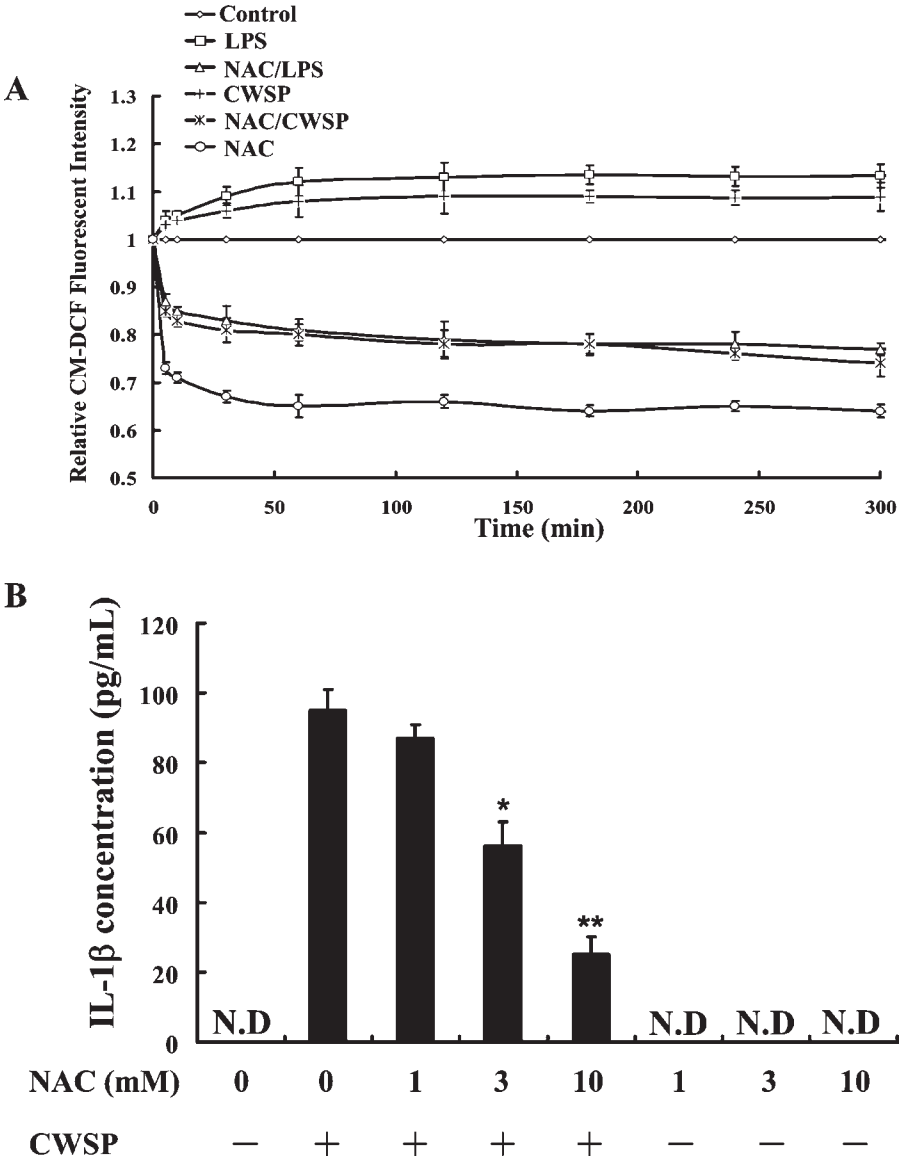

Figure 3. CWSP induce IL-1β expression via H_2O_2. (**A**) J774A.1 cells were preincubated with CM-H2DCFDA (2 μM) for 30 min, followed by substitution with medium containing CWSP or LPS in the presence or absence of N-acetyl-cysteine (NAC) for additional incubation for the indicated times. The relative fluorescence intensity of fluorophore CM-H2DCF was detected. (**B**) J774A.1 cells were stimulated with CWSP in the presence or absence of NAC for 24 h, and IL-1β secretion in cultured medium was measured by ELISA. Shown are the mean values of three experiments ± SD; *$p < 0.05$; **$p < 0.01$ versus CWSP alone. N.D: nondetectable.

stimulation (**Figure 1C**, samples 1−7). Similarly, CWSP induced proIL-1β expression in both human blood monocyte-derived primary macrophages (**Figure 1C**, samples 8 and 9) and human THP-1 macrophages (**Figure 1D**). Moreover, using a RT-PCR method we demonstrated that IL-1β mRNA expression was detected in J774A.1 macrophages 1 h subsequent to CWSP stimulation. After 12 h, IL-1β mRNA expression began to decline to the basal level (**Figure 1E**). Post-translational regulation and processing of proIL-1β protein into mature IL-1β via an IL-1β converting enzyme in J774A.1 macrophages has been reported previously (*15*). We found that IL-1β converting enzyme activity increased around 3-fold in CWSP-stimulated cells, compared to that in control cells (**Figure 1F**). Furthermore, the CWSP were observed to be primarily composed of rhamnose (31.8%), glucose (20.42%), galactose (13.28%), mannose (5.23%), and xylose (1.27%), as analyzed by GC-MS.

CWSP Induce IL-1β Expression via Toll-Like Receptor 4 (TLR4). We have demonstrated that TLR4 is involved in polysaccharide-mediated cytokine expression in macrophages (*13*, *15*). In this study, we chose two genetically specific TLR4 relevant murine macrophage cell lines, HeNC2 (with functional TLR4) and GG2EE (lacking functional TLR4), to examine the role of TLR4 in CWSP-mediated cytokine expression (*22*). HeNC2 cells produced proIL-1β upon CWSP stimulation, whereas there was no significant proIL-1β expression in CWSP-treated GG2EE cells (**Figure 2A**). In addition, significant proIL-1β expression was observed in LPS-treated HeNC2 cells but not in GG2EE cells (**Figure 2A**). These results suggested that TLR4 is one of the putative receptors for CWSP-mediated IL-1β expression.

Next, to investigate the potential interaction of TLR4 and CWSP leading to the induction of IL-1β secretion, experiments were conducted by preincubating human THP-1 macrophages with the TLR4 neutralizing antibody, which is known to specifically inhibit LPS-induced signaling through TLR4. As expected, TLR4 neutralizing antibody inhibited both CWSP- and

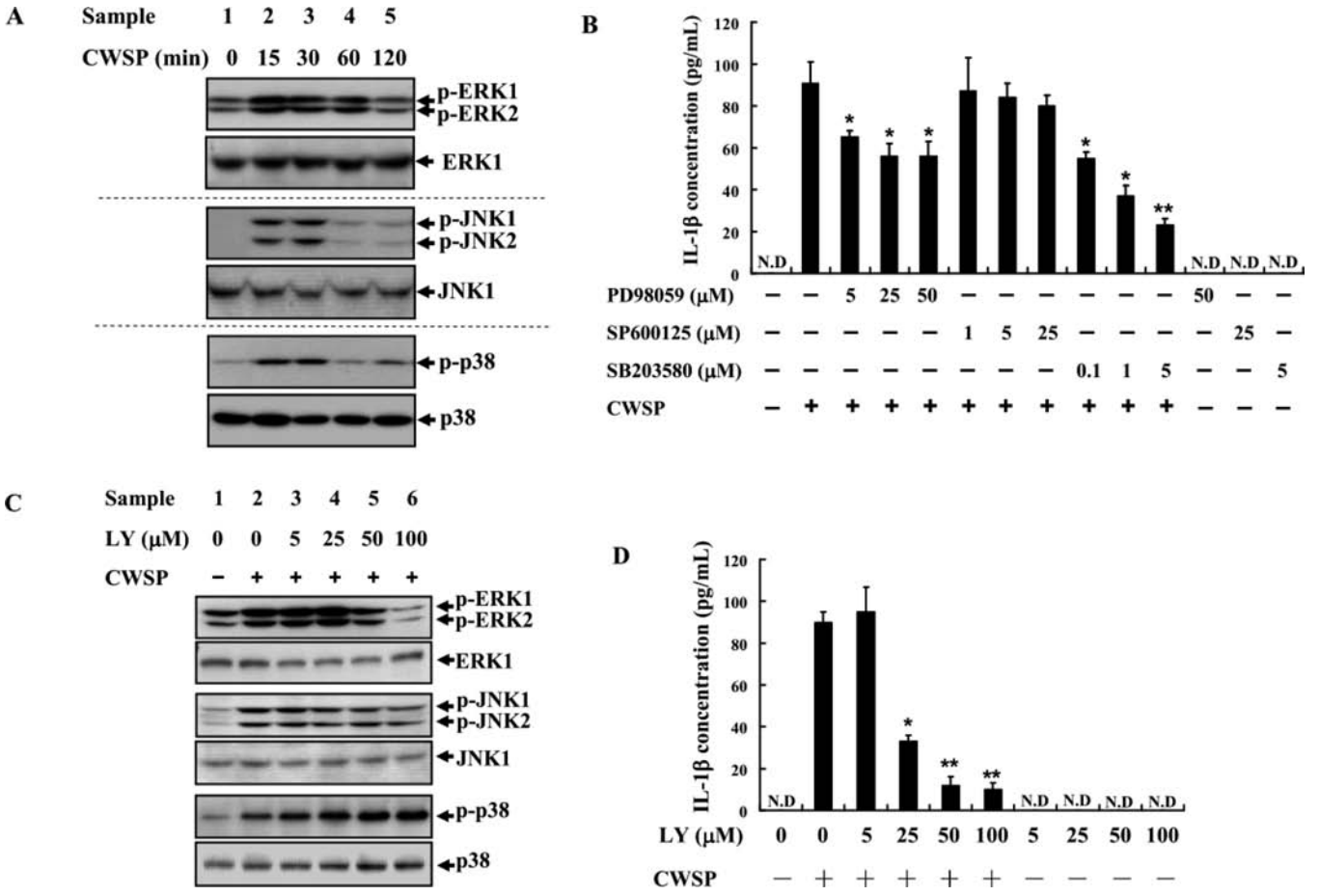

Figure 4. CWSP induces IL-1β secretion through MAPKs and PI3-kinase. (**A**) Time-course of ERK1/2, JNK1/2, and p38 phosphorylation in CWSP-stimulated J774A.1 macrophages. Cells were stimulated with CWSP (100 μg/mL) and the cell lysates collected at different periods of time. Cell lysates were analyzed by Western blot with antidiphosphorylated ERK1/2, antidiphosphorylated JNK1/2, and antidiphosphorylated p38 monoclonal antibody; one of three experiments is presented. (**B**) Effects of PD98059, SP600125, and SB203580 on IL-1β secretion in CWSP-stimulated J774A.1 macrophages. Cells were preincubated for 30 min with one of the following: PD98059 (5, 25, or 50 μM), SP600125 (1, 5, or 25 μM), and SB203580 (0.1, 1, or 5 μM), followed by CWSP (100 μg/mL) stimulation for an additional 24 h. Conditioned medium was assayed for IL-1β concentration using IL-1β ELISA. Shown are the mean values of three experiments $\pm$ SD; $^{**}p < 0.01$; $^{*}p < 0.05$ compared with CWSP alone. N.D: nondetectable. (**C**) Effect of PI3-kinase inhibitor LY294002 on ERK1/2, JNK1/2, and p38 phosphorylation in CWSP-stimulated J774A.1 macrophages. Cells were pretreated with various concentrations of LY294002 (5, 25, 50, and 100 μM) for 30 min prior to 20 min stimulation by CWSP (100 μg/mL), followed by the measurement of the phosphorylation of ERK1/2, JNK1/2, and p38; one of three experiments is presented. (**D**) LY294002 reduced IL-1β secretion in CWSP-stimulated J774A.1 macrophages. Cells were pretreated with LY294002 (5, 25, 50, and 100 μM) for 30 min followed by incubation with CWSP (100 μg/mL) for an additional 24 h; IL-1β secretion was analyzed by ELISA. Shown are the mean values of three experiments $\pm$ SD; $^{**}p < 0.01$; $^{*}p < 0.05$ compared with CWSP alone. N.D: nondetectable.

LPS-induced IL-1β secretion (**Figure 2B**). Furthermore, the expression level of IL-1β receptor-associated kinase-1, a rapidly degraded protein after TLR4 activation (*23*), was evaluated. The time course of IL-1β receptor-associated kinase-1 degradation in CWSP-treated cells was similar to that in LPS-treated cells (**Figure 2C**).

LPS is a cell wall component of Gram-negative bacteria, and is a potent stimulator of macrophages in regard to IL-1β expression (*13*). In order to rule out the possibility of CWSP-induced IL-1β expression being due to LPS contamination during CWSP preparation, we tested the effect of polymyxin B (PMB), an antibiotic, which was used to neutralize the activity of LPS (*15*) on CWSP- and LPS-induced proIL-1β expression in J774A.1 macrophages. We found that PMB obviously inhibited LPS-induced proIL-1β expression (**Figure 2D**, samples 10 and 11 vs samples 8 and 9); yet in contrast, PMB was not able to inhibit proIL-1β expression in CWSP-stimulated cells (**Figure 2D**, samples 5, 6, and 7 vs samples 2, 3 and 4). In addition, the LPS content of CWSP samples was determined by the Limulus amebocyte lysate assay, and an insignificant level of endotoxin (< 1 EU/mL^{-1}) was found in the tested CWSP samples (data not shown). Taken together, we consider that the effect of CWSP upon IL-1β expression was not due to LPS contamination of the CWSP.

CWSP Induce IL-1β Expression via H_2O_2. We have demonstrated that H_2O_2 is involved in TLR4-mediated IL-1β expression (*13*). In this study, we investigated whether H_2O_2 is involved in CWSP-mediated IL-1β expression in macrophages. Initially, we examined whether CWSP could induce H_2O_2 production in J774A.1 macrophages. Similar to the effect of LPS treatment on H_2O_2 production, CWSP rapidly induced significant H_2O_2 production in J774A.1 macrophages, compared to that in untreated control cells (**Figure 3A**). By contrast, pretreatment of J774A.1 macrophages for 30 min with *N*-acetyl-cysteine (NAC), a potent antioxidant, rapidly reduced CWSP-induced H_2O_2 production (**Figure 3A**). Furthermore, we found that NAC was able to elicit a dose-dependent inhibition of CWSP-induced IL-1β secretion, indicating that H_2O_2 is involved in IL-1β secretion in CWSP-stimulated J774A.1 macrophages (**Figure 3B**).

CWSP Induce IL-1β Expression via ERK1/2 and p38. In order to further examine CWSP-mediated signal transduction

pathways in the regulation of IL-1β expression, we tested whether CWSP could stimulate the phosphorylation of ERK1/2, p38, and JNK1/2 in J774A.1 macrophages. The level of ERK1/2 phosphorylation in J774A.1 macrophages appeared to increase at around 15 min post-CWSP stimulation and extended to 60 min post-CWSP stimulation (**Figure 4A**). The level of p38 phosphorylation in J774A.1 macrophages also increased significantly between 15 and 30 min subsequent to CWSP stimulation. After 60 min, the p38 phosphorylation level gradually returned to the basal level (**Figure 4A**). Furthermore, CWSP treatment of J774A.1 macrophages also increased the phosphorylation level of JNK1/2 in J774A.1 macrophages (**Figure 4A**).

Next, we examined whether MAPKs-related signaling constituted one of the main downstream signaling cascades in the regulation of IL-1β secretion in CWSP-stimulated J774A.1 macrophages. Cells were separately exposed to one of the pharmaceutical protein kinase inhibitors, PD98059, SP600125, or SB203580, such exposure inhibiting, respectively, MEK1, JNK1/2, and p38 activity. The results indicated that PD98059 and SB203580 inhibited IL-1β secretion, suggesting that the MEK1/ERK1/2 and p38 pathways are involved in IL-1β secretion in CWSP-stimulated J774A.1 macrophages (**Figure 4B**). In contrast, SP600125 appeared to exert no real effect upon IL-1β secretion, suggesting that JNK1/2-related signaling pathways are not involved in CWSP-induced IL-1β secretion (**Figure 4B**). Furthermore, the effects of these inhibitors on proIL-1 expression were similar to that on IL-1β secretion (data not shown).

PI3-Kinase Lies Upstream of ERK1/2 and JNK1/2 in CWSP-Stimulated J774A.1 Macrophages. We have demonstrated that PI3-kinase is involved in TLR4-mediated signal transduction in the regulation of IL-1β expression (*13*). In order to dissect the interrelationship between PI3-kinase and MAPKs, we used LY294002 to examine the role of PI3-kinase in the activation of ERK1/2, JNK1/2, and p38 in CWSP-stimulated J774A.1 macrophages. In the absence of LY294002, CWSP induced the phosphorylation of ERK1/2, JNK1/2, and p38; whereas CWSP-induced phosphorylation of ERK1/2 and JNK1/2, but not p38, was significantly inhibited by LY294002 (**Figure 4C**). These results indicate that PI3-kinase lies upstream of ERK1/2 and JNK1/2 in CWSP-stimulated J774A.1 macrophages. In addition, we found that LY294002 significantly reduced IL-1β secretion in CWSP-stimulated J774A.1 macrophages (**Figure 4D**).

CWSP Increases the Surface Expression of HLA-DA, -DB, and -DC, and HLA-DR, -DP, and -DQ as Well as the Expression of Costimulatory Molecules CD80 and CD86 in Human THP-1 Macrophages. A number of antigenic peptides presented to T cells by various HLA molecules, in order to promote clonal proliferation of antigen-specific T cells, play a central role in the process of an individual's adaptive immune response (*17*). Using the flow cytometry assay, we found that incubation of human THP-1 macrophages with CWSP or LPS for 24 h increased the cell surface expression of HLA-DA, -DB, and -DC and HLA-DR, -DP, and -DQ compared to that of control cells (**Figure 5A**). In addition, we found that both CWSP and LPS increased the cell surface expression of CD80 and CD86 in human THP-1 macrophages (**Figure 5B**).

CWSP Enhances Cytokine Expression in LPS-Stimulated J774A.1 Macrophages and Mice. It has been demonstrated that oral administration of certain hot-water extracts of *Chlorella vulgaris* reduced mortality via enhancing cytokine expression in *Listeria monocytogenes*-infected mice (*5*, *6*). These results prompted us to investigate the hypothesis that CWSP could modulate cytokine expression in LPS-stimulated macrophages and mice. We found that pretreatment of J774A.1 macrophages with CWSP for a period of 24 h substantially increased

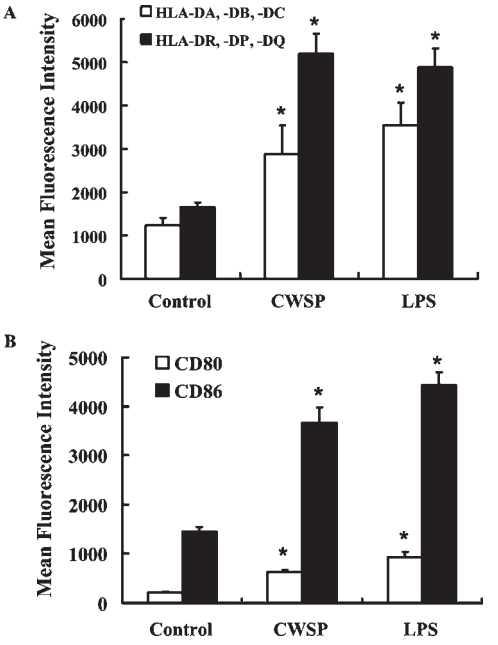

Figure 5. CWSP increases surface expression of HLA molecules and costimulatory molecules in human THP-1 macrophages. Human THP-1 macrophages were incubated with CWSP (100 μg/mL) for 24 h. Cell surface expression of (**A**) HLA-DA, -DB, and -DC, and HLA-DP, -DQ, and -DR, and (**B**) costimulatory molecules CD80 and CD86 were analyzed by flow cytometry. Shown are the mean values of three experiments $\pm$ SD; *$p < 0.05$ compared with the control sample.

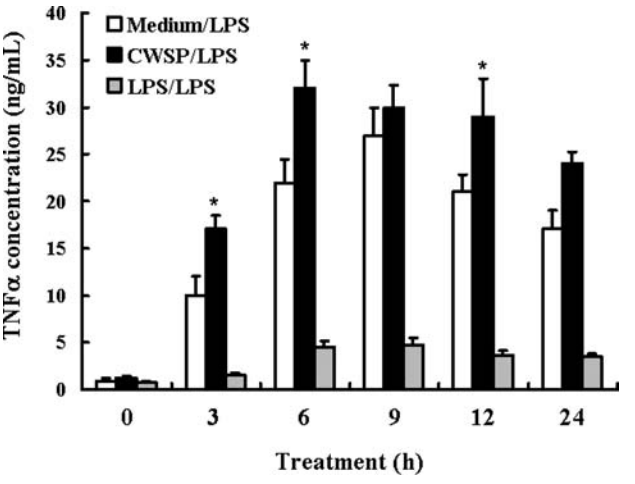

Figure 6. CWSP pretreatment increases TNFα secretion in LPS-stimulated J774A.1 macrophages. Cells were pretreated for 24 h with one of the following: medium, CWSP (100 μg/mL), or LPS (100 ng/mL). After washing, cells were challenged by LPS (1 μg/mL) for the indicated time points. TNFα concentration in conditioned media was measured by ELISA. Shown are the mean values of three experiments $\pm$ SD; *$p < 0.05$ compared with medium/LPS sample.

LPS-induced TNFα secretion compared to that of nonpretreated cells (**Figure 6**). Cells pretreated with LPS for 24 h showed diminished TNFα secretion in response to subsequent LPS challenge, demonstrating LPS tolerance (**Figure 6**).

As part of our further investigation, we tested the in vivo effect of CWSP on LPS-induced cytokine expression in C57BL/6J mice. In brief, C57BL/6J mice were initially intraperitoneally (IP) injected with one of the following: PBS, CWSP, or LPS. Then, 24 h later, the mice were again IP injected with PBS or LPS, 1.5 h

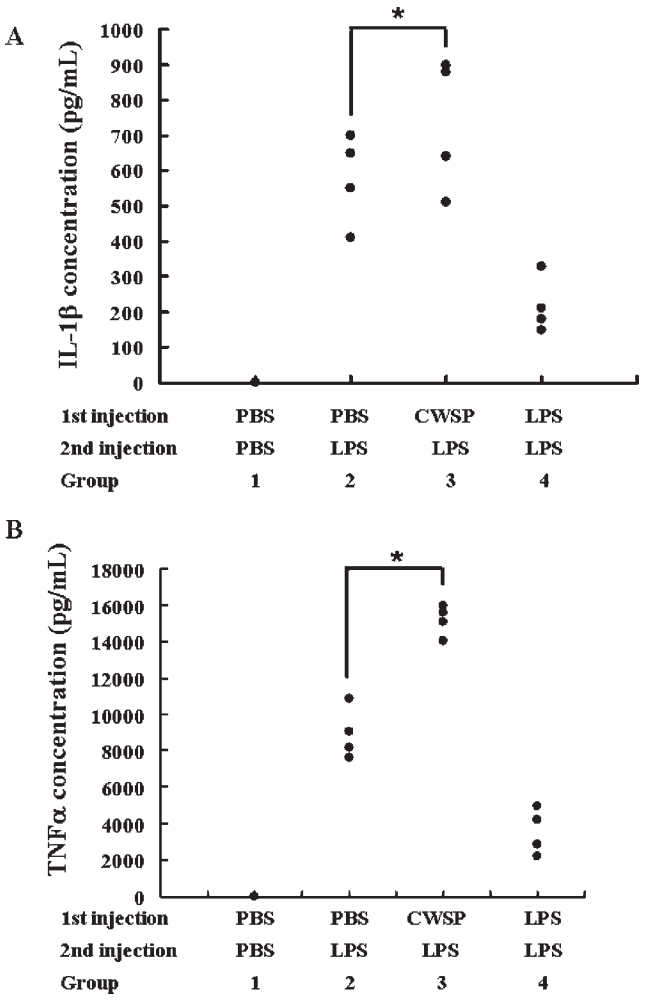

Figure 7. CWSP preinjection increases serum concentrations of cytokines in LPS-injected mice. (**A**) Male C57BL/6J mice were intraperitoneally (IP) injected with one of the following: PBS (0.1 mL), CWSP (100 mg/kg of body weight in 0.1 mL of PBS), or LPS (5 mg/kg of body weight in 0.1 mL of PBS). This was followed 24 h later by injection (IP) of PBS (0.1 mL) or LPS (10 mg/kg body weight in 0.1 mL of PBS). After 1.5 h, the IL-1β serum concentrations were measured using an IL-1β ELISA kit; $n = 4$; $^*p < 0.05$. (**B**) CWSP preinjection increases serum concentrations of TNFα in LPS-injected mice. The procedures of treatment were as shown in (**A**). TNFα serum concentrations were measured using a TNFα ELISA kit; $n = 4$; $^*p < 0.05$.

subsequent to which the serum concentrations of IL-1β and TNFα in the tested mice were measured by ELISA (**Figure 7A** and **B**). This revealed that C57BL/6J mice initially injected with PBS that received a second LPS injection showed a significant increase in baseline levels of IL-1β and TNFα secretion into the serum (**Figure 7A** and **B**, Group 2), more so than in the case of mice that received a second PBS injection (**Figure 7A** and **B**, Group 1). In addition, test mice were initially injected with CWSP, 24 h subsequent to which they received an injection of LPS; 1.5 h after the LPS injection, the serum levels of IL-1β and TNFα were measured by ELISA. The results of such a treatment indicated that CWSP slightly increased IL-1β secretion and significantly increased TNFα secretion in LPS-injected mice (**Figure 7A** and **B**, Group 3), compared to that of mice initially injected with PBS followed by a second injection with LPS (**Figure 7A** and **B**, Group 2). Other test mice were initially injected with LPS, followed 24 h later by a second injection of LPS. This second injection of LPS was responsible for the induction of lower levels of IL-1β and TNFα secretion into the serum (**Figure 7A** and **B**, Group 4), compared to that of mice injected with only LPS (**Figure 7A** and **B**, Group 2).

DISCUSSION

An increasing number of studies have been devoted to describing the immunological properties of polysaccharides containing materials derived from *Chlorella* (*3*−*7*, *24*−*27*). However, the signaling pathways related to cytokine expression in response to *Chlorella* polysaccharides in macrophages are less studied and still unclear. In this study, we found that the interaction of CWSP with TLR4 triggers the downstream signaling of protein kinases and related immune-modulating activities, including the expression of IL-1β as well as HLA and CD80/86 surface expression. Activation of macrophages by CWSP treatment provides support for the possible use of CWSP to modulate the innate and adaptive immune responses of humans and certain animals.

TLRs are involved in innate immunity by recognizing various components of bacteria, fungi, and viruses (*12*, *28*−*30*). In this study, we found that CWSP could induce proIL-1β expression in macrophages with functional TLR4; but CWSP only induced a small amount of proIL-1β expression in macrophages with deficient TLR4 (**Figure 2A**). Moreover, TLR4 neutralizing antibody could significantly block (but not completely) CWSP-mediated IL-1β secretion (**Figure 2B**). These results indicate that TLR4 is not the only receptor involved in the production of IL-1β in CWSP-stimulated macrophages. It has been reported that a glycoprotein purified from a culture medium of *Chlorella vulgaris* induced IL-12 secretion in spleen-adherent cells, partially through TLR2 (*12*). We could not rule out the possibility that TLR2 is involved in IL-1β expression in CWSP-stimulated macrophages. Furthermore, we were concerned about LPS contamination of CWSP samples because of the similarity of CWSP and LPS in IL-1β induction and IL-1β receptor-associated kinase-1 expression. On the basis of the results from the polymyxin B inhibition assay (**Figure 2D**) and the Limulus amebocyte lysate assay, we ruled out the possibility that CWSP-induced IL-1β expression is due to LPS contamination.

In addition, the JNK1/2 pathway played different roles in CWSP- and LPS-mediated IL-1β expression. In this study, we found that the JNK1/2 pathway was not involved in CWSP-mediated IL-1β expression (**Figure 4B**); in contrast, our previous study demonstrated that the JNK1/2 pathway played an important role in LPS-mediated IL-1β expression (*14*). The phenomenon was similar to that in previous research that had demonstrated that the alkali-soluble polysaccharides from *R. riparium* (RASP) were able of inducing IL-1β gene expression via protein kinase-mediated signal transduction pathways where the JNK and p38 MAPKs, but not ERK, play an important role in the regulation of IL-1 gene expression in RASP-stimulated J774A.1 cells (*16*).

TNFα is one kind of primary molecule that determines endotoxic shock. Our results showed that CWSP increased LPS-induced IL-1β and TNFα secretion in vitro and in vivo, which raised the possibility that CWSP may increase the risk of endotoxic shock in LPS-stimulated mice (**Figure 7**). However, no toxic effects of CWSP preinjection were observed after mice were injected with a sublethal dose of LPS (data not shown). The effect of CWSP on cytokine production in vitro and in vivo is similar to that in studies reporting that oral administration of hot-water extract of *Chlorella vulgaris* to mice enhances cytokine production (*5*, *6*). The adaptive immune responses are dependent on HLA molecules (*18*) and costimulatory molecules such as CD80, CD86, etc. (*19*, *20*), which are expressed by antigen-presenting cells, including monocytes and macrophages.

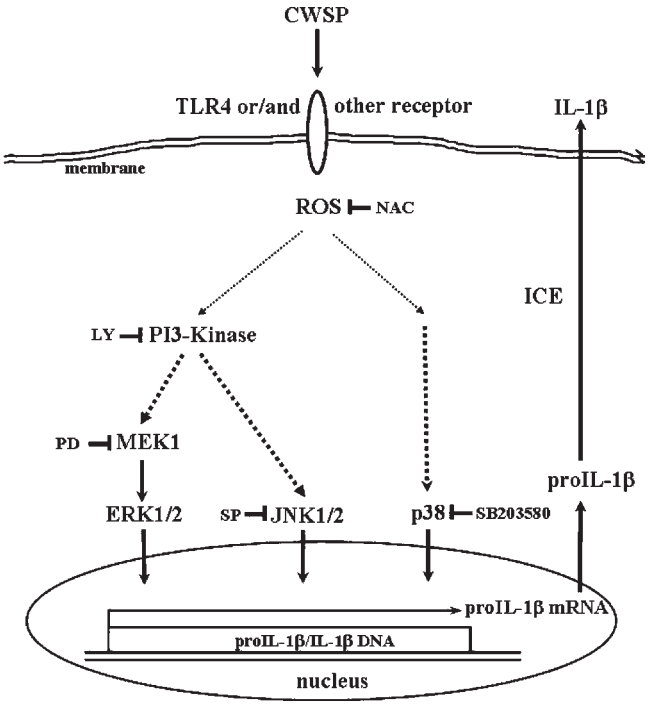

Figure 8. Proposed CWSP-mediated signal transduction pathways in the regulation of IL-1β expression.

Upregulation of HLA molecules and costimulatory molecules are taken as a hallmark of adjuvant effects (*18*−*20*). In the present study, we demonstrated that the expressions of HLA molecules and costimulatory molecules are upregulated in CWSP-stimulated human THP macrophages, suggesting the potential role of CWSP in the linkage between innate and adaptive immunity.

In summary, we have used a macrophage model to investigate, in vitro, the immunomodulating properties of water-soluble polysaccharides of *Chlorella pyrenoidosa* (CWSP). We found that CWSP activates IL-1 mRNA expression, proIL-1 protein expression, and IL-1 secretion within a macrophage model. Furthermore, we also dissected CWSP-mediated protein kinase signaling pathways involved in the regulation of IL-1 expression. In essence, JNK and p38, but not ERK, are involved in CWSP-mediated IL-1 expression. We also found that pretreatment of mice or macrophages with CWSP results in the upregulation of LPS-induced TNFα and IL-1β secretion. At the same time, we are the first to report that CWSP induced IL-1β expression via TLR4-mediated protein kinase activation in macrophages (**Figure 8**). In addition, we demonstrated that CWSP upregulated the surface expression of HLA molecules, CD80 and CD86, in macrophages. Furthermore, we found that pretreatment of mice or macrophages with CWSP results in the upregulation of LPS-induced TNFα and IL-1β secretion. Thus, our results may lead to the further development of CWSP for use as an agent to improve the integrity of the immune system, especially with regard to antimicroorganism activity and the continued enhancement of human immunity.

ABBREVIATIONS USED

CWSP, hot-water-soluble polysaccharides from *Chlorella pyrenoidosa*; CWE, hot-water extract of *Chlorella pyrenoidosa*; GC-MS, gas chromatography−mass spectrophotometer; HLA, human leukocyte antigen; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor-α; APCs, antigen-presenting cells; Th cells, T helper cells; MAPKs, mitogen-activated protein kinases; ERK1/2, extracellular signal-regulated kinases 1/2; JNK1/2, c-Jun N-terminal kinase 1/2; p38, p38 MAPK; PMB, polymyxin B; NAC, *N*-acetyl-cysteine; LPS, lipopolysaccharide.

ACKNOWLEDGMENT

We thank Christopher Salisbury for editing the English in this article.

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Received for review September 2, 2009. Revised manuscript received October 7, 2009. Accepted October 13, 2009. This work was supported by research grants from the National Science Council, Taiwan, of NSC 96-2116-M-039-001-MY3 (to L.K.C.), NSC 98-2320-B-197-003-MY2 (to K.-F.H.), and NSC 098-2811-B-197-001- (to K.-F.H.), from the China Medical University (CMU), CMU97-278 (to L.K.C.), from the "A grant from Ministry of Education, Aim for the Top University Plan" 98A-C-T148 (to H.-Y.H.), from the National Science Council, Taiwan, of NSC 96-2320-B-010-014-MY3 (to H.-Y.H.), and NSC 98-2627-M-010-004- (to H.-Y.H.).