

## 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-1 $\beta$  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- $\alpha$  and IL-1 $\beta$  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 INF $\gamma$  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-1 $\beta$  mRNA and TNF $\alpha$  mRNA expression and NF- $\kappa$ B 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-1 $\beta$ . It is well known that IL-1 $\beta$ , one of the critical cytokines for modulating immune responses, is secreted mainly from activated macrophages (8). Mice pretreated with recombinant IL-1 $\beta$  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-1 $\beta$  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-1 $\beta$  into bioactive IL-1 $\beta$  can be accomplished by the IL-1 $\beta$  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 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

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67 how *Chlorella* polysaccharides mediate MAPK activation in the  
68 regulation of cytokine expression is unclear. Therefore, in this  
69 research we will investigate the role of hot-water-soluble poly-  
70 saccharides from *Chlorella pyrenoidosa* (CWSP) in immune signal  
71 transduction in macrophages.

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

83 The potential beneficial immunological properties of polysac-  
84 charides derived from *Chlorella pyrenoidosa* algae have been less  
85 studied. Herein we report on how we have isolated and analyzed  
86 the chemical characteristics of CWSP and investigated their  
87 immune-modulation functions, including stimulation of IL-1 $\beta$   
88 expression in human and murine macrophages. In addition, we  
89 demonstrated that CWSP are able to increase cell surface  
90 expression of HLA as well as CD80 and CD86 in human  
91 macrophages. Further, we have found that pretreatment with  
92 CWSP of macrophages from C57BL/6J mice results in the  
93 upregulation of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and IL-1 $\beta$   
94 secretion (in cultured medium and mouse serum, respectively)  
95 after LPS stimulation. Thus, the results of this study may lead to  
96 the further development of CWSP as agents capable of contribu-  
97 ting to the modulation of cytokine expression.

## 98 MATERIALS AND METHODS

99 **Cell Cultures.** Murine macrophage J774A.1 cells (J774A.1 cells) were  
100 obtained from American Type Culture Collection (Manassas, VA).  
101 HeNC2 (with functional TLR4) and GG2EE (lacking functional TLR4)  
102 were from D. Radzioch (McGill University, Montreal, Canada). Human  
103 primary monocytes were obtained from normal blood donor buffy coats  
104 (Taipei Blood Center, Taipei, Taiwan). Buffy coat cells were mixed with an  
105 equal volume of PBS, layered on Histopaque-1077 (Sigma-Aldrich,  
106 St. Louis, MO, USA), and centrifuged at 400g for 30 min at 20 °C. The  
107 interface, containing mononuclear cells, was collected and washed twice  
108 with PBS. Thereafter, human primary monocytes (98% CD14<sup>+</sup>, analyzed  
109 by flow cytometry) were isolated from mononuclear cells by Monocyte  
110 Isolation Kit II (Miltenyi Biotech, Auburn, CA, USA). Human primary  
111 monocytes were cultured in RPMI-1640 supplemented with 10% fetal calf  
112 serum (HyClone, Logan, UT, USA). Human primary macrophages were  
113 obtained by culturing monocytes for 7 days in RPMI-1640 supplemented  
114 with 15% fetal calf serum at a density of  $1.5 \times 10^5$ /cm<sup>2</sup>. During the  
115 culturing period, nonadherent cells were removed by washing with PBS.  
116 After 7 days of incubation, the adherent cells were used as human blood  
117 monocyte-derived macrophages. Human THP-1 monocytes ( $1 \times 10^6$  cells/mL)  
118 were differentiated into macrophages in 60 mm dishes by culturing cells in  
119 RPMI-1640 supplemented with 10% fetal calf serum and 100 ng/mL  
120 phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA) for  
121 5 days. Nonadherent cells were removed by washing with PBS, and the  
122 remaining adherent cells were used as human THP-1 macrophages.  
123 Murine J774A.1 macrophages were obtained from ATCC (Rockville,  
124 MD, USA) and propagated in RPMI-1640 medium supplemented with  
125 10% heat-inactivated fetal calf serum and 2 mM L-glutamine (Life  
126 Technologies, Inc., Rockville, MD, USA), and then cultured in a 37 °C,  
127 5% CO<sub>2</sub> incubator.

128 **Materials.** *Chlorella pyrenoidosa* is a commercially available product  
129 from Taiwan *Chlorella* Manufacturing Co., Ltd. (Taipei, Taiwan). LPS  
130 (from *Escherichia coli* 0111: B4), antidi-phosphorylated extracellular  
131 signal-regulated kinase 1/2 (ERK1/2) antibody, antidi-phosphorylated  
132 c-Jun N-terminal kinase 1/2 (JNK1/2) antibody, antidi-phosphorylated  
133 p38-MAKP (p38) antibody, antiactin antibody, PD98059, Ro-31-8220,

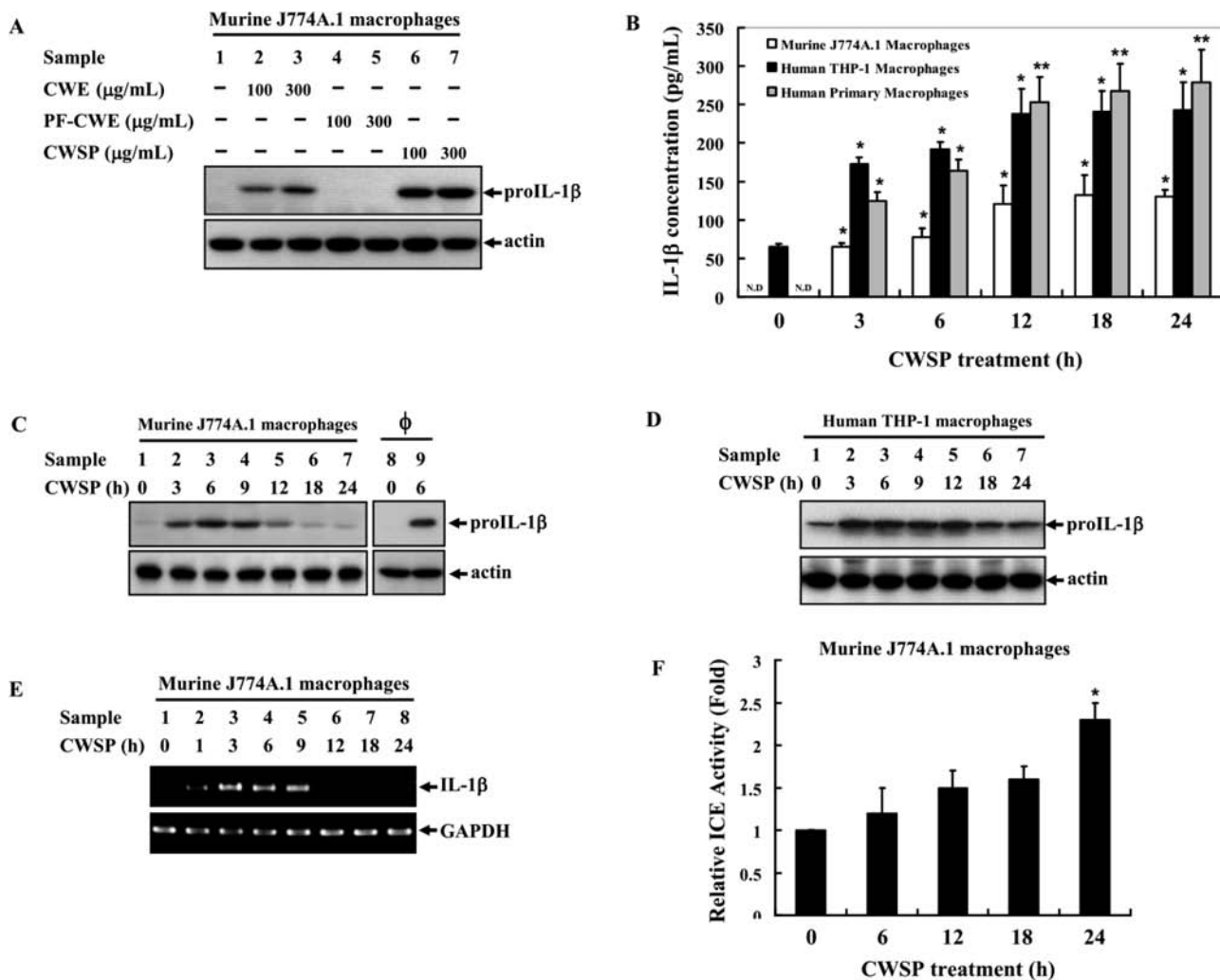
SB203580, curcumin, and LY294002 were purchased from Sigma Co. 134  
(St. Louis, MO, USA). Mouse IL-1 $\beta$  and TNF $\alpha$  ELISA kits were 135  
purchased from R&D Systems, Inc. (Minneapolis, MN, USA). A human 136  
IL-1 $\beta$  ELISA kit was purchased from BioSource International, Inc. 137  
(Camarillo, CA, USA). Anti-IL-1 $\beta$ , antirabbit IgG-HRP, and antimouse 138  
IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, 139  
CA, USA). The CaspACE Assay System, Fluorometric was purchased 140  
from Promega (Madison, WI, USA). 141

**Preparation of CWSP.** *Chlorella pyrenoidosa* samples were collected 142  
from aquaculture ponds at the Taiwan *Chlorella* Manufacturing Co., Ltd. 143  
After harvesting from the culture tank, the algae were cleaned with distilled 144  
water under a 200 mesh net and then air-dried. Twenty grams of *Chlorella* 145  
*pyrenoidosa* samples were extracted with distilled water (300 mL) for 146  
20 min at 121 °C by an autoclave. The extracts were filtered by a 0.2  $\mu$ m 147  
membrane, followed by vacuum-concentration at 50 °C, giving a final 148  
volume of 100 mL to which five volumes of 95% ethanol were added 149  
slowly at 4 °C. The mixture was centrifuged to produce the precipitates 150  
(CWSP, 1150 mg) and supernatants (polysaccharide-free *Chlorella pyre-* 151  
*noidosa* water-soluble extract, PF-CWE). 152

**Gel Filtration Chromatography and Sugar Composition Analysis** 153  
**of CWSP.** One hundred milligrams of CWSP was purified by gel filtra- 154  
tion chromatography using a Sephadex G-100 column (100  $\times$  1.6 cm) 155  
(Amersham Pharmacia Biotech, Inc., Uppsala, Sweden), with distilled 156  
deionized water as the eluent. The flow rate was set at 0.3 mL/min, and 157  
5.0 mL per tube was collected. Under the same conditions, a Shodex 158  
standard P-82 column was used as an internal standard for molecular 159  
weight. All chromatography processes were executed at 4 °C. After the 160  
chromatography processes, each fraction was subjected to analysis to 161  
determine its total carbohydrate content; this was done using the phenol- 162  
sulfuric acid colorimetric method (with glucose as the calibration 163  
standard) (21). For monosaccharide analysis, 0.5 mg of polysaccharide 164  
was methanolized with 0.2 mL of 0.5 N methanolic HCl (Supelco, 165  
Bellefonte, PA, USA) at 80 °C for 16 h; re-N-acetylated with 500 mL of 166  
methanol, 10 mL of pyridine, and 50 mL of acetic anhydride; and then 167  
treated with Sylon HTP trimethylsilylation reagent (Supelco, Bellefonte, 168  
PA, USA) for 20 min at room temperature, after which it was dried by 169  
nitrogen blowing and redissolved in hexane. GC-MS analysis of the 170  
trimethylsilylated derivatives was carried out using a Hewlett-Packard 171  
(HP) Gas Chromatograph 6890 connected to an HP 5973 mass selective 172  
detector. Samples were dissolved in hexane prior to splitless injection into 173  
an HP-5MS fused silica capillary column (30 m  $\times$  0.25 mm I.D.). The 174  
column head pressure was maintained at around 8.2 psi to give a constant 175  
flow rate of 1 mL/min using helium as the carrier gas. Initial oven 176  
temperature was held at 60 °C for 1 min; increased to 140 at 25 °C/min; 177  
then to 250 at 5 °C/min; and then to 300 at 10 °C/min. The CWSP 178  
investigated herein revealed a higher molecular-weight group comprising 179  
monosaccharides larger than 1,000 kDa (data not shown). 180

**Measurement of Intracellular H<sub>2</sub>O<sub>2</sub> Production.** Intracellular 181  
H<sub>2</sub>O<sub>2</sub> stimulated by CWSP and LPS was measured by detecting the 182  
fluorescent intensity of carboxyl-2',7'-dichlorofluorescein diacetate (CM- 183  
DCFH) (Molecular Probes, Inc., Eugene, OR, USA) oxidized product, 184  
CM-DCF. Briefly, J774A.1 cells ( $1 \times 10^6$ /mL) grown in serum- and phenol 185  
red-free RPMI medium for 24 h were then preincubated with CM-DCFH 186  
(2  $\mu$ M) and N-acetyl cysteine (NAC) (10 mM) at 37 °C for 30 min in the 187  
dark. This was followed by adding fresh starvation medium containing 188  
CWSP (100  $\mu$ g/mL) or LPS (1  $\mu$ g/mL) for an additional time, as indicated. 189  
The relative fluorescent intensity of the fluorophore CM-DCF, which was 190  
formed by peroxide oxidation of the nonfluorescent precursor, was 191  
detected at an excitation wavelength of 485 nm and an emission wave- 192  
length of 530 nm with a Cytofluor 2300 fluorometer (Millipore, Inc., 193  
Bedford, MA, USA). 194

**Mice Model for Testing CWSP.** All studies were approved by the 195  
Institutional Animal Care and Use Committee at National Yang-Ming 196  
University, Taipei, Taiwan, and met university guidelines for the use of 197  
experimental animals in research. Male, 6- to 8-week-old C57BL/6J mice 198  
(National Laboratory Animals Center, Taiwan) were used in all studies. 199  
Mice were housed in a monitored, light–dark cycled environment and 200  
provided standard lab chow and water. Mice were intraperitoneally (IP) 201  
injected with CWSP (100 mg/kg of body weight in 0.1 mL of PBS); control 202  
mice received PBS (0.1 mL) in the same regimen. After 24 h, all mice 203



**Figure 1.** CWSP increase IL-1 $\beta$  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-1 $\beta$  expression. (B) CWSP (100  $\mu\text{g/mL}$ ) stimulate IL-1 $\beta$  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  $\mu\text{g/mL}$ ) increase proIL-1 $\beta$  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  $\mu\text{g/mL}$ ) increase proIL-1 $\beta$  expression in human THP-1 macrophages. One of three experiments is presented. (E) CWSP (100  $\mu\text{g/mL}$ ) increase IL-1 $\beta$  mRNA expression in J774A.1 macrophages. GAPDH was used as an internal control. One of three experiments is presented. (F) CWSP (100  $\mu\text{g/mL}$ ) increase IL-1 $\beta$  converting enzyme (ICE) activity in J774A.1 macrophages. Shown are the mean values of three experiments  $\pm$  SD \* $p$  < 0.05 versus control.

204 received a challenge dose of LPS (10 mg/kg, IP). Serum was collected from  
205 test mice after 1.5 h of LPS challenge for further assessment of cytokine  
206 expression by ELISA.

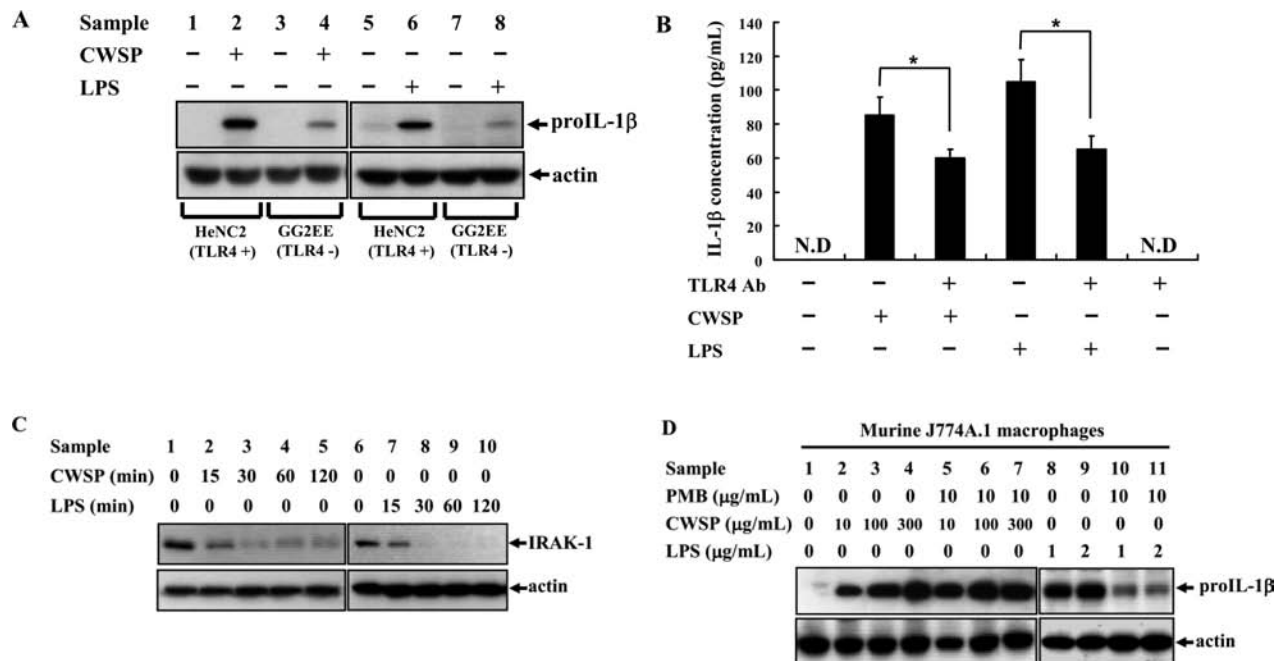
207 **Monitoring of LPS Contamination of CWSP in Experiments.**  
208 Our reagents and utensils for the preparation of CWSP were either of LPS-  
209 free grade or were washed with PBS containing 50  $\mu\text{g/mL}$  polymyxin B  
210 (PMB), then rinsed with PBS. In order to rule out possible LPS  
211 contamination of CWSP samples, J774A.1 cells were preincubated with  
212 or without PMB (10  $\mu\text{g/mL}$ ) for 30 min, followed by treatment for 6 h with  
213 CWSP (10, 100, or 300  $\mu\text{g/mL}$ ) or LPS (1 or 2  $\mu\text{g/mL}$ ), respectively. ProIL-  
214  $\beta$  expression was analyzed by Western blotting. In addition, LPS  
215 concentration in the CWSP samples was analyzed using a Limulus  
216 amoebocyte lysate PYROCHROME assay kit (Associates of Cape Cod,  
217 Inc., Falmouth, MA, USA).

218 **Flow Cytometry Analysis.** For cell surface expression experiments of  
219 HLA and costimulatory molecules, THP-1 macrophages were incubated  
220 with medium (control), CWE (100  $\mu\text{g/mL}$ ), CWSP (100  $\mu\text{g/mL}$ ), poly-  
221 saccharide-free CWE (100  $\mu\text{g/mL}$ ), or LPS (1  $\mu\text{g/mL}$ ) for 24 h. Cells were  
222 fixed, and cell surface expression of HLA and costimulatory molecules  
223 were measured by staining cells for 30 min with specific fluorescence-

224 conjugated antibodies on ice. After washing, cells were subjected to flow  
225 cytometry analysis on FACSCalibur using CellQuest software (Becton  
226 Dickinson, San Jose, CA, USA).

227 **RT-PCR (Reverse Transcription–Polymerase Chain Reaction).**  
228 Total RNA was isolated by the REzol C&T method according to the  
229 manufacturer's instructions. For reverse transcription, the reaction was  
230 performed at 42  $^{\circ}\text{C}$  for 30 min, at 99  $^{\circ}\text{C}$  for 5 min, and then cooled to 4  $^{\circ}\text{C}$ .  
231 For future PCR, the PCR mixture was held at 94  $^{\circ}\text{C}$  for 2 min and then  
232 cycled 30 times at 94  $^{\circ}\text{C}$  for 30 s, 55  $^{\circ}\text{C}$  for 30 s, and 72  $^{\circ}\text{C}$  for 2 min  
233 followed by 10 min at 72  $^{\circ}\text{C}$  at the final cycle. The products from the PCR  
234 reaction were examined by 1% agarose gel electrophoresis with ethidium  
235 bromide (EtBr) and normalized by comparison to RT-PCR of mRNA of  
236 GAPDH, a constitutively expressed gene. Each EtBr-stained band was  
237 quantified using ImageQuaNT software from a PhosphorImager from  
238 Molecular Dynamics (Sunnyvale, CA).

239 **Western Blotting.** Whole cell lysates were separated by 12% sodium  
240 dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and  
241 electrotransferred to a PVDF membrane. The membrane was incubated in  
242 blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room  
243 temperature for 1 h. The membrane was incubated with anti-IL-1 $\beta$



**Figure 2.** CWSP induce IL-1 $\beta$  expression via TLR4. (A) CWSP (100  $\mu$ g/mL) induce proIL-1 $\beta$  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  $\mu$ g/mL) blocks CWSP (100  $\mu$ g/mL)-induced IL-1 $\beta$  secretion in human THP-1 macrophages. Shown are the mean values of three experiments  $\pm$  SD; \* $p$  < 0.05. (C) CWSP (100  $\mu$ g/mL) and LPS (1  $\mu$ g/mL) downregulate IL-1 $\beta$  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-1 $\beta$  expression in J774A.1 macrophages. One of three experiments is presented.

244 antibody or anti-MAPKs antibody at room temperature for 2 h. After  
245 washing three times in PBS with 0.1% Tween 20, the membrane was  
246 incubated with an HRP-conjugated secondary antibody directed against  
247 the primary antibody. The membrane was developed by an enhanced  
248 chemiluminescence Western-blotting detection system (DuPont NEN  
249 Research Product Co, Boston, MA) according to the manufacturer's  
250 instructions.

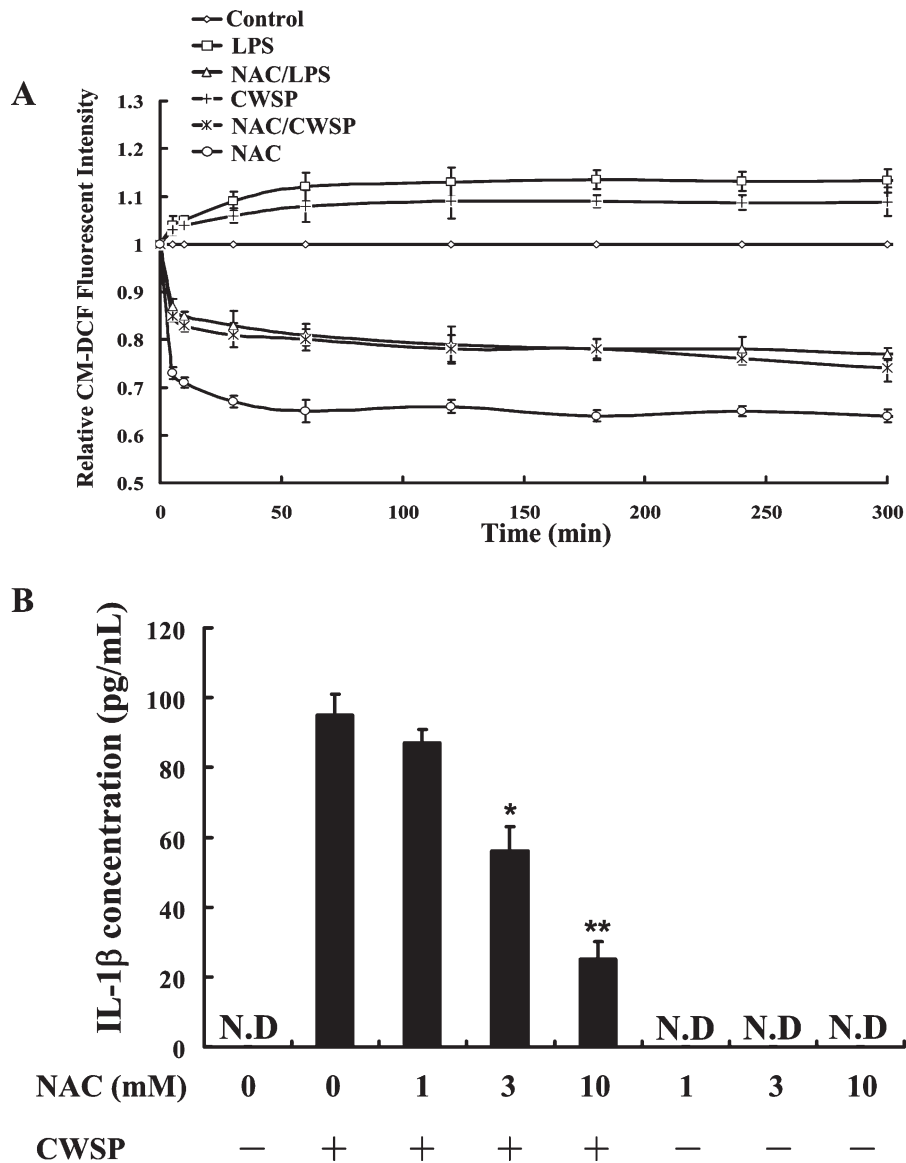
251 **ELISA (Enzyme Linked Immunosorbent Assay).** The J774A.1 cell,  
252 THP-1 cells, and human primary cells ( $1 \times 10^6$ /mL) were stimulated with  
253 CWSP (100  $\mu$ g/mL) for the indicated time points within 24 h. The IL-1  
254 concentration in the condition medium was analyzed by ELISA according  
255 to the protocol from R & D Mouse IL-1beta ELISA System (R & D  
256 Systems, Inc.). In brief, 50  $\mu$ L of biotinylated antibody reagent and 50  $\mu$ L  
257 of supernatant were added to an antimouse IL-1 pre-coated stripwell plate  
258 and incubated at room temperature for 3 h. After washing the plate three  
259 times with washing buffer, 100  $\mu$ L of diluted streptavidin-HRP concentra-  
260 rate was added to each well and incubated at room temperature for  
261 30 min. The washing process was repeated, and 100  $\mu$ L of a premixed TMB  
262 substrate solution was added to each well and developed at room  
263 temperature in the dark for 30 min. Following the addition of 100  $\mu$ L of  
264 provided stop solution to each well to stop the reaction, the absorbance of  
265 the plate was measured by a MRX microplate reader (DyneX Tech. Inc.) at  
266 a 450–550 nm wavelength.

267 **Statistical Analysis.** All values are given as the mean  $\pm$  SD. Data  
268 analysis involved one-way ANOVA with a subsequent Scheffé test.

## 269 RESULTS

270 **CWSP Induce IL-1 $\beta$  Expression in Macrophages and Sugar**  
271 **Composition Analysis.** The immune-modulation activity of *Chloro-*  
272 *ella pyrenoidosa* was monitored by its proIL-1 $\beta$  (precursor of IL-  
273 1 $\beta$ ) induction activity in J774A.1 macrophages. The hot-water  
274 extract of *Chlorella pyrenoidosa* (CWE) induced proIL-1 $\beta$  expres-  
275 sion in a dose-dependent manner (Figure 1A, samples 2 and 3).  
276 In order to investigate the role of polysaccharides on proIL-1 $\beta$   
277 expression in CWE-stimulated J774A.1 macrophages, the poly-  
278 saccharides in CWE were removed. We found that polysaccharide-

free CWE was unable to induce proIL-1 $\beta$  expression (Figure 1A, 279  
samples 4 and 5). Furthermore, we isolated CWSP and found that 280  
CWSP induced proIL-1 $\beta$  expression to a greater level than CWE 281  
did (Figure 1A, samples 6 and 7), indicating that polysaccharides 282  
play important roles in *Chlorella pyrenoidosa*-induced proIL-1 $\beta$  283  
expression. In addition, in order to test whether CWSP are able to 284  
stimulate macrophages to secrete IL-1 $\beta$ , J774A.1 macrophages 285  
were stimulated with CWSP during a testing period of 0 to 24 h. 286  
IL-1 $\beta$  was detected in conditioned media as early as 3 h after 287  
CWSP stimulation of J774A.1 macrophages, and the IL-1 $\beta$  288  
concentration remained elevated for up to 24 h post-CWSP 289  
stimulation (Figure 1B, white bars). We have tested the effect of 290  
dextran and the polysaccharides from *Isochrysis galbana* 291  
(M.W. > 1 M Da) on IL-1 $\beta$  expression, and we found that both 292  
of them were unable to induce IL-1 $\beta$  expression in murine 293  
macrophages (data not shown). In addition, the effect of CWSP 294  
on IL-1 $\beta$  expression in human THP-1 macrophages was investi- 295  
gated. IL-1 $\beta$  protein levels of around 170, 190, 210, 240, and 296  
250 pg/mL were detected in the conditioned medium of CWSP- 297  
treated THP-1 macrophages at poststimulation times of, respec- 298  
tively, 3, 6, 9, 12, 18, and 24 h (Figure 1B, black bars). This IL-1 $\beta$  299  
induction activity of CWSP was also confirmed in human blood 300  
monocyte-derived primary macrophages (Figure 1B, gray bars). 301  
These results indicate that CWSP could induce IL-1 $\beta$  secretion in 302  
both murine and human macrophages. We further investigated 303  
the molecular mechanism controlling the secretion of mature IL- 304  
1 $\beta$  in CWSP-stimulated J774A.1 macrophages. The expression of 305  
proIL-1 $\beta$  was detected between 3 and 12 h subsequent to CWSP 306  
stimulation (Figure 1C, samples 1–7). Similarly, CWSP induced 307  
proIL-1 $\beta$  expression in both human blood monocyte-derived 308  
primary macrophages (Figure 1C, samples 8 and 9) and human 309  
THP-1 macrophages (Figure 1D). Moreover, using a RT-PCR 310  
method we demonstrated that IL-1 $\beta$  mRNA expression was 311  
detected in J774A.1 macrophages 1 h subsequent to CWSP 312



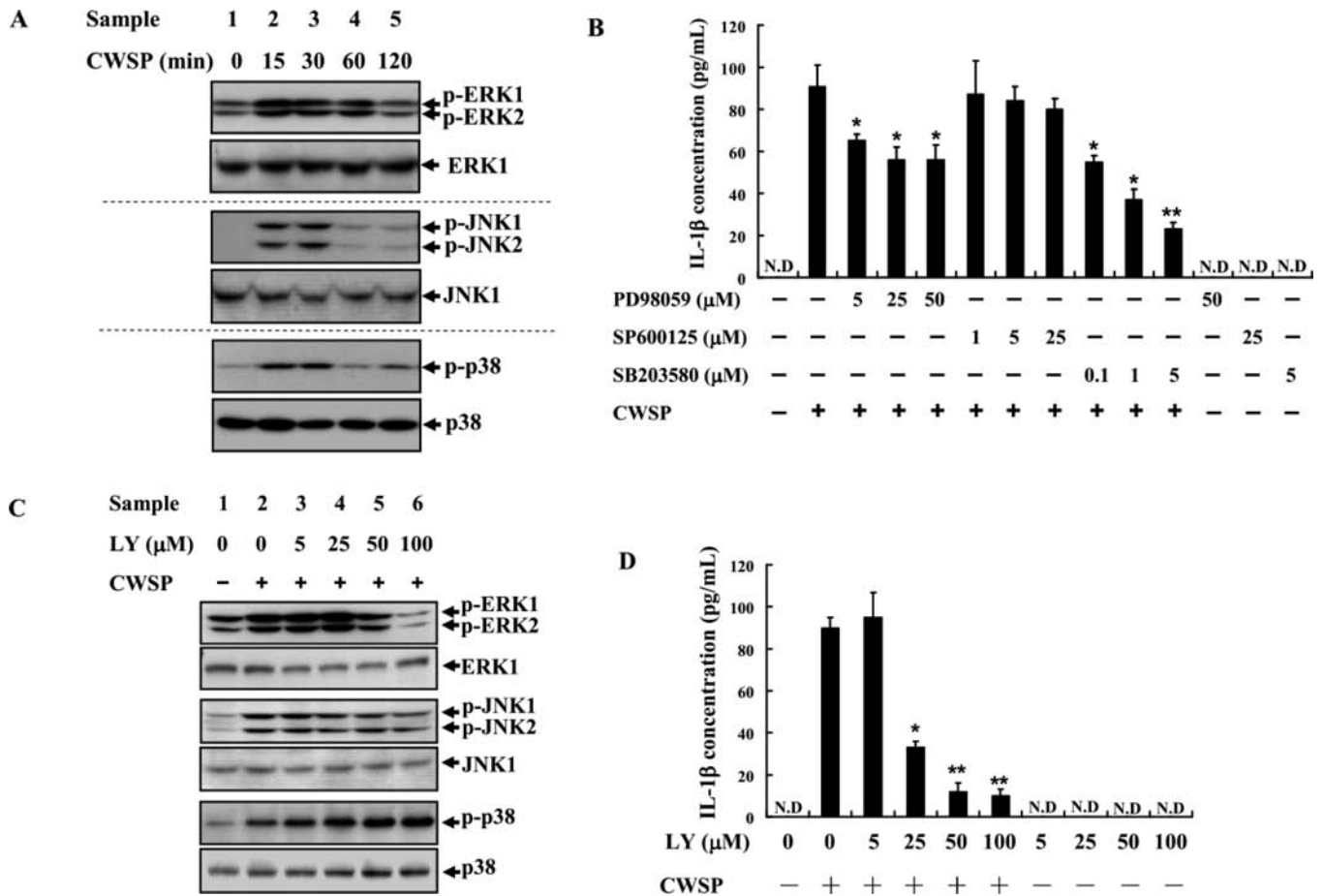
**Figure 3.** CWSP induce IL-1 $\beta$  expression via H<sub>2</sub>O<sub>2</sub>. **(A)** J774A.1 cells were preincubated with CM-H2DCFDA (2  $\mu$ M) for 30 min, followed by substitution with medium containing CWSP or LPS in the presence or absence of N-acetyl-cysteine (NAC) for additional incubation 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-1 $\beta$  secretion in cultured medium was measured by ELISA. Shown are the mean values of three experiments  $\pm$  SD; \* $p$  < 0.05; \*\* $p$  < 0.01 versus CWSP alone. N.D: nondetectable.

313 stimulation. After 12 h, IL-1 $\beta$  mRNA expression began to decline  
 314 to the basal level (**Figure 1E**). Post-translational regulation and  
 315 processing of proIL-1 $\beta$  protein into mature IL-1 $\beta$  via an IL-1 $\beta$   
 316 converting enzyme in J774A.1 macrophages has been reported  
 317 previously (15). We found that IL-1 $\beta$  converting enzyme activity  
 318 increased around 3-fold in CWSP-stimulated cells, compared to  
 319 that in control cells (**Figure 1F**). Furthermore, the CWSP were  
 320 observed to be primarily composed of rhamnose (31.8%), glucose  
 321 (20.42%), galactose (13.28%), mannose (5.23%), and xylose  
 322 (1.27%), as analyzed by GC-MS.

323 **CWSP Induce IL-1 $\beta$  Expression via Toll-Like Receptor 4**  
 324 **(TLR4).** We have demonstrated that TLR4 is involved in  
 325 polysaccharide-mediated cytokine expression in macrophages  
 326 (13, 15). In this study, we chose two genetically specific TLR4  
 327 relevant murine macrophage cell lines, HeNC2 (with functional  
 328 TLR4) and GG2EE (lacking functional TLR4), to examine the  
 329 role of TLR4 in CWSP-mediated cytokine expression (22).  
 330 HeNC2 cells produced proIL-1 $\beta$  upon CWSP stimulation,

whereas there was no significant proIL-1 $\beta$  expression in CWSP-  
 331 treated GG2EE cells (**Figure 2A**). In addition, significant proIL-  
 332 **F2** 1 $\beta$  expression was observed in LPS-treated HeNC2 cells but not  
 333 in GG2EE cells (**Figure 2A**). These results suggested that TLR4 is  
 334 one of the putative receptors for CWSP-mediated IL-1 $\beta$  expression.  
 335  
 336

Next, to investigate the potential interaction of TLR4 and  
 337 CWSP leading to the induction of IL-1 $\beta$  secretion, experiments  
 338 were conducted by preincubating human THP-1 macrophages  
 339 with the TLR4 neutralizing antibody, which is known to speci-  
 340 fically inhibit LPS-induced signaling through TLR4. As expected,  
 341 TLR4 neutralizing antibody inhibited both CWSP- and LPS-  
 342 induced IL-1 $\beta$  secretion (**Figure 2B**). Furthermore, the expression  
 343 level of IL-1 $\beta$  receptor-associated kinase-1, a rapidly degraded  
 344 protein after TLR4 activation (23), was evaluated. The time  
 345 course of IL-1 $\beta$  receptor-associated kinase-1 degradation in  
 346 CWSP-treated cells was similar to that in LPS-treated cells  
 347 (**Figure 2C**).  
 348



**Figure 4.** CWSP induces IL-1 $\beta$  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  $\mu$ g/mL) and the cell lysates collected at different periods of time. Cell lysates were analyzed by Western blot with antidi-phosphorylated ERK1/2, antidi-phosphorylated JNK1/2, and antidi-phosphorylated p38 monoclonal antibody; one of three experiments is presented. **(B)** Effects of PD98059, SP600125, and SB203580 on IL-1 $\beta$  secretion in CWSP-stimulated J774A.1 macrophages. Cells were preincubated for 30 min with one of the following: PD98059 (5, 25, or 50  $\mu$ M), SP600125 (1, 5, or 25  $\mu$ M), and SB203580 (0.1, 1, or 5  $\mu$ M), followed by CWSP (100  $\mu$ g/mL) stimulation for an additional 24 h. Conditioned medium was assayed for IL-1 $\beta$  concentration using IL-1 $\beta$  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  $\mu$ M) for 30 min prior to 20 min stimulation by CWSP (100  $\mu$ g/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-1 $\beta$  secretion in CWSP-stimulated J774A.1 macrophages. Cells were pretreated with LY294002 (5, 25, 50, and 100  $\mu$ M) for 30 min followed by incubation with CWSP (100  $\mu$ g/mL) for an additional 24 h; IL-1 $\beta$  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.

349 LPS is a cell wall component of Gram-negative bacteria, and is  
 350 a potent stimulator of macrophages in regard to IL-1 $\beta$  expres-  
 351 sion (13). In order to rule out the possibility of CWSP-induced IL-  
 352 1 $\beta$  expression being due to LPS contamination during CWSP  
 353 preparation, we tested the effect of polymyxin B (PMB), an  
 354 antibiotic, which was used to neutralize the activity of LPS (15)  
 355 on CWSP- and LPS-induced proIL-1 $\beta$  expression in J774A.1 mac-  
 356 rophages. We found that PMB obviously inhibited LPS-induced  
 357 proIL-1 $\beta$  expression (Figure 2D, samples 10 and 11 vs samples  
 358 8 and 9); yet in contrast, PMB was not able to inhibit proIL-1 $\beta$   
 359 expression in CWSP-stimulated cells (Figure 2D, samples 5, 6,  
 360 and 7 vs samples 2, 3 and 4). In addition, the LPS content of  
 361 CWSP samples was determined by the Limulus amoebocyte lysate  
 362 assay, and an insignificant level of endotoxin (< 1 EU/mL<sup>-1</sup>) was  
 363 found in the tested CWSP samples (data not shown). Taken  
 364 together, we consider that the effect of CWSP upon IL-1 $\beta$   
 365 expression was not due to LPS contamination of the CWSP.

366 **CWSP Induce IL-1 $\beta$  Expression via H<sub>2</sub>O<sub>2</sub>.** We have demon-  
 367 strated that H<sub>2</sub>O<sub>2</sub> is involved in TLR4-mediated IL-1 $\beta$  expres-  
 368 sion (13). In this study, we investigated whether H<sub>2</sub>O<sub>2</sub> is involved

in CWSP-mediated IL-1 $\beta$  expression in macrophages. Initially,  
 we examined whether CWSP could induce H<sub>2</sub>O<sub>2</sub> production in  
 J774A.1 macrophages. Similar to the effect of LPS treatment on  
 H<sub>2</sub>O<sub>2</sub> production, CWSP rapidly induced significant H<sub>2</sub>O<sub>2</sub> pro-  
 duction 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<sub>2</sub>O<sub>2</sub> production  
 (Figure 3A). Furthermore, we found that NAC was able to elicit a  
 dose-dependent inhibition of CWSP-induced IL-1 $\beta$  secretion,  
 indicating that H<sub>2</sub>O<sub>2</sub> is involved in IL-1 $\beta$  secretion in CWSP-  
 stimulated J774A.1 macrophages (Figure 3B).

**CWSP Induce IL-1 Expression via ERK1/2 and p38.** In order to  
 further examine CWSP-mediated signal transduction pathways  
 in the regulation of IL-1 $\beta$  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

389 J774A.1 macrophages also increased significantly between 15 and  
390 30 min subsequent to CWSP stimulation. After 60 min, the p38  
391 phosphorylation level gradually returned to the basal level  
392 (Figure 4A). Furthermore, CWSP treatment of J774A.1 macro-  
393 phages also increased the phosphorylation level of JNK1/2 in  
394 J774A.1 macrophages (Figure 4A).

395 Next, we examined whether MAPKs-related signaling consti-  
396 tuted one of the main downstream signaling cascades in the  
397 regulation of IL-1 $\beta$  secretion in CWSP-stimulated J774A.1  
398 macrophages. Cells were separately exposed to one of the  
399 pharmaceutical protein kinase inhibitors, PD98059, SP600125,  
400 or SB203580, such exposure inhibiting, respectively, MEK1,  
401 JNK1/2, and p38 activity. The results indicated that PD98059  
402 and SB203580 inhibited IL-1 $\beta$  secretion, suggesting that the  
403 MEK1/ERK1/2 and p38 pathways are involved in IL-1 $\beta$  secre-  
404 tion in CWSP-stimulated J774A.1 macrophages (Figure 4B). In  
405 contrast, SP600125 appeared to exert no real effect upon IL-1 $\beta$   
406 secretion, suggesting that JNK1/2-related signaling pathways are  
407 not involved in CWSP-induced IL-1 $\beta$  secretion (Figure 4B).  
408 Furthermore, the effects of these inhibitors on proIL-1 expression  
409 were similar to that on IL-1 $\beta$  secretion (data not shown).

410 **PI3-Kinase Lies Upstream of ERK1/2 and JNK1/2 in CWSP-**  
411 **Stimulated J774A.1 Macrophages.** We have demonstrated that  
412 PI3-kinase is involved in TLR4-mediated signal transduction in  
413 the regulation of IL-1 $\beta$  expression (13). In order to dissect the  
414 interrelationship between PI3-kinase and MAPKs, we used  
415 LY294002 to examine the role of PI3-kinase in the activation of  
416 ERK1/2, JNK1/2, and p38 in CWSP-stimulated J774A.1 macro-  
417 phages. In the absence of LY294002, CWSP induced the phos-  
418 phosphorylation of ERK1/2, JNK1/2, and p38; whereas CWSP-  
419 induced phosphorylation of ERK1/2 and JNK1/2, but not p38,  
420 was significantly inhibited by LY294002 (Figure 4C). These  
421 results indicate that PI3-kinase lies upstream of ERK1/2 and  
422 JNK1/2 in CWSP-stimulated J774A.1 macrophages. In addition,  
423 we found that LY294002 significantly reduced IL-1 $\beta$  secretion in  
424 CWSP-stimulated J774A.1 macrophages (Figure 4D).

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

439 **CWSP Enhances Cytokine Expression in LPS-Stimulated**  
440 **J774A.1 Macrophages and Mice.** It has been demonstrated that  
441 oral administration of certain hot-water extracts of *Chlorella*  
442 *vulgaris* reduced mortality via enhancing cytokine expression  
443 in *Listeria monocytogenes*-infected mice (5, 6). These results  
444 prompted us to investigate the hypothesis that CWSP could  
445 modulate cytokine expression in LPS-stimulated macrophages  
446 and mice. We found that pretreatment of J774A.1 macrophages  
447 with CWSP for a period of 24 h substantially increased LPS-  
448 induced TNF $\alpha$  secretion compared to that of nonpretreated cells  
449 (Figure 6). Cells pretreated with LPS for 24 h showed diminished  
450 TNF $\alpha$  secretion in response to subsequent LPS challenge, demon-  
451 strating LPS tolerance (Figure 6).

452 As part of our further investigation, we tested the in vivo effect  
453 of CWSP on LPS-induced cytokine expression in C57BL/6J mice.

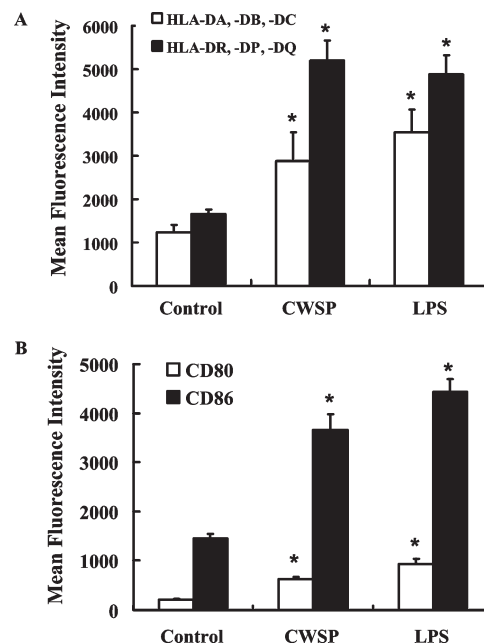


Figure 5. CWSP increases surface expression of HLA molecules and costimulatory molecules in human THP-1 macrophages. Human THP-1 macrophages were incubated with CWSP (100  $\mu$ g/mL) for 24 h. Cell surface expression of (A) HLA-DA, -DB, and -DC, and HLA-DR, -DP, and -DQ, and (B) costimulatory molecules 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.

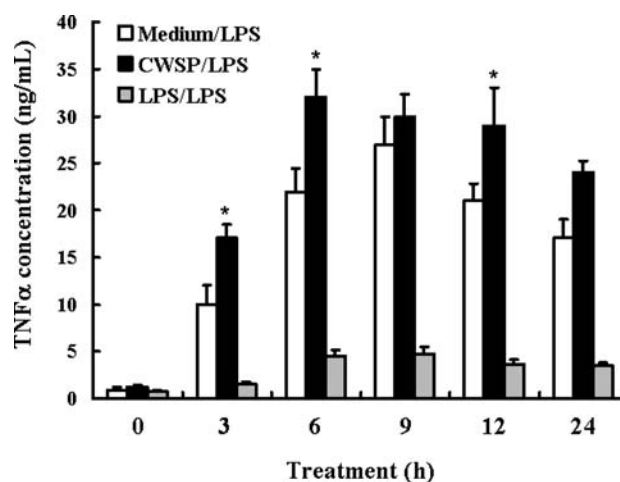
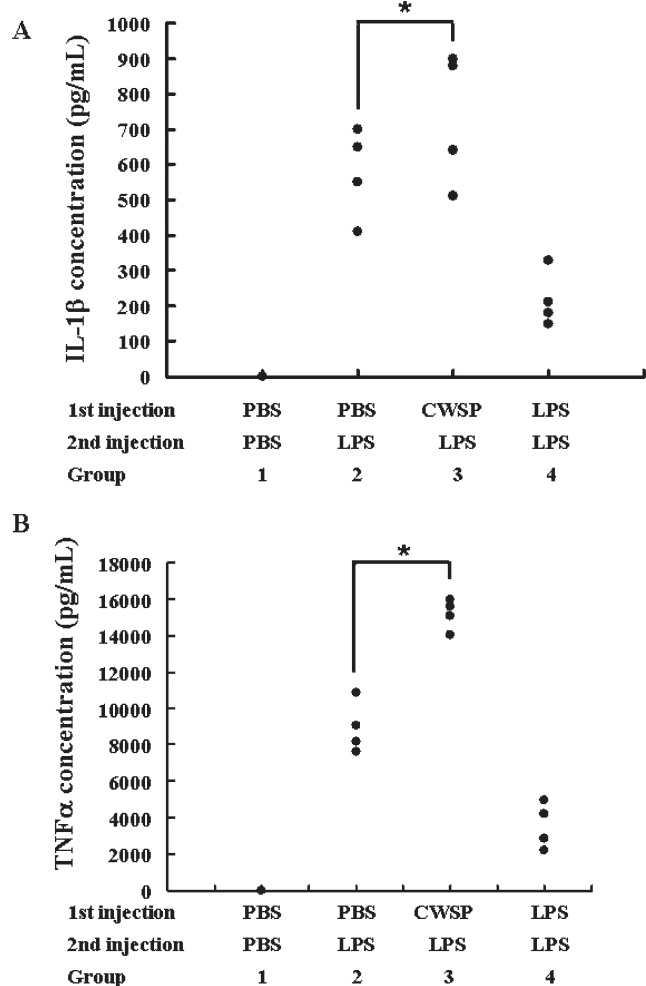


Figure 6. CWSP pretreatment increases TNF $\alpha$  secretion in LPS-stimulated J774A.1 macrophages. Cells were pretreated for 24 h with one of the following: medium, CWSP (100  $\mu$ g/mL), or LPS (100 ng/mL). After washing, cells were challenged by LPS (1  $\mu$ g/mL) for the indicated time points. TNF $\alpha$  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.

In brief, C57BL/6J mice were initially intraperitoneally (IP) 454  
injected with one of the following: PBS, CWSP, or LPS. Then, 455  
24 h later, the mice were again IP injected with PBS or LPS, 1.5 h 456  
subsequent to which the serum concentrations of IL-1 $\beta$  and TNF $\alpha$  457  
in the tested mice were measured by ELISA (Figure 7A and B). 458 F7  
This revealed that C57BL/6J mice initially injected with PBS that 459  
received a second LPS injection showed a significant increase in 460  
baseline levels of IL-1 $\beta$  and TNF $\alpha$  secretion into the serum 461  
(Figure 7A and B, Group 2), more so than in the case of mice that 462



**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-1 $\beta$  serum concentrations were measured using an IL-1 $\beta$  ELISA kit;  $n = 4$ ;  $*p < 0.05$ . **(B)** CWSP preinjection increases serum concentrations of TNF $\alpha$  in LPS-injected mice. The procedures of treatment were as shown in **(A)**. TNF $\alpha$  serum concentrations were measured using a TNF $\alpha$  ELISA kit;  $n = 4$ ;  $*p < 0.05$ .

463 received a second PBS injection (**Figure 7A and B**, Group 1). In  
 464 addition, test mice were initially injected with CWSP, 24 h sub-  
 465 sequent to which they received an injection of LPS; 1.5 h after the  
 466 LPS injection, the serum levels of IL-1 $\beta$  and TNF $\alpha$  were measured  
 467 by ELISA. The results of such a treatment indicated that CWSP  
 468 slightly increased IL-1 $\beta$  secretion and significantly increased  
 469 TNF $\alpha$  secretion in LPS-injected mice (**Figure 7A and B**, Group  
 470 3), compared to that of mice initially injected with PBS followed  
 471 by a second injection with LPS (**Figure 7A and B**, Group 2). Other test  
 472 mice were initially injected with LPS, followed 24 h later by a  
 473 second injection of LPS. This second injection of LPS was  
 474 responsible for the induction of lower levels of IL-1 $\beta$  and TNF $\alpha$   
 475 secretion into the serum (**Figure 7A and B**, Group 4), compared to  
 476 that of mice injected with only LPS (**Figure 7A and B**, Group 2).

## 477 DISCUSSION

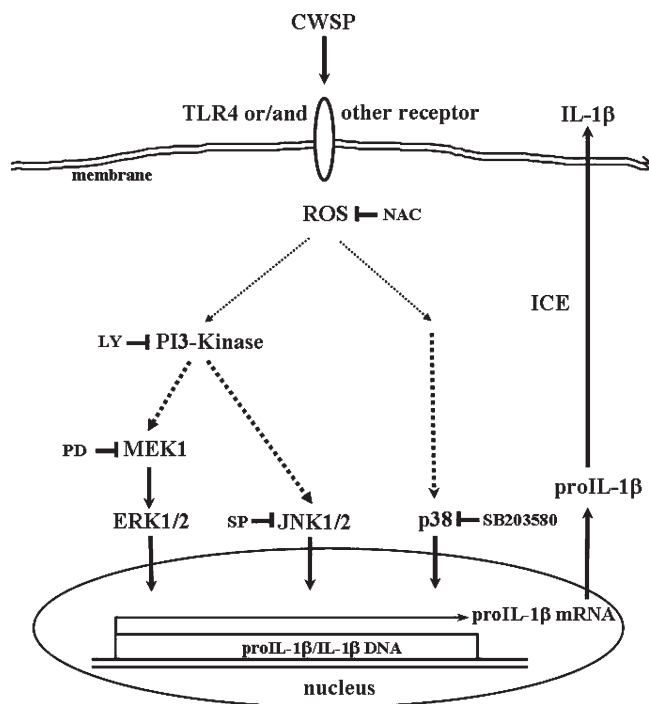
478 An increasing number of studies have been devoted to describ-  
 479 ing the immunological properties of polysaccharides containing

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 expres-  
 sion of IL-1 $\beta$  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-1 $\beta$  expression in  
 macrophages with functional TLR4; but CWSP only induced a  
 small amount of proIL-1 $\beta$  expression in macrophages with  
 deficient TLR4 (**Figure 2A**). Moreover, TLR4 neutralizing anti-  
 body could significantly block (but not completely) CWSP-  
 mediated IL-1 $\beta$  secretion (**Figure 2B**). These results indicate that  
 TLR4 is not the only receptor involved in the production of IL-1 $\beta$   
 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-1 $\beta$  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-1 $\beta$  induction and IL-1 $\beta$  receptor-associated kinase-1 expres-  
 sion. On the basis of the results from the polymyxin B inhibition  
 assay (**Figure 2D**) and the Limulus amoebocyte lysate assay, we  
 ruled out the possibility that CWSP-induced IL-1 $\beta$  expression is  
 due to LPS contamination.

In addition, the JNK1/2 pathway played different roles in  
 CWSP- and LPS-mediated IL-1 $\beta$  expression. In this study, we  
 found that the JNK1/2 pathway was not involved in CWSP-  
 mediated IL-1 $\beta$  expression (**Figure 4B**); in contrast, our previous  
 study demonstrated that the JNK1/2 pathway played an impor-  
 tant role in LPS-mediated IL-1 $\beta$  expression (14). The phenom-  
 enon was similar to that in previous research that had  
 demonstrated that the alkali-soluble polysaccharides from  
*R. riparium* (RASP) were able of inducing IL-1 $\beta$  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).

TNF $\alpha$  is one kind of primary molecule that determines  
 endotoxic shock. Our results showed that CWSP increased  
 LPS-induced IL-1 $\beta$  and TNF $\alpha$  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 macro-  
 phages. 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.



**Figure 8.** Proposed CWSP-mediated signal transduction pathways in the regulation of IL-1 $\beta$  expression.

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 TNF $\alpha$  and IL-1 $\beta$  secretion. At the same time, we are the first to report that CWSP induced IL-1 $\beta$  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 TNF $\alpha$  and IL-1 $\beta$  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; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; 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.

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