0026-895X/06/7002-637–644$20.00
MOLECULAR PHARMACOLOGY
Vol. 70, No. 2
22327/3125769
Mol Pharmacol 70:637–644, 2006
Printed in U.S.A.

Polysaccharide Purified from *Ganoderma lucidum* Induces Gene Expression Changes in Human Dendritic Cells and Promotes T Helper 1 Immune Response in BALB/c Mice

Yu-Li Lin, Shiuh-Sheng Lee, Shin-Miao Hou, and Bor-Luen Chiang

Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China (Y.-L.L., S.-M.H., B.-L.C.); and Department of Biochemistry, National Yang-Ming University, Taipei, Taiwan, Republic of China (S.-S.L.)

Received January 6, 2006; accepted May 2, 2006

ABSTRACT

Ganoderma lucidum is a medicinal mushroom in China and other Asian countries. The polysaccharide from *G. lucidum* (PS-G) is a branched (1→6)-β-D-glucan moiety. In this study, we examined the effects of PS-G on human monocyte-derived dendritic cells (DCs) with microarray analysis by Human Genome U133 Plus 2.0 GeneChip. In comparing mean signal values between PS-G-treated DCs with untreated DCs, 3477 (17%) probe sets were up-regulated, and 4418 (19%) probe sets were down-regulated after PS-G treatment. These results demonstrate that genes associated with phagocytosis (CD36, CD206, and CD209) are decreased and genes associated with proinflammatory chemokines (CCL20, CCL5, and CCL19), cytokines [interleukin (IL)-27, IL-23A, IL-12A, and IL-12B], and costimulatory molecules (CD40, CD54, CD80, and CD86) are increased. To confirm the microarray data, we further investigated the effect of PS-G on antigen-specific antibody and cytokine production in BALB/c mice. Immunization with ovalbumin (OVA)/PS-G showed that the anti-OVA IgG2a levels were significantly increased compared with OVA alone in BALB/c mice. Together, our data demonstrate that PS-G could effectively promote the activation and maturation of immature DCs, preferring a T helper 1 response. Furthermore, the results also demonstrate that the data from microarray analysis could be correlated with the in vivo effect of the immune-enhancing compound.

Ganoderma lucidum, a mushroom, has been widely used in China and other Asian countries. *G. lucidum* has been reported to be effective in modulating immune functions, promoting antitumor activity, and yielding antiviral effects. It also has been used in the treatment of asthma, chronic hepatopathy, hypertension, and hyperglycemia (Miyazaki and Nishijima, 1981). The polysaccharide from *G. lucidum* (PS-G) is a branched (1→6)-β-D-glucan moiety. Studies have demonstrated the antineoplastic action of *G. lucidum* and attributed it to the activated host immune response (Wang et al., 1997). PS-G has been reported to enhance the cytotoxic activity of natural killer cells and to increase tumor necrosis factor-α and interferon (IFN)-γ release, from macrophages and lymphocytes, respectively (Lee et al., 1995). The polysaccharide component from *G. lucidum* has also been reported to elicit antiapoptotic effects on neutrophils, and this action primarily depends on the activation of Akt-regulated signaling pathways (Hsu et al., 2002).

Dendritic cells (DCs) are the most potent antigen-presenting cells, whose primary function is to capture, process, and present antigens to naive T cells (Banchereau and Steinman, 1998). Immature DCs reside in nonlymphoid tissues where they can capture and process antigens. Thereafter, DCs migrate to the T-cell areas of lymphoid organs where they lose antigen-processing activity and mature to become potent immunostimulatory cells (Cella et al., 1997). The induction of DCs maturation is critical for the induction of antigen-specific T-cell responses and may be essential for the development of human vaccines relying on T-cell immunity. Fully mature DCs show high surface expression of MHC class II and costimulatory molecules, and the secretion of cytokines and chemokines, but a decreased capacity to internalize antigens (Cella et al.,

This study was supported by the National Science Council (Taipei, Taiwan, Republic of China).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.022327.

ABBREVIATIONS: PS-G, polysaccharide from *G. lucidum*; IFN, interferon; DC, dendritic cell; CC, chemokine; MHC, major histocompatability complex; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; Th, T helper; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; ISG20, interferon-stimulated gene of 20 kDa; IRF, interferon regulatory factor.

1997). Up-regulation of CD83, a specific marker for DC maturation, also occurs (Sallusto et al., 1995). Various stimuli, such as proinflammatory cytokines (e.g., tumor necrosis factor-α and IL-1), CD40 ligation, bacterial products [e.g., lipopolysaccharide (LPS) and unmethylated DNA CpG motif], and contact sensitizers, can induce DC maturation in vivo and in vitro (Caux et al., 1998; Jakob et al., 1998). Several studies have already indicated that nuclear transcription factor NF-κB also plays an important role in DC maturation (Yoshimura et al., 2001).

Specific immune responses that can be differentiated into Th1 and Th2 responses have distinct roles in the immune system (Mossmann and Coffman, 1989). Th1 cells modulate cellular immunity by producing IL-2 and IFN-γ, whereas Th2 cells are implicated in humoral response by secreting IL-4, IL-5, and IL-6. In addition, IFN-γ suppresses Th2 immune responses (Dickensheets et al., 1999), whereas IL-4 and IL-10 down-regulate Th1 responses (Yin et al., 1997). Antigen-presenting cells such as dendritic cells also play a major role in T helper cell differentiation. IL-12 derived from antigen-presenting cells stimulates IFN-γ production from T cells, thereby favoring a Th1 pattern of response (Romagnani, 1994). There is clear evidence that IL-12 can suppress IL-4 mRNA induction, both directly and indirectly, through induction of IFN-γ (Finkelman et al., 1994). In the antibody response, IgG2a responses are induced by IFN-γ and suppressed by IL-4 (Snapper and Paul, 1987). IFN-γ promotes isotype switching to IgG2a. IL-12 also stimulates the production of IgG2a, presumably through induction of IFN-γ from T cells and natural killer cells (McKnight et al., 1994). Switching to IgG1 antibody is regulated by IL-4 and inhibited by IFN-γ (Schmitt et al., 1994). Thus, Th1 cells are involved in the differentiation of B lymphocytes and production of the IgG2a isotype (Schmitt et al., 1994). Th2 cells help antibody-producing cells to induce class switching of IgG1.

We have reported that PS-G can induce important changes in the phenotype and function of DCs (Lin et al., 2005) To date, there are no published reports describing genomic-scale analysis of these changes. Therefore, to explore further the concept of PS-G-induced DC maturation and perhaps identify novel genes that are regulated after the interaction of PS-G with DCs, we examined at the transcriptional level the effects of PS-G exposure to DCs. In addition, information gained by transcript profiling may prove useful in the development of endpoint measures that can serve as the basis for an in vitro method to identify potential immune-enhancing compound.

In the present study, to evaluate the ability of PS-G to modulate the Th1/Th2 balance, we analyzed the gene expression changes in immature human monocyte-derived DCs with PS-G, using the Human Genome U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) oligonucleotide microarrays. Because IFN-γ increase and Th1 polarization are usually the consequences of IL-12 production by DCs, we used anti-IL-12 antibody in mixed leukocyte reaction to clarify this point. We also investigated its in vivo effect on antigen-specific IgG2a/IgG1 antibodies production and ex vivo effect on Th1/Th2 cytokine production by cultured splenocytes derived from OVA-immunized BALB/c mice.

Materials and Methods

PS-G Purification from *G. lucidum*. As in our previous study (Wang et al., 1997), fruiting bodies of *G. lucidum* were washed, disintegrated, and extracted with boiling water for 8 to 12 h. Hot water extract of *G. lucidum* was fractionated into a polysaccharide fraction (alcohol insoluble) and nonpolysaccharide fraction (alcohol soluble). The crude polysaccharide obtained was then passed through a gel-filtration Sephadex G 50 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and was further purified by anion exchange chromatography with a column of diethylaminoethyl-cellulose (Miyazaki and Nishijima, 1981). The PS-G was a protein-bound polysaccharide consisting of approximately 95% polysaccharide and 5% peptides. To rule out possible endotoxin LPS contamination of PS-G samples, we determined LPS content by the chromogenic *Limulus* amebocyte lysate assay. We found that there was no detectable level of endotoxin (<0.10 endotoxin units/ml) in the PS-G samples.

Generation of Human DCs. DCs were generated from PBMCs, as described previously (Zhou and Tedder, 1996; Lin et al., 2005), with some modification. In brief, PBMCs were obtained from healthy donors by centrifugation with the Ficoll-Hypaque method (GE Healthcare). PBMCs were incubated with anti-$CD14^+$ microbeads in conjunction with the MiniMACS system by following the manufacturer's instructions (Miltenyi Biotec Inc., Auburn, CA). The $CD14^+$ cells were at 95 to 99% pure, as assessed by flow cytometry. The $CD14^+$ cells were cultured at 1×10^6 cells per 1 ml of RPMI 1640 medium, 2 mM L-glutamine, streptomycin/penicillin, and 10% fetal calf serum supplemented with 800 U/ml granulocyte macrophage–colony-stimulating factor and 500 U/ml IL-4 in 24-well plates (Corning Life Sciences, Acton, MA) to obtain immature dendritic cells. Fresh medium containing granulocyte macrophage–colony-stimulating factor and IL-4 was added every 2 to 3 days. Human monocyte-derived DCs were routinely used at day 6 of culture.

RNA Preparation. Cells were harvested 16 h after stimulation. Total RNA from DCs was immediately isolated with a TRIzol kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and was used to generate cRNA probes. Preparation of cRNA, hybridization, and scanning of the microarrays were performed according to the manufacturer's protocol (Affymetrix). In brief, 10 μg/ml RNA was converted into double-stranded cDNA by reverse transcription using a cDNA synthesis kit (SuperScript Choice; Invitrogen) with an oligo$(dT)_{24}$ primer containing a T7 RNA polymerase promoter site-added 3′ of poly(T) (Genset, La Jolla, CA). After second-strand synthesis, labeled cRNA was generated from the cDNA sample by an in vitro transcription reaction supplemented with the Bioarray High Yield RNA transcription labeling kit (Enzo Diagnostics, Farmingdale, NY). The labeled cRNA was purified using RNeasy spin columns (QIAGEN, Valencia, CA) and denatured at 94°C before hybridization.

Microarray Hybridization and Data Analysis. Labeled cRNA was hybridized to the Affymetrix U133 Plus 2.0 GeneChip while rotating at 60 rpm for 16 h at 45°C. After hybridization, the microarray was washed using the Affymetrix Fluidics Station in buffer containing biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) for 10 min at 25°C and stained with streptavidin-PE (final concentration, 10 μg/ml; Invitrogen) for 10 min at 25°C. Thereafter, the microarray was washed, restained with streptavidin-PE for 10 min at 25°C, and washed again before measuring fluorescence at 570 nm in an Affymetrix scanner. Data were normalized by global scaling using Affymetrix software.

Initial data analysis was performed with the Microarray Facility using Affymetrix Microarray Suite 5.0 to determine gene expression levels. Data analysis was conducted using GeneSpring (Silicon Genetics, Redwood City, CA), and -fold change values for genes were calculated as the ratio of the signal values of the PS-G-treated group compared with the control group. Only those changes in gene expres-

sion with 2-fold significance changes were considered to be caused by PS-G treatment.

Autologous Mixed Leukocyte Reaction. PBMCs were obtained as described above, and naive $CD4^+$ T cells were purified by a naive $CD4^+$ T cell isolation kit (Miltenyi Biotec). The autologous $CD4^+$ T cells obtained were distributed at 10^5 cells per well and incubated for 3 or 5 days in the presence of 10^4 cells per well of PS-G-treated or nontreated DCs with or without 20 μg/ml anti-IL-12 monoclonal antibody. On day 3, the culture supernatants were harvested for IL-5 and IFN-γ analysis. On day 5, tritiated thymidine (1 μCi/well; PerkinElmer Life and Analytical Sciences, Boston, MA) was added, and the cells were incubated for another 16 h. The cells were harvested on a cell harvester (PerkinElmer Life and Analytical Sciences), and the incorporated radioactivity was measured using a beta-counter (PerkinElmer Life and Analytical Sciences).

Mice and Immunizations. Female BALB/c mice were obtained from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China. Mice receiving only antigen were immunized by i.p. injections with 0.2 ml of solution containing 50 μg of OVA (Sigma-Aldrich, St. Louis, MO) in saline. The group receiving experimental adjuvant was immunized with 0.2 ml of solution containing 1.0 mg of PS-G admixed with 50 μg of OVA in saline. The mice given intraperitoneal injection of 1× PBS in each immunization were regarded as the negative control group. Mice immunized with 0.2 ml of solution containing 50 μg of OVA admixed with 4 mg of alum (Pierce Chemical, Rockford, IL) as an adjuvant in saline were regarded as the positive control group. Animals were immunized on days 0, 14, and 28. Blood was collected by retro-orbital puncture at various time points after immunization.

OVA-Specific Antibody Assay. Sera anti-OVA IgG1 and IgG2a antibody titers were determined by ELISA. In brief, 96-well flat-bottomed plates were coated with 10 μg/ml OVA. After overnight incubation at 4°C, plates were washed and blocked with 3% bovine serum albumin in PBS for 2 h at 37°C. Serum samples were diluted and added to each well overnight at 4°C. Then, the plates were washed, and biotin-conjugated anti-mouse IgG1 (1:5000; BD PharMingen, San Diego, CA) or IgG2a (1:1000; BD PharMingen) was added for 1 h at 37°C. Streptavidin-conjugated horseradish peroxidase (1:10,000) was added for an additional 2 h at room temperature. Finally, the reaction was developed by H_2O_2 and tetramethylbenzidine, followed by 50 μl/well H_2SO_4 stop solution. A_{450} was measured using a microplate reader (Anthos reader 2010; Anthos Labtec Inc., Salzburg, Austria). The results were expressed in ELISA units (EU):

$$EU = (A_{sample} - A_{blank})/(A_{positive} - A_{blank}).$$

Determination of Cytokine Levels. To measure the levels of cytokines, splenocytes (5×10^6/well) of immunized mice treated with or without PS-G were cultured in RPMI 1640 medium supplemented with 2% TCM in the presence of 10 μg/ml OVA in 24-well microtiter plates at 37°C for 48 h. The culture supernatants were collected and centrifuged at 400*g* at 4°C. The cell-free supernatants were stored at −70°C until they were used for the cytokine assay. The IFN-γ and IL-5 in the culture supernatants were assayed with an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical Analysis. The Student's *t* test was used to analyze the results, and a *P* value of less than 0.05 was considered to be statistically significant.

Results

Global Characteristics of Gene Expression in PS-G-Treated Human Monoctye-Derived DCs. To obtain monocytes with high purity, we preferred magnetic sorting of $CD14^+$ cells rather than enrichment by adherence, because the latter often results in inhomogeneous cell populations. After differentiation to immature DCs, the human DCs were cultured with PS-G for 16 h. Changes in gene expression were analyzed using the Human Genome U133 Plus 2.0 GeneChip, which contains ~38,500 genes and more than 54,000 probe sets. Comparing mean signal values between PS-G-treated with untreated control DCs, 3477 (17%) probe sets were up-regulated and 4418 (19%) probe sets were down-regulated after PS-G treatment. The entire control and PS-G raw databases can be found at http://ntuh.mc.ntu.edu.tw/allergy/english/plane/file/PS-G%20raw%20data.xls and http://ntuh.mc.ntu.edu.tw/allergy/english/plane/file/control%20raw%20data.xls.

Cytokines and Cytokine Receptors. We demonstrated that transcript levels for cytokines IL-27, IL-12A, IL-12B, IL-23A, and Epstein-Barr virus-induced gene 3 were significantly

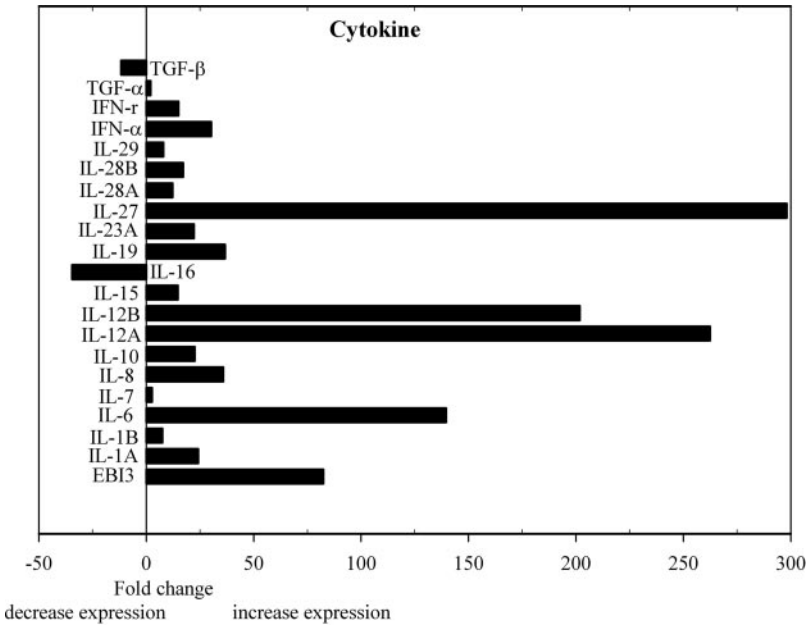

Fig. 1. List of cytokine genes that were significantly changed (more than 2-fold) in PS-G-treated compared with untreated human monocyte-derived dendritic cells. Human dendritic cells were treated with PS-G or untreated for 16 h. RNA was prepared and processed for hybridization to Affymetrix Human Genome U133 Plus 2.0 GeneChip and analyzed as described under *Materials and Methods*.

increased after PS-G-treated human dendritic cells, whereas only transcripts for IL-16 and transforming growth factor-β were reduced (Fig. 1). Transcript levels for cytokine receptors IL-2RA, IL-3RA, IL-4R, IL-6R, IL-7R, IL-15R, and IL-22RA1 were increased after PS-G treatment, whereas transcripts for type II IL-1 receptor (IL1R2) were reduced (Fig. 2).

Chemokines and Chemokines Receptors. Chemokines are a diverse superfamily of small secreted proteins. The regulated expression of chemokines and chemokine receptors is an important component of an integrated immune response. Dendritic cells secrete chemokines and express chemokine receptors. We found the levels of transcripts for CCL20, CCL19, CCL5, CXCL9, CXCL10, and CXCL11, and the levels for CCR7 were higher in PS-G-treated DCs than in immature DCs (Fig. 3). Of special importance, the transcript level of CCL20 (MIP-3α) was 1363-fold higher than that of untreated DCs.

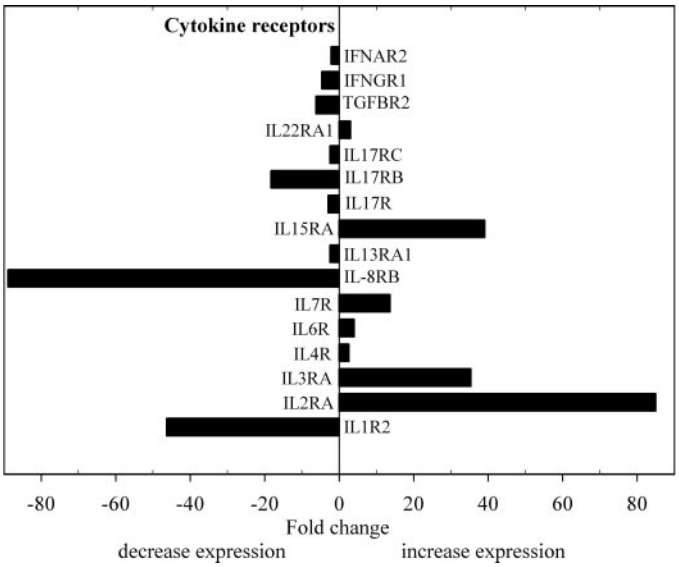

Fig. 2. List of cytokine receptors genes that were significantly changed (more than 2-fold) in PS-G-treated compared with untreated human monocyte-derived dendritic cells. Human dendritic cells were treated with PS-G or untreated for 16 h. RNA was prepared and processed for hybridization to Affymetrix Human Genome U133 Plus 2.0 GeneChip and analyzed as described under *Materials and Methods*.

Transcripts for Cell Surface Proteins. DC maturation induced high levels of costimulatory molecules. Transcript levels for MHC class I and II molecules were largely affected by maturation. We demonstrated that transcript levels for CD80, CD83, and CD86 were higher (more than a 2-fold increase) in PS-G-treated DCs, whereas the levels for CD1A, CD1B, and CD1C were lower (more than a 2-fold decrease). Of particular note was maturation associated with reduction of transcript levels for human leukocyte antigen-DM, a molecule that mediates loading of antigenic peptides into MHC class II molecules, a process more active in immature DCs (Fig. 4).

Transcripts for Signal Transduction. We have demonstrated that PS-G go through the Toll-like receptor 4 and rapidly induce the significant activation and maturation of human DCs by the NF-κB and p38 MAPK pathways (Lin et al., 2005). In this study, we found that transcript levels for NFKB1, NFKB2, RELA, RELB, and MAPK11 were increased in PS-G-treated DCs (Fig. 5). The results also show a marked effect of PS-G on the expression of numerous IFN-regulated genes (IFI27, IFI35, IFI44, IFIT1, IFIT2, IFIT4, and IFITM3) in human DCs. In this set of microarray data, interferon-stimulated gene of 20 kDa (ISG20) was the most highly expressed (1522-fold) gene in PS-G-treated DCs.

PS-G-Stimulated DCs Polarize Naive T Cells for Th1 Response, and IL-12 Neutralization Reduces IFN-γ Production. In the current study, we demonstrated that treatment of DCs with PS-G resulted in the enhanced expression of IL-12 p35 and IL-12 p40 mRNA by reverse transcription-polymerase chain reaction and enhanced production of IL-12 p70 and p40 by the ELISA method (Lin et al., 2005). IFN-γ increase and Th1 polarization are usually the consequences of IL-12 production by dendritic cells. To clarify whether PS-G-stimulated DCs can induce Th1 polarization, and whether the Th1 was reversed by anti-IL-12 antibody, the mixed leukocyte reaction was studied.

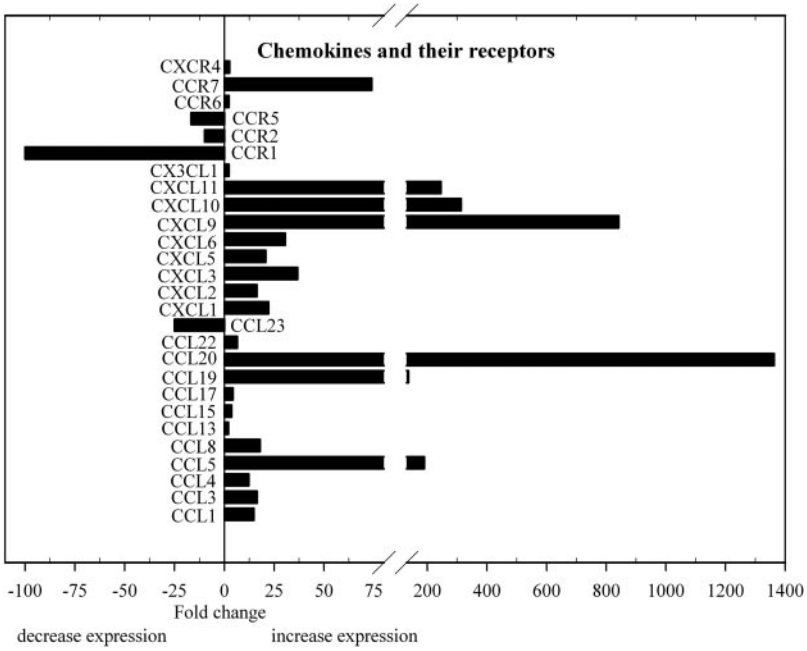

Fig. 3. List of chemokines and their receptors genes that were significantly changed (more than 2-fold) in PS-G-treated compared with untreated human monocyte-derived dendritic cells. Human dendritic cells were treated with PS-G or untreated for 16 h. RNA was prepared and processed for hybridization to Affymetrix Human Genome U133 Plus 2.0 GeneChip and analyzed as described under *Materials and Methods*.

DCs were treated with PS-G, and these cells were then used to activate $CD4^+$ naive T cells. The results presented in Fig. 6A show that PS-G-treated DCs significantly enhanced T-cell activation compared with untreated DCs ($p = 0.00029$). The IFN-γ production induced under these experiment conditions was far higher than in untreated DCs ($p = 0.01489$) (Fig. 6B), and the IL-5 production was not effected (Fig. 6C). With the addition of an anti-IL-12 antibody to the coculture of PS-G-treated DCs with T cells, we demonstrated that the secretion of IFN-γ was markedly decreased compared with no addition of an anti-IL-12 antibody ($p = 0.016291$), indicating that IL-12 production by PS-G-treated DCs is critical for the Th1 polarization.

Effect of PS-G on Serum Anti-Ovalbumin Antibody Levels. To confirm the microarray data, we studied the effect of PS-G on antigen-specific IgG1 and IgG2a in OVA-immunized BALB/c mice. We obtained serum from OVA immunized 38 and 49 days after first immunization. In PS-G admixed with OVA-immunized mice, antigen-specific IgG2a production was significantly increased at the time point ($p = 0.000045$, day 38; $p = 0.000145$, day 49) examined after immunization compared with mice immunized with OVA alone (Fig. 7A). However, the anti-OVA IgG1 antibody levels showed no significant differences among the OVA-immunized mice (Fig. 7B).

Regulatory Effect of PS-G on the Balance of Th1/Th2 Cell Responses in OVA-immunized Mice. It has been reported that cytokines play an important role in the antibody response. Therefore, we examined the regulatory effect of PS-G on Th1/Th2 cell responses in OVA-immunized mice. Mice were immunized with OVA plus PS-G on days 0, 14, and 28. IFN-γ (Th1 cytokine) and IL-5 (Th2 cytokine) production in splenocytes stimulated with OVA was assayed (Fig. 8). The production of IFN-γ significantly increased in PS-G admixed with OVA-immunized mice compared with OVA-immunized mice ($p = 0.026$) (Fig. 8A). The production of IL-5 significantly decreased in PS-G admixed with OVA-immunized mice compared with OVA-immunized mice ($p = 0.036$) (Fig. 8B). These results indicate that PS-G changed the balance of Th1/Th2 cell immune responses from Th2-dominant to Th1-dominant in OVA-immunized mice.

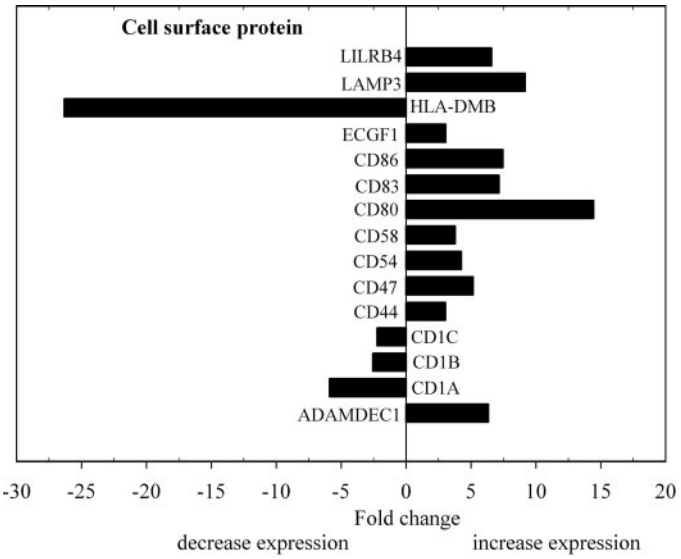

Fig. 4. List of cell surface proteins genes that were significantly changed (more than 2-fold) in PS-G-treated compared with untreated human monocyte-derived dendritic cells. Human dendritic cells were treated with PS-G or untreated for 16 h. RNA was prepared and processed for hybridization to Affymetrix Human Genome U133 Plus 2.0 GeneChip and analyzed as described under *Materials and Methods*.

Discussion

In the current study, we demonstrated that PS-G induced morphological, phenotypical, and functional changes in human monocyte-derived DCs (Lin et al., 2005). However, there are no published reports describing genomic-scale analysis of the changes induced in human DCs resulting from PS-G treatment. In this study, we examined changes in gene expression in human monocyte-derived DCs that were treated by exposure to PS-G using Affymetrix GeneChip microar-

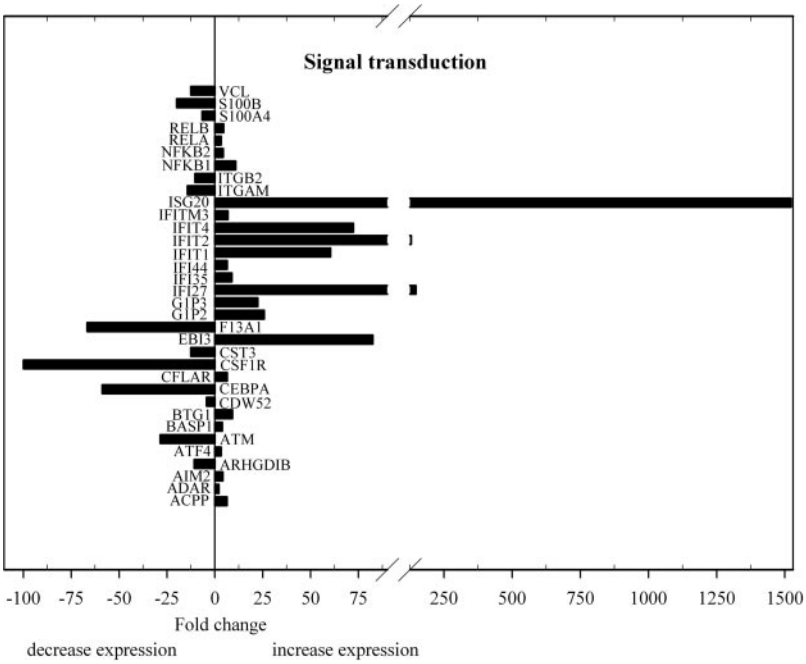

Fig. 5. List of signal transduction genes that were significantly changed (more than 2-fold) in PS-G-treated compared with untreated human monocyte-derived dendritic cells. Human dendritic cells were treated with PS-G or untreated for 16 h. RNA was prepared and processed for hybridization to Affymetrix Human Genome U133 Plus 2.0 GeneChip and analyzed as described under *Materials and Methods*.

rays. In a comparison of mean signal values between PS-G-treated DCs with untreated control DCs, 3477 (17%) probe sets were 2-fold up-regulated and 4418 (19%) probe sets were 2-fold down-regulated after PS-G treatment. We focused our attention only on those genes for which expression was found to be significantly different from control DCs.

The biological process of DC maturation represents a crucial step in the initiation of adaptive immune responses. This process is regulated by various extracellular stimuli, including cytokines, bacterial products, and membrane-bound ligands (O'Sullivan and Thomas, 2002). DC maturation is accompanied by changes in their morphological, phenotypic, and functional properties (Sallusto et al., 1995). PS-G promoted the maturation of DCs, whereas mature DCs demonstrated characteristic morphology, with enlarged size and numerous cytoplasmic processes that gave rise to a stellate appearance (data not shown). Maturation of DCs was characterized by a decreased antigen processing capacity and an increased cell surface expression of MHC class II molecules and costimulatory molecules, and the secretion of IL-12, which primed a strong stimulation of T lymphocytes growth and differentiation. The CD83 marker for mature human DCs was also increased. In this study, we demonstrated that genes associated with phagocytosis (CD36, CD206, and CD209) were down-regulated (data not shown) and that the association with proinflammatory chemokines (CCL20, CCL5, and CCL19), cytokines (IL-27, IL-23A, IL-12A, and IL-12B), and costimulatory molecules (CD40, CD80, and CD86) were increased. These results were correlated with our current report, and we found that treatment of DCs with PS-G resulted in the enhanced cell surface expression of CD80, CD86, CD83, CD40, CD54, and human leukocyte antigen-DR by flow cytometry, as well as enhanced production

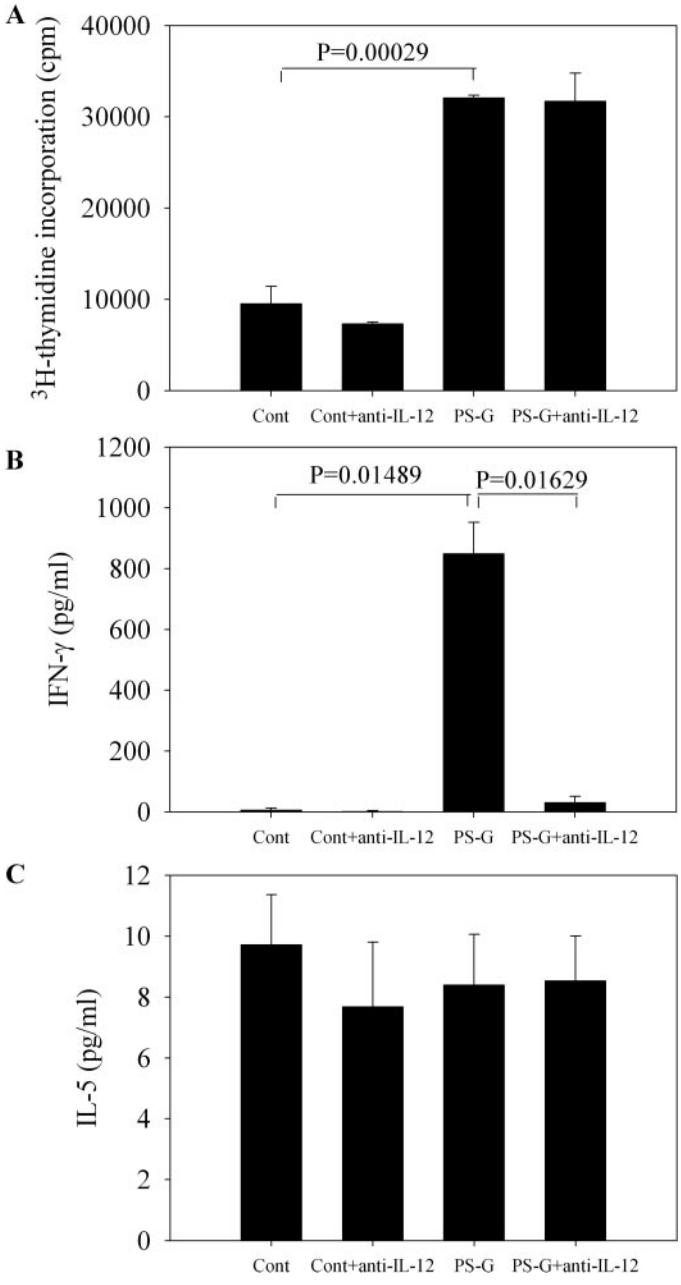

Fig. 6. T cells primed with DCs treated with anti-IL-12 release lower amount of IFN-γ. A, immature dendritic cells (10^6 cells/ml) were treated with or without PS-G (10 μg/ml) for 24 h and then washed and cocultured with naive $CD4^+$ autologous T cells (10^5 cells/well) with or without 20 μg/ml anti-IL-12 monoclonal antibody at DC/T-cell ratio of 1:10. After 5 days, proliferation of autologous T cells was measured by [^{3}H]thymidine incorporation. Supernatants were analyzed for IFN-γ (B) and IL-5 (C), produced by activated T cells after 2 days of culture. Data represent the mean ± S.E. of triplicates.

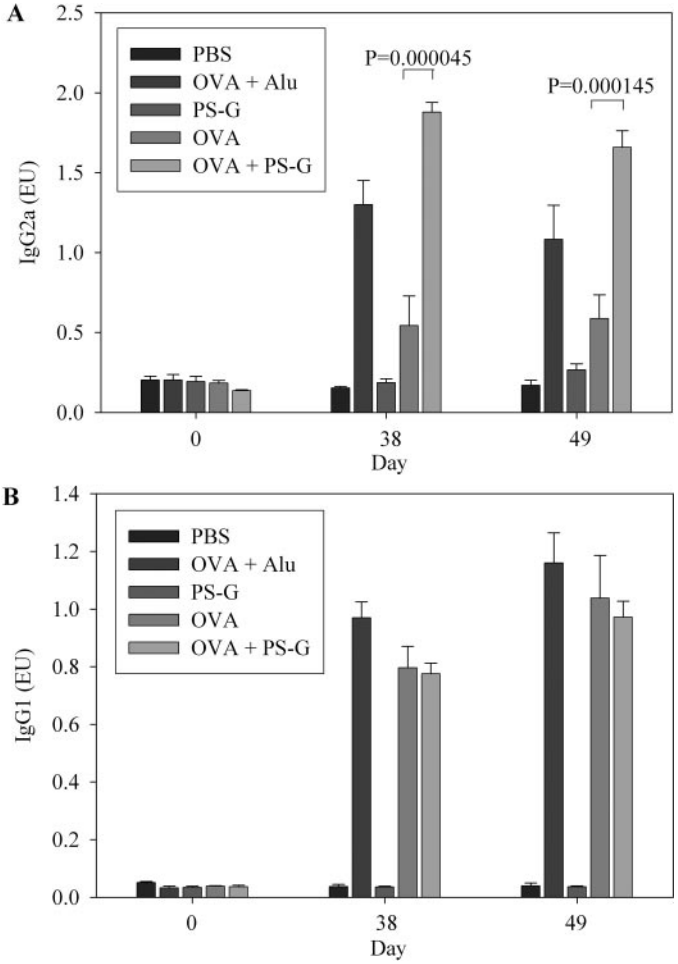

Fig. 7. Serum anti-OVA IgG2a (A) and IgG1 (B) responses after intraperitoneal immunization of BALB/c mice with OVA or OVA/PS-G. Mice were immunized with 50 μg of OVA i.p. plus 1.0 mg of PS-G i.p. or with PBS on days 0, 14, and 28, and serum samples were collected on days 0, 38, and 49 after the first immunization. Data represent the mean ± S.E., and each group had six mice.

of IL-12p70 and p40 by the ELISA method (Lin et al., 2005). Significant activation and maturation of human DCs is by the NF-κB and p38 MAPK pathways.

In immune responses, IL-12 plays a central role as a link between the innate and adaptive immune systems (Trinchieri, 1998). Thus, IL-12 induces and promotes natural killer and T cells to generate IFN-γ and lytic activity. In addition, IL-12 polarizes the immune system toward a Th1 response. Moreover, IL-12 has been shown to prevent development of the Th2 immune response in several mice models of immune activation and infection (Lee et al., 2001). Some studies indicate that IL-23 and IL-27, two cytokines that are closely related to IL-12, also regulate Th1-cell responses (Hunter, 2005). IL-23, comprising a p19 subunit and IL-12p40, is produced by activated macrophages and dendritic cells (Langrish et al., 2004). IL-27 is a heterodimeric cytokine composed of p28, a newly identified IL-12p35-related protein, and Epstein-Barr virus-induced gene 3, an IL-12p40-related protein. IL-27 is produced primarily from activated dendritic cells and induces an early phase of T helper type I differentiation. Chiyo et al. (2005) suggested that expressed IL-27 in tumors produces T-cell-dependent and-independent antitumor effects and is a possible therapeutic strategy for cancer. A number of studies have demonstrated that secretion of Th1-type cytokines from tumors activated host defense mechanisms have consequently produced antitumor effects. Transfer of cytokine genes is thereby a possible strategy for cancer treatment and is currently being investigated for its clinical feasibility (Chiyo et al., 2004; Triozzi et al., 2005).

Chemokines cause recruitment and polarization of T cells

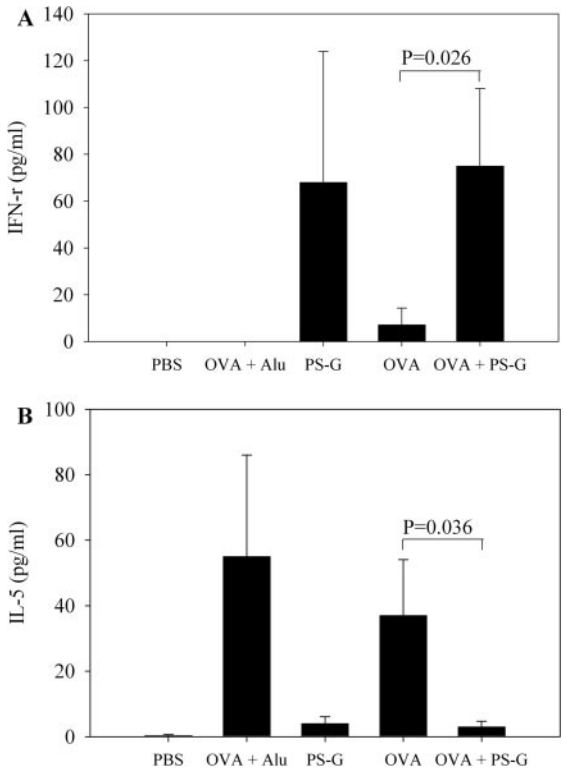

Fig. 8. IFN-γ and IL-5 production by murine splenocytes after immunization of BALB/c mice with OVA plus PS-G. Mice were immunized as described in Fig. 7. The splenocytes were prepared on day 49 and were incubated at 37°C with 10 μg/ml OVA for 48 h. The culture supernatants were collected and used to determine IFN-γ and IL-5 production. The data shown are means ± S.E.

(Wong and Fish, 2003). Furthermore, certain chemokines such as CCL5 (regulated on activation normal T cell expressed and secreted) are able to costimulate T-cell proliferation. In this study, we report that PS-G can induce the transcripts of CCL20, CCL19, CCL5, CXCL9, CXCL10, and CXCL11 in human DCs. Of particular importance, PS-G induced the highest transcript of CCL20, approximately 1363-fold compared with untreated DCs. CCL20 (macrophage inflammatory protein-3α) is the only chemokine known to interact with CCR6, a property shared with the antimicrobial β-defensins. The CCL20-CCR6 is responsible for the chemoattraction of immature DCs, effect/memory T cells and B cells, and plays a role in skin and mucosal surfaces under homeostatic and inflammatory conditions, and in pathology, including cancer and rheumatoid arthritis (Schutyser et al., 2003). Adenovirus-mediated gene transfer of human CCL20 cDNA by injection in a variety of preformed mice subcutaneous tumors led to intratumor expression of CCL20 (Fushimi et al., 2000). This strategy induced a local accumulation of immature DCs, resulting in tumor-specific cellular immunity and significant growth suppression of established tumors. *G. lucidum* has been reported to have antitumor activity (Lee et al., 1995; Wang et al., 1997). Recent clinical studies have demonstrated that the polysaccharide fractions of *G. lucidum* polysaccharides have potential antitumor activity and enhance host immune functions (Gao et al., 2005). Therefore, we suggest that PS-G from *G. lucidum* could induce transcripts of CCL20, IL-27, IL-23A, IL-12A, and IL-12B in human DCs. As such, these genes might play an important role in the treatment of cancer.

In this study, we demonstrated that TRIF-dependent genes, including IRF-1 (8-fold), IRF-7 (11-fold), Mx1 (12-fold), Mx2 (23-fold), and ISG20 (1522-fold) were significantly upregulated in PS-G-treated DCs, which have been implicated in the generation of antiviral immune responses or viral replication (Horisberger, 1995; Sato et al., 2000). IRF-1 has also been reported to be required for Th1 responses (Lohoff et al., 1997). ISG20 is a 3′→5′ exonuclease whose gene is transcriptionally induced by both type I and type II IFN (Espert et al., 2003). Its induction by IFN is strictly dependent upon the activation and binding of IRF-1 to a specific interferon-stimulated response element on the Isg20 promoter. Moreover, the TATA-less Isg20 promoter contains one E-box and putative NF-κB and Sp-1 binding sites, suggesting that it could be induced by other stimuli. ISG20 has an antiviral activity, supporting the idea that it might represent a novel antiviral pathway (Espert et al., 2004). *G. lucidum* has been reported to have antiviral activity (Li et al., 2005). From our results, we found that ISG20 is the most highly expressed in PS-G-treated DCs, whereas PS-G also can induce transcripts of type I and type II IFN. Therefore, we inferred that one reason for PS-G could involve the antiviral function by type I, type II IFN, and ISG20 production.

Recently, several researchers have demonstrated the immunomodulatory effects of polysaccharides purified from *G. lucidum* on T lymphocytes (Gao et al., 2003). However, there are only a limited number of studies on the adjuvant effects of polysaccharides purified from *G. lucidum* on antibody production. Therefore, we further investigated the adjuvant effects of PS-G on antigen-specific antibody and cytokine production using BALB/c mice immunized with OVA antigen. This study demonstrates that PS-G seems to have marked

induction effects on Th1 responses because treatment of mice with PS-G was followed by an increase in Th1 response, including anti-ovalbumin IgG2a and IFN-γ production. Moreover, PS-G had no effect on the anti-OVA IgG1 levels. These findings demonstrate that PS-G could be used as adjuvant to induce Th1 immunity in BALB/c mice. Together, our data demonstrated that PS-G could effectively promote the activation and maturation of immature DCs and prefer a Th1 response, suggesting that PS-G may possess the potential capacity in regulating immune responses. The precise mechanism by which PS-G induced Th1 responses in vivo might be that the PS-G can induce DC activation and maturation, and thus induce Th1-related cytokines and chemokines production.

In conclusion, we demonstrated that PS-G effectively and rapidly induced the significant activation and maturation of human DCs. PS-G also is an adjuvant-active molecule that stimulates Th1 response. As a nontoxic and very stable compound, it could find its application as an adjuvant for vaccines. Therefore, PS-G is a good and potential part of the treatment regimen to regulate host immune responses, and this study may provide information for the further design of PS-G-treated-DC-based immunotherapies for many diseases. In addition, it is hoped that some of the transcript changes identified with microarray analysis will be shown to be suitable for use in the development of an in vitro predictive assay for Chinese herbs.

Acknowledgments

We thank National Taiwan University (Center for Genomic Medicine) for the microarray analysis. We extend gratitude to Dr. Sung-Liang Yu for discussion and comments on this work and manuscript.

References

Banchereau J and Steinman RM (1998) Dendritic cells and the control of immunity. *Nature (Lond)* **392:**245–252.

Caux C, Massacrier C, Vanbervliet B, Dubois B, van Kooten C, Durand I, and Banchereau J (1998) Activation of human dendritic cells through CD40 cross-linking. *J Exp Med* **180:**1263–1272.

Cella M, Pinet Engering A, Pieters J, and Lanzavecchia A (1997) Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature (Lond)* **388:**782–787.

Cella M, Sallusto F, and Lanzavecchia A (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol* **9:**10–16.

Chiyo M, Shimozato O, Iizasa T, Fujisawa T, and Tagawa M (2004) Antitumor effects produced by transduction of dendritic cells-derived heterodimeric cytokine genes in murine colon carcinoma cells. *Anticancer Res* **24:**3763–3767.

Chiyo M, Shimozato O, Yu L, Kawamura K, Iizasa T, Fujisawa T, and Tagawa M (2005) Expression of IL-27 in murine carcinoma cells produces antitumor effects and induces protective immunity in inoculated host animals. *Int J Cancer* **115:** 437–442.

Dickensheets HL, Venkataraman C, Schindler U, and Donnelly RP (1999) Interferons inhibit activation of STAT6 by interleukin 4 in human monocytes by inducing SOCS-1 gene expression. *Proc Natl Acad Sci USA* **96:**10800–10805.

Espert L, Degols G, Gongora C, Blondel D, Williams BR, Silverman RH, and Mechti N (2003) ISG20, a new interferon-induced RNase specific for single-stranded RNA, defines an alternative antiviral pathway against RNA genomic viruses. *J Biol Chem* **278:**16151–16158.

Espert L, Rey C, Gonzalez L, Degols G, Chelbi-Alix MK, Mechti N, and Gongora C (2004) The exonuclease ISG20 is directly induced by synthetic dsRNA via NF-κB and IRF1 activation. *Oncogene* **23:**4636–4640.

Finkelman FD, Madden KB, Cheever AW, Katona IM, Morris SC, Gately MK, Hubbard BR, Gause WC, and Urban JF Jr (1994) Effects of interleukin 12 on immune responses and host protection in mice infected with intestinal nematode parasites. *J Exp Med* **179:**1563–1572.

Fushimi T, Kojima A, Moore MA, and Crystal RG (2000) Macrophage inflammatory protein 3α transgene attracts dendritic cells to established murine tumors and suppresses tumor growth. *J Clin Investig* **105:**1383–1393.

Gao Y, Tang W, Dai X, Gao H, Chen G, Ye J, Chan E, Koh HL, Li X, and Zhou S (2005) Effects of water-soluble *Ganoderma lucidum* polysaccharides on the immune functions of patients with advanced lung cancer. *J Med Food* **8:**159–168.

Gao Y, Zhou S, Jiang W, Huang M, and Dai X (2003) Effects of ganopoly (a *Ganoderma lucidum* polysaccharide extract) on the immune functions in advanced-stage cancer patients. *Immunol Investig* **32:**201–215.

Horisberger MA (1995) Interferons, Mx genes and resistance to influenza virus. *Am J Respir Crit Care Med* **152:**S67–S71.

Hsu MJ, Lee SS, and Lin WW (2002) Polysaccharide purified from *Ganoderma lucidum* inhibits spontaneous and Fas-mediated apoptosis in human neutrophils through activation of the phosphatidylinositol 3 kinase/Akt signaling pathway. *J Leukoc Biol* **72:**207–216.

Hunter CA (2005) New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* **5:**521–531.

Jakob T, Walker PS, Krieg AM, Udey MC, and Vogel JC (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J Immunol* **161:**3042–3049.

Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA, and Cua DJ (2004) IL-12 and IL-23: master regulators of innate and adaptive immunity. *Immunol Rev* **202:**96–105.

Lee SS, Wei YH, Chen CF, Wang SY, and Chen KY (1995) Antitumor effects of *Ganoderma lucidum*. *J Clin Med* **6:**1–12.

Lee YL, Ye YL, Yu CI, Wu YL, Lai YL, Ku PH, Hong RL, and Chiang BL (2001) Construction of single-chain interleukin-12 DNA plasmid to treat airway hyperresponsiveness in an animal model of asthma. *Hum Gene Ther* **12:**2065–2079.

Li Z, Liu J, and Zhao Y (2005) Possible mechanism underlying the antiherpetic activity of a proteoglycan isolated from the mycelia of *Ganoderma lucidum* in vitro. *J Biochem Mol Biol* **38:**34–40.

Lin YL, Liang YC, Lee SS, and Chiang BL (2005) Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human monocyte-derived dendritic cells by the NF-κB and p38 mitogen-activated protein kinase pathways. *J Leukoc Biol* **78:**533–543.

Lohoff M, Ferrick D, Mittrücker H-W, Duncan GS, Bischof S, Röllinghoff M, and Mak TW (1997) Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. *Immunity* **6:**681–689.

McKnight AJ, Zimmer GJ, Fogelman I, Wolf SF, and Abbas AK (1994) Effects of IL-12 on helper T cell-dependent immune responses in vivo. *J Immunol* **152:**2172–2179.

Miyazaki T and Nishijima M (1981) Studies on fungal polysaccharides, XXVII. Structural examination of a water-soluble, anti-tumor polysaccharide of *Ganoderma lucidum*. *Chem Pharm Bull* **29:**3611–3616.

Mossmann TR and Coffman RL (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* **7:**145–173.

O'Sullivan BJ and Thomas R (2002) CD40 ligation conditions dendritic cell antigen-presenting function through sustained activation of NF-κB. *J Immunol* **168:**5491–5498.

Romagnani S (1994) Lymphokine production by human T cells in disease states. *Annu Rev Immunol* **12:**227–257.

Sallusto F, Cella M, Danieli C, and Lanzavecchia A (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* **182:**389–400.

Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, et al. (2000) Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity* **13:**539–548.

Schmitt E, Hoehn P, Huels C, Goedert S, Palm N, Rude E, and Germann T (1994) T helper type 1 development of naive CD4+ T cells requires the coordinate action of interleukin-12 and interferon-gamma and is inhibited by transforming growth factor-beta. *Eur J Immunol* **24:**793–798.

Schutyser E, Struyf S, and Damme JV (2003) The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* **14:**409–426.

Snapper CM and Paul WE (1987) Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science (Wash DC)* **236:**944–947.

Trinchieri G (1998) Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol* **70:**83–243.

Triozzi PL, Allen KO, Carlisle RR, Craig M, LoBuglio AF, and Conry RM (2005) Phase I study of the intratumoral administration of recombinant canarypox viruses expressing B7.1 and interleukin 12 in patients with metastatic melanoma. *Clin Cancer Res* **11:**4168–4175.

Wang SY, Hsu ML, Hsu HC, Tzeng CH, Lee SS, Shiao MS, and Ho CK (1997) The anti-tumor effect of *Ganoderma lucidium* is mediated by cytokines released from activated macrophages and T lymphocytes. *Int J Cancer* **70:**699–705.

Wong MM and Fish EN (2003) Chemokines: attractive mediators of the immune response. *Semin Immunol* **15:**5–14.

Yin Z, Braun J, Neure L, Wu P, Liu L, Eggens U, and Sieper J (1997) Crucial role of interleukin-10/interleukin-12 balance in the regulation of the type 2 T helper cytokine response in reactive arthritis. *Arthritis Rheum* **40:**1788–1797.

Yoshimura S, Bondeson J, Foxwell BM, Brennan FM, and Feldmann M (2001) Effective antigen presentation by dendritic cells is NF-kappaB dependent: coordinate regulation of MHC, co-stimulatory molecules and cytokines. *Int Immunol* **13:**675–683.

Zhou LJ and Tedder TF (1996) CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc Natl Acad Sci USA* **93:**2588–2592.

Address correspondence to: Dr. Bor-Luen Chiang, Department of Pediatrics, National Taiwan University Hospital, 7, Chung-Shan South Rd., Taipei, Taiwan, Republic of China. E-mail: gicmbor@ha.mc.ntu.edu.tw