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過敏原對樹突細胞活化及訊息傳導的影響(1/2)

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計畫參與人員：蔡演忠

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共同主持人：
計畫參與人員： 蔡演忠先生

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中文摘要

樹突細胞被認為是在免疫反應的產生上扮演著一個最關鍵的角色，有愈來愈多的證據顯示樹突細胞可能在決定身體免疫系統接觸到外來抗原時的免疫反應的走向。也因此，如果我們能夠對樹突細胞的活化和成熟機轉，和活化後凋亡的機制有更進一步的了解，將有助於我們研究免疫反應的機轉。尤其是在調控免疫反應的走向上，有著關鍵的影響。所以我們將分別研究不同刺激下的樹突細胞，是否會產生不同型的樹突細胞。目前知道樹突細胞如果接受不同的刺激，也會在輔助分子和細胞激素的分泌上有著不同的表現，而這些不同表現的樹突細胞便會主導不同免疫反應的進行。而這些不同表現型的樹突細胞可能在活化途徑和凋亡現象上可能有著相當大的差異，如果能夠釐清這些現象將讓我們對疾病的機轉有更清楚的認識。本研究計畫將分成二年來加以執行：

在第一年的計畫中我們建立樹突細胞的培養系統來分析樹突細胞在培養過程和利用如 lipopolysaccharide 或是 CD40L 刺激後的輔助分子和細胞激素表現的情形。我們將分別利用在免疫反應不同的刺激物如 CpG motif(能夠促進第一型的 T 輔助細胞反應)和過敏原(mite allergens,能夠促進第二型 T 輔助細胞反應)。我們已經順利地培養樹突細胞，再利用靈芝的萃取物和塵 mite 來刺激樹突細胞，了解樹突細胞分泌細胞激素的形式。結果發現靈芝的萃取物已經有效地刺激樹突細胞分泌細胞激素如 IL-10 和 IL-12，由於可以分泌高量的 IL-12，所以對 T 細胞的免疫反應有調節的效果。而在 mite 的刺激下，的確能夠可以分泌高量的細胞激素。所以我們目前正在繼續相關的研究中，也希望能夠在第二年完成所有的訊息傳導研究。

相信未來樹突細胞不論在免疫反應的機轉上將會有更多的研究，同時，這些新的知識也將可望應用在新的免疫療法的研發上。而本研究的另一個目標是著重在利用過敏原來了解樹突細胞的活化途徑，經由此一研究計畫我們將能夠結合基礎和臨床的研究，對免疫反應的調控機制有更進一步的了解。

關鍵詞: 樹突細胞、訊息傳導、過敏原

Abstract

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) in the immune system, which can trigger the immune response. They not only express high level of the MHC-peptide complex, but also the other accessory molecules that interact with the receptors on the T cells to enhance adhesion and signalling. More and more evidences suggest that dendritic cells play the critical role in the initiation and development of immune response. In this project, we aim to clarify the activation pathway involved in the different types of dendritic cells with the stimulation of mite allergens. Our first year's data suggested that *in vitro* culture and characterization of dendritic cells can be established for the activation of dendritic cells with various stimuli such as lipopolysaccharide, and CD40L. Antigens from two different stimuli including Th1-related immune response (CpG DNA motif) and Th2-related immune response (mite allergen) will be used to study the cytokine profiles and accessory molecules of the dendritic cells. In addition, intracellular cAMP activity and kinases involved in the activation of the dendritic cells will be also studied. We have already established the culture system for the dendritic cells and stimulation with either Ganoderma lucidum extract or mite antigen. The preliminary data suggested that both Ganiderma lucidum and mite allergen can stimulate dendritic cells in producing IL-12 and IL-10 cytokines. In addition, we have also investigated the signal pathway of dendritic cells stimulated by Ganoderma lucidum extract and finished the manuscript recently. We plan to continue the study on the stimulation of dendritic cells with either Ganoderma lucidum or mite allergen to clarify the activation pathway of dendritic cells.

In this project, we aim to clarify the functional changes and activation signals of dendritic cells after activation with mite allergens. The information generated in the project will help us in understanding the basic mechanisms of activation pathway of the dendritic cells and also their roles in the pathogenesis of the diseases.

Key words: dendritic cells, signal pathway, mite allergens

一、 前言

最近幾年來過敏疾病有逐年增加的趨勢，同時並未見到減緩的趨勢，所以進一步研究出過敏疾病的機轉，已經是件刻不容緩的課題。在過敏疾病的發生中一直以來都認為第二型 T 輔助細胞扮演著一個重要的功能，由第二型 T 輔助細胞所製造的細胞激素，介白質-4 和介白質-5，會進一步促進 IgE 抗體的製造和嗜伊紅性白血球的增加，而導致嚴重的過敏反應。但是，在過去的研究中，一直忽略了抗原呈現細胞所扮演的重要性。因為抗原呈現細胞是第一個接觸到過敏原的細胞，如果過敏原與抗原呈現細胞接觸時便已經改變了一些基本的細胞激素製造，而這些細胞激素如果有利於第二型 T 輔助細胞的發展，是否這是造成過敏原特別容易導致第二型 T 輔助細胞的發生和過敏疾病發生的原因。而在這些抗原呈現細胞中又以樹突細胞所扮演的角色更為重要，所以我們研究的對象便以樹突細胞為主。

所以，在這個研究中，我們將特別著重於塵 mite 過敏原是否會誘發較高的與第二型 T 輔助細胞的分子，以及製造較高量的 IL-10，而會造成較高活性的第二型 T 輔助細胞活性。同時，我們也將進一步研究這些樹突細胞受到過敏原活化後的訊息傳導，以了解是否與一般的細菌內毒素是否有所不同。在第一年的計畫中，我們已經分別完成了對樹突細胞的基本刺激和活化，同時測定了細胞激素所製造的分泌情形。我們也研究了與這些樹突細胞一道培養後是否會改變 T 細胞的活性。在接下來的研究中，我們將更進一步研究樹突細胞活化後的訊息傳導途徑，以了解樹突細胞受到過敏原刺激後是否會產生特殊的活化機轉，與未來過敏疾病的發生是否有相關。

這是個兩年的研究計畫，我們相信由這個研究計畫所得到的研究結果，將有助於我們對過敏疾病的發生機轉有更進一步的了解，對這些過敏原的特性能夠了解得更清楚。

二、 研究目的

計畫目標： 我們要建立一個系統來研究過敏原對樹突細胞的刺激，以了解是否過敏原能夠對樹突細胞刺激，而進一步研究其中的訊息傳導。

三、 文獻探討

Specific Aims

The project here is to investigate the activation pathway of dendritic cells, which has been thought to play a critical in initiating and regulating immune response. We aim to explore the signal pathway of the dendritic cells with the stimulation of lipopolysaccharide, CpG DNA motif and mite allergens. Both activation pathway-related kinase and certain nuclear factors such as NF-kB of the dendritic cells after stimulation will be investigated. To clarify the effect of mite allergens on the function of the dendritic cells and the pathogenesis of the diseases, two different immune-mediated diseases including CpG motif (Th1) and allergic disease (Th2) will be used as the model to study the role of dendritic cells in the regulation of immune responses. We believe that the project here will provide a novel insight into understanding the regulation of mite allergens and also allergic diseases.

Background and Significance

It is well known that BM-DCs play a central role in the induction of immunity in T cells as well as B cells *in vivo* (Banchereau et al 1998). They not only express high level of the MHC-peptide complex, but also the other accessory molecules that interact with the receptors on the T cells to enhance adhesion and signalling (Flores-Romo 2001, Lane et al 1999, Rescigno et al 2001). In addition, immature BM-DCs can take up and present antigens so efficient that picomolar and

nanomolar concentrations of antigen suffice, much less than the micromolar levels typically employed by B cells or macrophages (Banchereau et al 1998; Banchereau et al 2000) Because BM-DCs are the most potent antigen-presenting cells (APCs) known, it can be applied as the therapeutic tools for many immunological diseases (Bell et al 1999, Cella et al 1997, Ingulli et al 1997; Knight et al 1998).

The development of immune response is suggested to play the major role in the pathogenesis of the diseases. It has been well documented that two different types of T helper cells exert the different effect in the defense mechanisms. Type 1 T helper (Th1) cells are important for cell-mediated immunity such as activation of cytotoxic T cells and natural killer cells which are critical for the clearance of viral infection, tumor immunity and transplantation. By contrast, type 2 T helper (Th2) cells play a critical role in eliciting allergic immune response and against parasite infestation. The recent data also suggested the existence of two different types of dendritic cells during the activation and development which could decide the types of immune response (Cella et al 1999). It has documented that certain stimuli such as lipopolysaccharide or CpG motif can stimulate a type 1 dendritic cells and subsequent type 1 T helper (Th1) response. By contrast, stimuli such as cholera toxin, parasite antigen or prostaglandin could direct a type 2 dendritic cells response and Th2 activity (Gagliardi et al 2000, Whelan et al 2000). Further, the data also dendritic cells isolated form mucosal Patcher's patch tended to be a type 2 dendritic cells which secrete much lower IL-12 activity (Huang et al 2000, Iwasaki et al 1999, Iwasaki et 2000, Williamson et al 1999). More and more evidences suggested that dendritic cells and the key cytokine such as IL-12 might play the most critical role in the development of immune responses (Schulz et al 2000, Steinman et al 2000, Viney et al 1998). Therefore, it becomes necessary to clarify the determinants in driving the development of dendritic cells (Tang et al 1999). The researchers suggested the phenotypes of the dendritic cells actually determine the development of the immune responses (Langenkamp et al 2000, Moser et al 2000). Certain stimuli such as tumor necrosis factor-脉 (TNF-脉), IL-1刍, bacterial or viral components (lipopolysaccharide, CpG DNA motif, dsRNA) can activate dendritic cells to express higher levels of accessory molecules and secrete more cytokines for the development of T cells (Patterson et al 2000, Palucka et al 1999a, Palucka et al 1999b).

It is documented that certain receptors such as toll-like receptor on the surface of dendritic cells might play the critical role in the activation of dendritic cells by bacterial or viral components. The expression of CD40 antigen has been found to play a critical role in the activation of dendritic cells and cytokine secretion (Cella et al 1996, Chen et al 1999, Goerdt et al 1998, Koch et al , Kooten et al 1997).In addition, different stimuli could activate different phenotypes of dendritic cells and affect the development of T helper cells. Certain TLRs such as TLR4 has been found to be the major receptor for LPS stimulation while dendritic cells encounter the pathogens (Brightbill et al 2000, Kaisho et al 2001, Muzio et al 2000). By contrast, TLR2 is important for the stimuli such as lipoproteins derived from Mycobacteria or certain Gram-positive bacteria. It has been documented that the stimulation of bacterial CpG motif is mediated via the involvement of TLR9. Several recent studies showed certain molecules such as myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinaase (IRAK) and tumor necrosis factor receptor-associated factor (TRAF) are important for upstream signaling pathway for the TLRs activation (Kaisho et al

2001, Wong et al 1999). Furthermore, downstream kinases and nuclear factors are also important for the signal transduction (Baeuerle et al 1996).

The frequency of allergic diseases such as asthma and allergic rhinitis has increased rapidly during the past decade, however, the exact mechanisms have still not been established. Both air pollution and change of diet habit have been thought to play an important role in increasing prevalence of atopic diseases (Hsieh et al 1988). Atopic diseases were mediated predominantly by type 2 T helper (Th2)-mediated activity including allergen-specific IgE antibody and eosinophils. It has been suggested dysregulation between Th1 and Th2 immunity might be the major problem among the modern population. Therefore, it is important to clarify the role of dendritic cells in the pathogenesis of the allergic diseases. After the establishment of the in vitro cultured dendritic cells, they have been extensively applied for the treatment of disease such as tumor and pathogenesis of disease (Inaba et al 1992, Inaba et al 1998, Gallucci et al 1999, Girolomoni et al 1997, Ludewig et al 1998, Melero et al 2000, Porgador et al 1996, Schuler et al 1997). However, only few reports on the role of dendritic cells in the pathogenesis of allergic diseases have been reported.

In this project, we plan to apply two different kinds of stimuli including Th1-related antigens (CpG DNA motif) and Th2-related antigens (mite allergens) for the activation of dendritic cells and characterization of the signal pathway. In addition, we plan to identify the receptors for these different antigens in order to further understand the initiation of immune responses in the disease models.

First year: *In vitro* culture and characterization of dendritic cells will be established for the activation of dendritic cells with various stimuli such as lipopolysaccharide, and CD40L. Antigens from two different disease models including Th1-related immune response (CpG DNA motif) and Th2-related immune response (mite allergens) will be used to study the activation signals such as intracellular cAMP activity and kinases involved in the activation of the dendritic cells.

四、 結果與討論

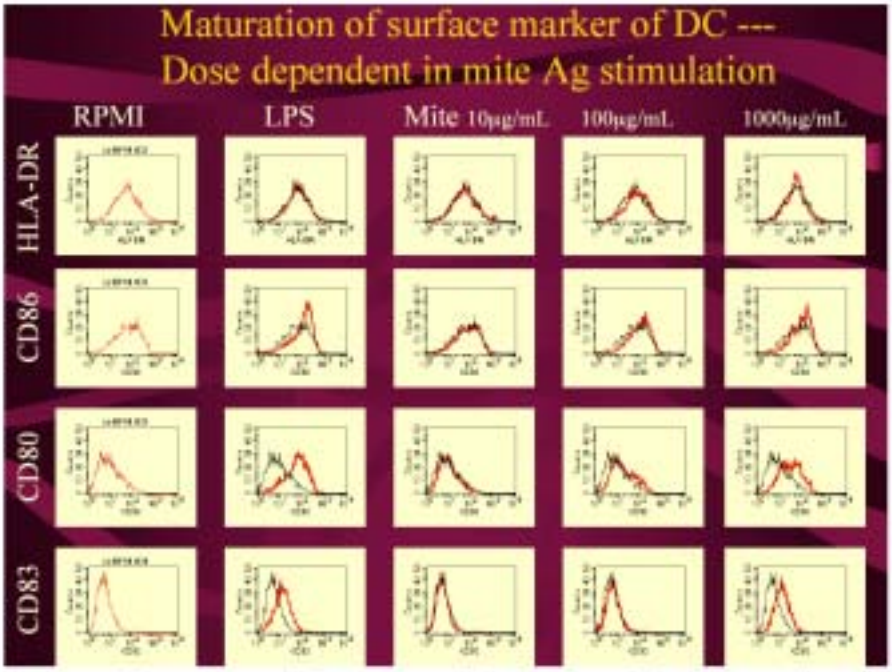
這是二年計畫中的第一年計畫，我們建立了樹突細胞培養，同時也利用 mite 來進行刺激，目前已經分析了相關的細胞激素分泌的情形。

1．我們對樹突細胞的刺激，利用 mite 來刺激後，再觀察其細胞表面標記的變化。其結果整理在(圖一)。同時，也利用樹突細胞來進行細胞吞噬的研究，發現受到活化後的樹突細胞的確其吞噬能力變得較差(圖二)。

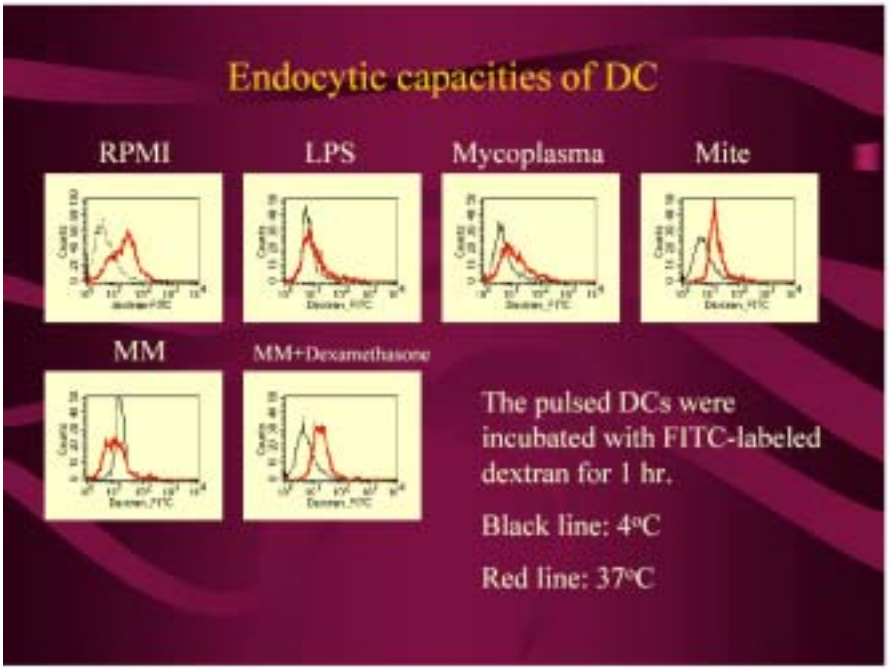
2．我們的研究顯示樹突細胞受到刺激後，可以分泌出 IL-12p40、IL-12p70 和 IL-10 等細胞激素(圖三)。同時，這些樹突細胞與這些 mite 過敏原培養，再與 T 細胞一道培養後會讓 T 細胞的增殖反應能力變得較差(圖四)。而且這些 T 細胞所分泌的細胞激素以第二型 T 輔助細胞的細胞激素要比 LPS 所刺激的 T 細胞要來得高。

3．至於為了建立樹突細胞的活化和訊息傳導，所以我們利用靈芝的分離成份來進行這部份的研究，我們已經加以完成，並且已經撰寫成論文投稿。

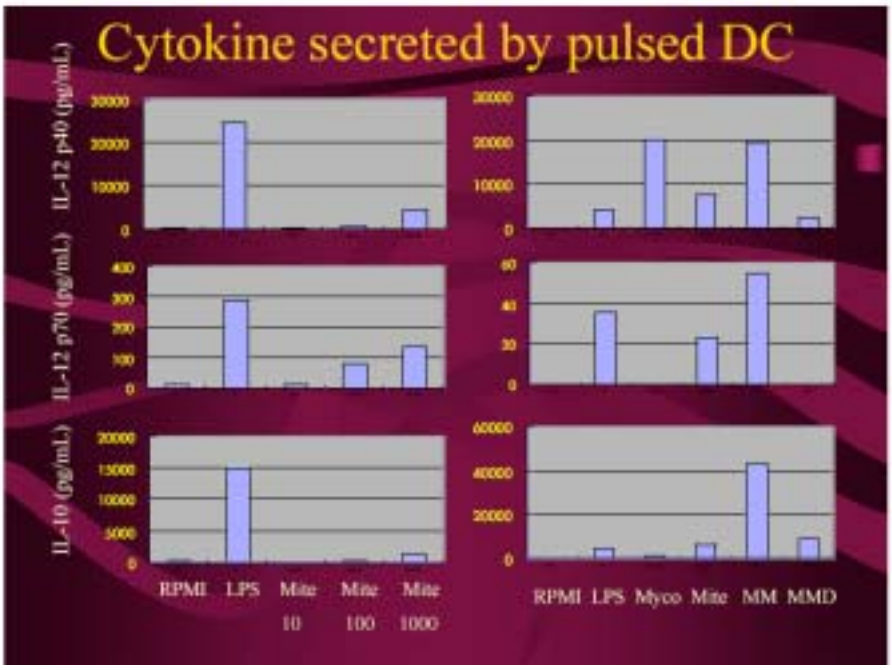
Lin YL et al: Polysaccharide purified from Ganoderma lucidum induced activation and maturation of human monocyte-derived dendritic cells by the NF-kB and p38 mitogen-activated protein kinase pathways. (Submitted)



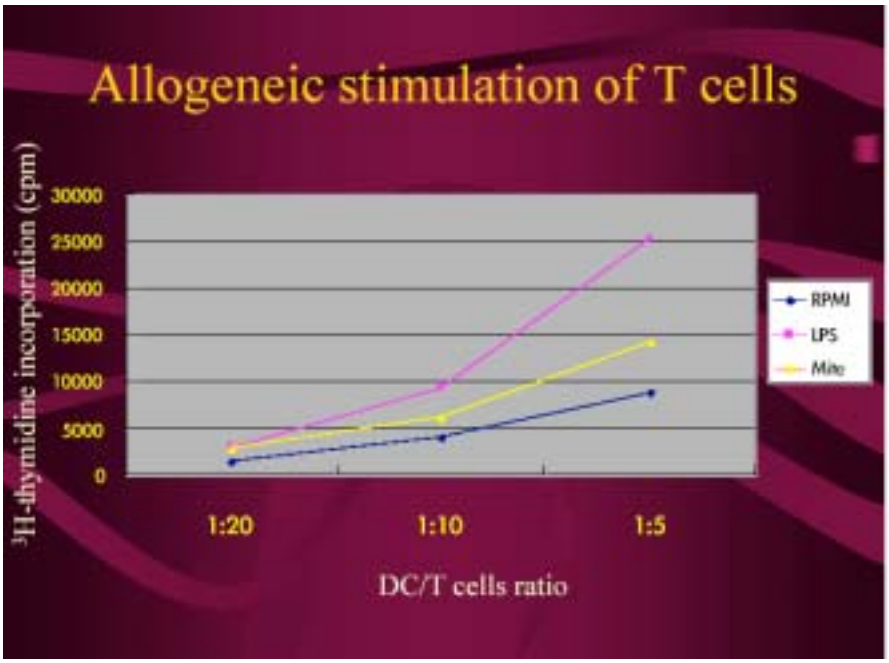
圖一．已經活化的 B 細胞(>45%)會有較高的細胞增殖反應。



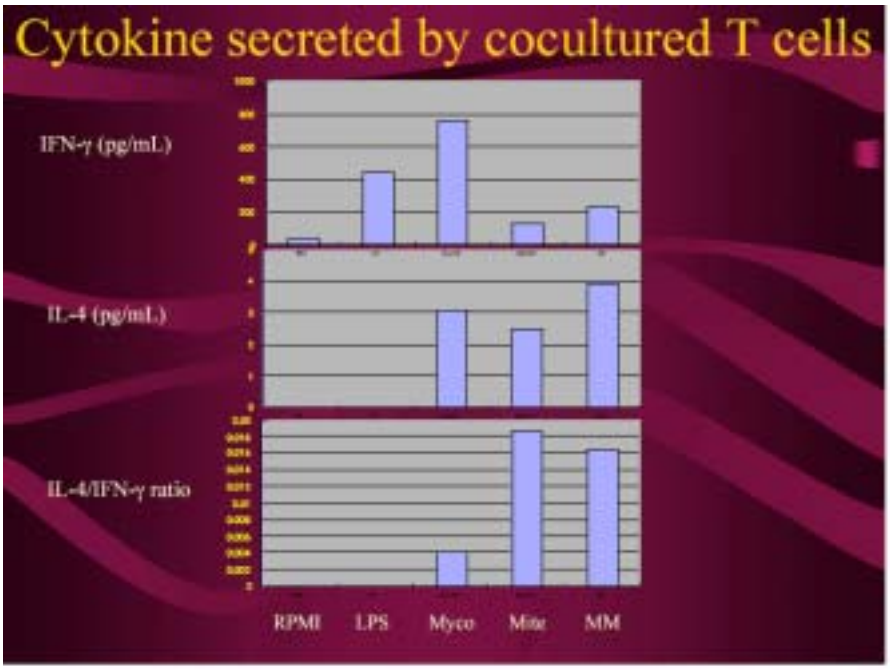
圖二．ML.lpr/lpr 小鼠會自發性地分泌較高量的 IgM 抗體，而此一現象在正常小鼠則不會出現。



圖三．已經受到活化的 B 細胞在 LPS 及自發的情形下都會分泌較高量的 IgM 抗體。



圖四



圖五

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

Yu-Li Lin[*], Yu-Chih Liang[†], Shiuh-Sheng Lee[‡], and Bor-Luen Chiang[*]

[*]Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, R.O.C.; [†]Graduate Institute of Biomedical Technology, College of Medicine, Taipei Medical University, Taipei, Taiwan, R.O.C.; and [‡]Department of Biochemistry, National Yang-Ming University, Taipei, Taiwan, R.O.C.

Please send all correspondence to:

Dr. Bor-Luen Chiang

Department of Pediatrics

National Taiwan University Hospital

No. 7, Chungshan South Road, Taipei, Taiwan, R.O.C.

TEL: 886-2-2312-3456 ext. 7299 or 7302

FAX: 886-2-2397-2031

E-mail: gicmbor@ha.mc.ntu.edu.tw

Running title: Polysaccharide from Ganoderma lucidum activation of dendritic cell

Keywords: Dendritic Cells, Ganoderma lucidum, NF-κB, p38 MAPK, Signal Transduction

Abstract

Ganoderma lucidum has been widely used to promote health and longevity in Chinese. The polysaccharide component with a branched (1→3)-β-D-glucan moiety from G. lucidum (PS-G) has been reported to exert anti-tumor activity and activation of natural killer cells. In this study, we investigated the effects of PS-G on human monocyte-derived dendritic cells (DC). Treatment of DC with PS-G results in enhanced cell surface expression of CD80, CD86, CD83, CD40, and HLA-DR, enhanced production of IL-12 p70, IL-12 p40, and IL-10, enhanced induction of T-cell stimulatory capacity, and enhanced T-cell secretion of IFN-γ and IL-10 in the culture supernatant, whereas the capacities for the endocytosis is suppressed in these cells. In analysis of IL-12 p35, IL-12 p40, and IL-10 mRNA expression by RT-PCR, we found that significantly higher levels of these mRNA were expressed in PS-G-treated DC. Further study showed that PS-G was able to augment IKK and nuclear factor-κB (NF-κB) activity, as determined by IKK kinase and gel mobility shift assay. PS-G was also able to promote IκBα and p38 mitogen-activated protein kinase (MAPK) phosphorylation. Inhibition of NF-κB by helenalin and p38 MAPK by SB98059 can prevent the effects of PS-G in the expression of CD80, CD86, CD83, CD40, and HLA-DR, and production of IL-12 p70, IL-12 p40, and IL-10 in various degrees. Taken together, our data demonstrate that PS-G can effectively and rapidly promote the activation and maturation of immature DC, suggesting that PS-G may possess a potential capacity in regulating immune responses.

Introduction

Ganoderma lucidum, a China herb, has been widely used in China and other Asian countries. G. lucidum has been reported to be effective in modulating immune functions, inhibiting tumor growth, and effective in the treatment of chronic hepatopathy, hypertension, and hyperglycemia (1). The polysaccharide from Ganoderma lucidum (PS-G) is a branched (1→3)-β-D-glucan moiety. Studies have demonstrated the antineoplastic action of Ganoderma lucidum and attributed it to the activated host immune response (2, 3). PS-G has been reported to enhance cytotoxic activity of natural killer cells and to increase tumor necrosis factor-α and interferon-γ release, respectively, from macrophages and lymphocytes (4, 5).

Dendritic cells (DC) are the most professional antigen presenting cells (APCs) whose primary function is to capture, process, and present Ags to unprimed T cells (6, 7). Immature DC reside in non-lymphoid tissues where they can capture and process Ags. Thereafter, DC migrate to the T cell areas of lymphoid organs where they lose Ag-processing activity and mature to become potent immunostimulatory cells (8). The induction of DC maturation is critical for the induction of Ag-specific T lymphocyte responses and may be essential for the development of human vaccines relying on T cell immunity. Fully mature DC show a high surface expression of MHC class II and costimulatory molecules (CD40, CD80 and CD86) but a decreased capacity to internalize Ags (9). Up-regulation of CD83, a specific marker for DC maturation, also occurs (10). Various stimuli, such as proinflammatory cytokines (eg. TNF-α and IL-1), CD40 ligation, bacterial products (eg. LPS and unmethylated DNA CpG motif), and contact sensitizers, can induce DC maturation in vivo and in vitro (11-14). Several reports have already indicated that nuclear transcription factor NF-κB plays an important role in DC maturation (15, 16). Another intracellular component involved in DC maturation, the three major mitogen-activated protein kinases (MAPK) signaling pathways in mammals, including the p38 MAPK, extracellular signal-regulated kinases (ERK), and the c-Jun N-terminal kinases (JNK), are activated in DC on maturation induced by LPS or TNF-α (17, 18).

The exact effects of PS-G on human DC are yet to be defined. In the present study, we first examined the molecular mechanisms of PS-G on activation and maturation of human monocyte-derived dendritic cells.

Materials and Methods

Reagents

Escherichia coli LPS (L8274, Sigma Chemical Co.) were purchased from Sigma Chemical Co. (St. Louis, MO). Isotopes were obtained from Amersham Corp. Helenalin, SB203580, PD98059, and JNF inhibitor II were purchased from Calbiochem (Germany). Treatment of immature DC with these inhibitors (helenalin, SB203580, PD98059, and JNF inhibitor II) before stimulation was performed for 60 min. These inhibitors were dissolved in DMSO, a 0.1% (v/v) concentration of DMSO was used as a negative control where indicated.

PS-G purification from G. lucidum

As in our previous study (2), fruiting bodies of G. lucidum were washed, disintegrated, and extracted with boiling water for 8-12 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

(Pharmacia, Upsala, Sweden) and was further purified by anion exchange chromatography with a column of diethylaminoethyl-cellulose (1). The PS-G was a protein-bound polysaccharide consisting of about 95% polysaccharide and 5% peptides. To rule out possible endotoxin lipopolysaccharide (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 PS-G samples.

Human DC generation

DC were generated from PBMC, as described previously (19, 20), with some modification. Briefly, PBMC were obtained from healthy donors by centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and the light density fraction from the 42.5-50% interface was recovered. $CD14^+$ cells were purified by positive selection using anti-$CD14^+$ microbeads in conjunction with the MiniMACS system by following the manufacturers instructions (Miltenyi Biotech, Auborn, CA). The $DC14^+$ cells were cultured at 1×10^6 cells per 1 ml cRPMI in 24-well plates (Costar, Cambridge, MA) with GM-CSF (800 U/ml) and IL-4 (500 U/ml). Fresh medium containing GM-CSF and IL-4 was added every 2 to 3 days. Human monocyte-derived DC were routinely used at day 6 of culture.

Determination of cytokine levels

The IL-12 p70, IL-12 p40, IL-10, and IFN-γ in culture supernatant from DC or T-cell were assayed with an ELISA kit (R&D) as per the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from DC using TRIzol reagent (Life Technologies) following the instructions of the manufacturer. Total RNA was converted to cDNA with Moloney-murine leukemia virus reverse transcriptase (Life Technologies) at 42°C for 1 h. The amplification of IL-12 p35, IL-12 p40, and IL-10 cDNA was performed by incubating equivalents of cDNA with Super Taq DNA polymerase. The IL-12 p35 primers used were forward primer 5'-GAGTCCCGGGAAAGTCCTGCC-3' and reverse primer 5'-TCTGGCCTTCTGGAGCATGTT-3'. The IL-12 p40 primers used were forward primer 5'-GGGGTGACGTGCGGAGCTGCT-3' and reverse primer 5'-TCTTGCCCTGGACCTGAACGC-3'. The IL-10 primers used were forward primer 5'-TTTCTCTTGGAGCTTATTAAAG-3' and reverse primer 5'-AAGACTTTCTTTCAAATGAAGG-3' (Invitrogen). The cDNA sequence of GAPDH was also amplified as a control in a similar way using the following primers: 5'-CTCATGACCACAGTCCATGC-3' and 5'-CCCTGTTGCTGTAGCCAAAT-3'; these primers produced a 450-bp product. A thermal cycle of 30 sec at 94°C, 30 sec at 52°C and 1 min at 72°C was used for 35 cycles for IL-12 p35 and IL-12 p40. A thermal cycle of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C was used for 35 cycles for IL-10.

Flow cytometric analysis

DC were harvested and washed with cold buffer (PBS containing 2% FCS and 0.1% sodium azide). Cells were then incubated in cold buffer. Subsequent stainings with mAbs or isotype-matched controls were performed for 30 min on ice. Stained cells were then washed twice and resuspended in cold buffer and analyzed with a FACSort cell analyzer (Becton-Dickinson). More than 1×10^4 cells were analyzed for each sample, and the results were processed by using Cellquest software (Becton-Dickinson).

FITC-labeled dextran uptake

Cultured DC were washed twice and resuspended in 1 ml of RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 25 mM HEPES. Cells were then incubated with FITC-labeled dextran (0.2 mg/ml) either at 4°C or 37°C for 1 h. Cells were washed thrice with cold buffer and analyzed with a FACSort cell analyzer, as described above.

Allogeneic mixed leukocyte reaction (MLR)

PBMC were obtained as described above, and nonadherent cells were passed through a nylon wool column. Thus, allogeneic T cells obtained were distributed at 1×10^5 cells per well and incubated for 5 days in the presence of graded numbers of irradiated DC (3000 rad, ^{137}Cs source). Tritiated thymidine (1 μCi/well; New England Nuclear, Boston, MA) incorporation for 6 h was determined with a liquid counter.

IκB kinase (IKK) assay.

The kinase assay was performed as described by Spiecker et al. with some modifications (21). Whole cell extract was lysed with Gold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris-HCl pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 30 min at 4°C. The cell lysate was clarified by centrifugation at 12,000g for 10 min at 4°C. Equal amounts of total cellular protein (100 μg) were immunoprecipitated with IKK1 and IKK2 specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A/G-PLUS agarose for 12 h at 4°C. Kinase assay was carried out in 45 μl of kinase buffer (40 mM Tris-NaOH pH 7.5, 500 mM NaCl, 0.1% NP-40, 6 mM EDTA, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM sodium orthovanadate, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1mM DTT) containing 5 μM cold ATP, 10 μCi [γ-^{32}P] ATP (5000Ci/mmol, Amersham), and 1 μg GST-IκBα fusion protein (Santa Cruz Biotechnology) as substrate, and incubated for 20 min at 25°C. Each sample was mixed with 8 μl of 5× Laemmli's loading buffer to stop the reaction, heated for 10 min at 100°C, and subjected to 10% SDS-PAGE. The gels were dried, visualized by autoradiography, and quantified by densitometry (IS-1000 Digital Imaging System).

Western blotting

Total cellular extract was prepared using Gold lysis buffer. Total protein (50 μg) was separated on 10% SDS-polyacrylamide minigels, and transferred to Immobilon PVDF membrane (Millipore). The membrane was incubated overnight at 4°C with 10% bovine serum albumin in PBS to block non-specific immunoglobulins, and then incubated with anti-IκB-P polyclonal, anti-α-tubulin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p38-P, anti-p42/44-P, anti-p46/54-P, anti-total p38 polyclonal antibody (Cell Signaling).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear and cytoplasmic extracts were prepared as previously described (22). At the end of the culture, the cells were suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 10 min on ice and vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12,000g for 20 sec. The supernatants containing cytosolic proteins

were collected. Pellets containing nuclei were resuspended in buffer C (20 mM HEPES, pH 7.6, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSE) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12,000g for 20 min and stored at -70°C. For EMSA, each 5 μg nuclear extract was mixed with the labeled double-stranded NF-κB oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and incubated at room temperature for 20 min. The incubation mixture included 1 μg of poly (dI-dC) in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl). The DNA-protein complex was electrophoresed on 4.5% non-denaturing polyacrylamide gels in 0.5× TBE buffer (0.0445 M Tris, 0.0445 M borate, 0.001 M EDTA). A double-stranded mutated oligonucleotide, 5′-AGTTGAGGCGACTTTCCCAGGC-3′, was used to examine the specificity of the binding of NF-κB to DNA. The specificity of binding was also examined by comparison with the unlabelled oligonucleotide.

Statistical analysis

Individual experimental values were compared by the student's t test.

Results

PS-G induces maturation of human monoctye-derived DC

LPS has been described as an inducer of DC activation and maturation (23). Therefore, we use LPS as a positive control in this study. To determine whether PS-G also can modulate the development of human DC *in vitro*, we compared the phenocyte of human DC treated with or without PS-G for 24 h. Our data demonstrated that PS-G increased the presentation of CD80, CD86, CD83, CD40, and MHC class II molecules on the cell membrane of human DC (Fig. 8).

PS-G induces IL-12 p70, IL-12 p40, and IL-10 production in human DC

IL-12 production is an important marker for DC maturation and can be used as a method for selecting Th1-inducing adjuvants. IL-10, a cytokine that inhibits inflammatory and cell-mediated immune responses (24), has enormous potential for the treatment of inflammatory and autoimmune disorders. To determine whether PS-G can affect the cytokine production in human DC, we compared the cytokine concentrations in the supernatants of DC cultured with different doses of PS-G. It is clear that PS-G enhanced the production of IL-12 p70, IL-12 p40, and IL-10 (Fig. 1A). When human DC were treated with 10 μg/ml of PS-G at 3, 6, 18, 24, and 48 h, we found that PS-G significantly enhanced the production of IL-12 p70, IL-12 p40, and IL-10 at 24 and 48 h, respectively (Fig. 1B). It was clear that the stimulatory effect of PS-G on IL-12 p70, IL-12 p40, and IL-10 production was dose- and time-dependent in manner. To determine whether PS-G could affect IL-12 p35, IL-12 p40, and IL-10 mRNA expression, human DC were activated with PS-G at indicated periods of time and assayed for IL-12 p35, IL-12 p40, and IL-10 mRNA expression by RT-PCR. We found that significantly higher levels of IL-12 p35, IL-12 p40, and IL-10 mRNA were expressed at 6, 6, and 18 h, respectively, in human DC, especially IL-12 p40 mRNA highly expressed (Fig. 1C). In unstimulated DC, there were no detectable IL-12 p35, IL-12 p40, and IL-10 mRNA.

PS-G downregulation of endocytotic activity of human DC

Immature DC capture and process Ags via their high endocytic capacity, and they lose their endocytic/processing activities of Ags and mature into potent immunostimulatory APCs during differentiation (25). The uptake of FITC-dextran is known to be maximal in the immature monocyte-derived DC and occurs by a combination of macropinocytosis and binding to the mannose receptor. Previous studies have shown that the endocytic capacity of DC are suppressed by LPS during their maturation process. Thus, we tested whether PS-G affected the uptake of FITC-labeled dextran by human DC. In our study, we demonstrated a reduction in FITC-dextran uptake when human DC were matured with PS-G (Fig. 2).

Enhancement of T cell activation by PS-G-treated human DC

Mature DC have the capacity to induce proliferation in allogeneic T cells at a much higher level than immature DC (25). In human DC, we found that PS-G up-regulates cell surface markers, increases IL-12 production, and induces activation of NF-κB. To test whether this maturation is sufficient to promote activation of naive T cells, DC were treated with LPS or PS-G. These cells were then used to activate allogeneic naive T cells. The results presented in Fig. 3A show that PS-G-treated DC enhanced T cell activation as evidenced by the secretion of IFN-γ in the culture supernatant (Fig. 3B). The IFN-γ production induced under these experimental conditions was similar to that seen following LPS treatment of DC. Interestingly, we demonstrated that PS-G-treated DC enhanced T cells secretion of IL-10, except for LPS-treated DC that were not able to enhance T cells secretion of IL-10 in the supernatant (Fig. 3C). In the LPS group or PS-G-treated group, DC could not induce T cells secretion of IL-2 and IL-4 cytokine (data not shown).

PS-G induces IKK activity and phosphorylation of IκBα in human DC

Since the activation of IKK activity is necessary for IκBα phosphorylation, the effect of PS-G on IKK activity was studied. Human DC were treated with PS-G (10 μg/ml) for the indicated periods of time. In order to directly measure IKK1 activity in human DC, IKK1 and IKK2 proteins were immuno-precipitated from cell extracts, and the kinase activity in the immunocomplex was assayed using recombinant GST-IκBα (1-317) as a substrate. Figure 4A illustrates the relative effect on IKK activity. After stimulation with PS-G, the GST-IκBα fusion protein was strongly phosphorylated at 30 min, indicating the stimulation of IKK activity in human DC.

NF-κB is one molecular family whose activation is associated with DC maturation. NF-κB normally binds to IκBα, which impedes NF-κB nuclear translocation from the cytoplasm to the nucleus. Once cells are exposed to inflammatory stimuli, including LPS and TNF-α, IκBα is phosphorylated, leading to IκBα degradation and nuclear translocation of NF-κB. We thus examined whether PS-G had any affect on IκBα phosphorylation. The cytoplasmic levels of IκBα-P protein were examined by Western blot analysis. Both LPS and PS-G induced the phosphorylation of IκBα. After 60 min activation of human DC with PS-G, the cytosolic IκBα protein was significantly phosphorylated (Fig. 4B).

PS-G induces NF-κB activation

DC maturation derived by LPS has been clearly associated with NF-κB activation. To determine whether PS-G uses similar activation pathways, we monitored its ability to activate NF-κB translocation into the nucleus. DC were cultured in the presence of PS-G for 2 h and nuclear extracts were analyzed for NF-κB binding by the electrophoretic mobility shift assay. As shown in Figure 5, PS-G was able to induce NF-κB translocation and activation. Identical results were obtained after treatment of DC with LPS. The binding of NF-κB was specific and could be blocked by unlabelled competing NF-κB oligonucleotide.

PS-G induce phosphorylation of members of the three families of MAPK in human DC

MAPK is a serine and threonine protein kinase whose activities are up regulated through tyrosine and threonine residue phosphorylation by its upstream regulators (26-29). We further characterized the MAPK activation pathways involved in PS-G signaling. This experiment focused on p38 MAPK, p42/44 ERK, and p46/54 JNK. Human DC were unstimulated or stimulated with PS-G, and the level of MAPKs phosphorylations were assessed by Western blotting with respective anti-tyrosine phosphorylated MAPKs mAb, and total-p38 mAb was used for internal control. Results presented in Figure 6 show that PS-G induced the phosphorylation of all MAPKs tested, especially inducing a higher p38 phosphorylation at 30 min. The total amount of p38 was unchanged following stimulation.

Inhibition of NF-κB and MAPKs prevents the maturation changes induced by PS-G

PS-G-treated DC produced many cytokines, including IL-12 p70, IL-12 p40, and IL-10, during maturation (Fig. 1A and 1B). We wondered whether PS-G induced secretion of IL-12 p70, IL-12 p40, and IL-10 were affected by the inhibitors of NF-κB, p38 MAPK, p42/44 ERK, and p46/54 JNK. Immature human DC were pretreated with helenalin (a specific blocker of NF-κB), SB203580 (a specific blocker of p38 MAPK), PD98059 (an inhibitor of the ERK pathway), or JNK inhibitor II (an inhibitor of the JNK pathway) for 1 h and subsequently stimulated with PS-G for 24 h. The production of IL-12 p70, IL-12 p40, and IL-10 were quantified by means of ELISA. PS-G induced significant production of IL-12 p70, IL-12 p40, and IL-10, and these cytokine productions were abrogated significantly by helenalin and SB 203580 (Fig. 7). In contrast, both PD98059 and JNK inhibitor II also down-regulated IL-12 p70 and IL-10 production, only having little effect on the inhibition of IL-12 p40 production induced by PS-G.

To further examine the involvement of NF-κB, p38 MAPK, ERK, and JNK in the PS-G induced expression of costimulatory and antigen-presenting molecules, NF-κB inhibitor, helenalin, p38 MAPK inhibitor, SB203580, ERK pathway inhibitor, PD98059, and JNK pathway inhibitor, JNK inhibitor II in the expression of costimulatory and adhesion molecules as well as HLA-DR was investigated. Blocking the NF-κB pathway with helenalin significantly inhibited the PS-G induced up-regulation of CD80, DC86, CD83, CD40, and HLA-DR (Fig. 8). In contrast, blocking the p38 MAPK and JNK pathway with SB203580 and JNK inhibitor II, respectively, had little effect on CD80, CD86, CD83, and HLA-DR expression. Using PD98059, a specific inhibitor of ERK, had no effect on these costimulatory molecules and MHC class II expression. These results show that certain features of human monocyte derived-DC maturation are regulated

by signaling via NF-κB and p38 MAPK and imply that different aspects of the maturation process induced by PS-G may be regulated by distinct signal transduction pathways.

Discussion

The biological process of DC maturation represents a crucial step in the initiation of adaptive immune response (30). This process is regulated by various extracellular stimuli, including cytokines, bacterial products, and membrane-bound ligands (10, 31, 32). DC maturation is accompanied by changes of their morphological, phenotypic, and functional properties (24). Recently, several researchers have demonstrated the immunomodulatory effects of polysaccharide purified from *Ganoderma lucidum* on T lymphocytes (33). However, little is known about the molecular mechanisms responsible for the regulation of DC in their activation and maturation states by PS-G. In this study, we were the first to demonstrate the PS-G induced morphological, phenotypical, and functional changes in human monocyte-derived DC. PS-G promoted the maturation of DC, while mature DC demonstrated characteristic morphology, with enlarged size and numerous cytoplasmic processes giving rise to a stellate appearance (data not shown). Maturation of DC is characterized by a decreased antigen processing capacity, an increased cell surface expression of MHC class II molecule and co-stimulatory molecules CD80, CD86, and CD40, and secretion of IL-12, priming strong stimulation of T lymphocytes growth and differentiation. The CD83 marker for mature human DC was also increased.

In immune responses, IL-12 plays a central role as a link between the innate and adaptive immune systems (34). Thus, IL-12 induces and promotes natural killer (NK) and T cells to generate interferon (IFN)-γ and lytic activity. In addition, IL-12 polarizes the immune system toward a primary T-helper cell (Th)-1 response. In this study, we found that PS-G can induce IL-10 and IL-12 production in human DC. IL-10 is a pleiotropic cytokine produced by DC, T cells, and macrophages and possesses both anti-inflammatory and immunosuppressive properties (23). In Figure 1B and 1C, showing the time course of IL-12 and IL-10 protein production and mRNA expression levels, the data shows that PS-G induced first IL-12 and then IL-10 production. This may be important in keeping homeostasis. Thus, our results imply that IL-12 and IL-10 reciprocally controlled activation and maturation states of DC *in vivo*.

IFN-γ and IL-10 cytokines were induced in MLR by PS-G-treated human DC. In contrast to PS-G, only IFN-γ cytokine was induced in MLR by LPS-treated human DC. Therefore, LPS was described as a Th1 inducer. Our experimental data show that under some conditions PS-G can induce a Th1 differentiation or promote the differentiation of naïve T cells into T-regulatory cells. However, although DC are widely regarded as the most potent antigen-presenting cells, recent evidence also indicates that DC play an important role in inducing immune tolerance (35) and regulating Th1/Th2 immunity balance (36). Furthermore, these immature DC may also alternatively be activated and induced to exert suppressive effects (37).

Recent reports show that NF-κB is responsible for LPS-induced DC maturation in an *in vitro* murine model (38), and that cytokine-induced maturation of human DC results in increased NF-κB nuclear translocation (39). Many proinflammatory cytokines display NF-κB–responsive elements in their promoters, conferring a major role on immune responses (40, 41). Moreover, the p38 MAPK pathway has been shown to contribute to NF-κB–mediated transactivation (42-44).

Little is known about the signal transduction pathways involved in the maturation of human monocyte-derived DC by PS-G. We demonstrated that the NF-κB pathway, the p38 MAPK, the ERK1/2, and the p46/54 JNK pathways are all activated when immature human DC are exposed to PS-G, suggesting a role of these pathways in the maturation process. The promoters of hIL-12 p35 and hIL-12 p40 gene contain κB binding sites (45). It likely that NF-κB is involved in IL-12 p35 and IL-12 p40 expression. The lack of κB binding sites in the hIL-10 promoter makes it unlikely that NF-κB is involved in IL-10 regulation (46). Recently, it has been suggested that p38 MAPK is involved in the regulation of IL-10 production (47).

Early phosphorylation of p38 MAPK, ERK1/2, and p46/54 JNK were investigated in PS-G-treated human monocyte-derived DC. Our results corroborate recent reports using murine models, as well as human DC *in vitro* models showing activation of all three MAPKs pathways during maturation (38, 48, 49). The availability of specific inhibitory drugs for the NF-κB, p38 MAPK, ERK, and JNK pathways prompted us to investigate the respective roles of the NF-κB and these MAPKs in DC maturation. In cytokines analysis, pretreatment of both helenalin and SB203580 significantly inhibited the IL-12 p70, IL-12 p40, and IL-10 production in PS-G-treated human DC. In contrast, PD98059 and JNK inhibitor II were shown to inhibit IL-12 p70 and IL-10 production, although we only observed a little inhibitory effect of these compounds in the up-regulation of IL-12 p40 in the process of DC maturation triggered by PS-G. Concerning costimulatroy molecules and MHC class II expression, helenalin pretreated human DC were able to completely suppress these molecules expression induced by PS-G. The inhibition of the p38 MAPK and p46/54 JNK by SB 203580 and JNK inhibitor II, respectively, before PS-G stimulation had a weak effect on the CD80, CD86, CD83, and MHC class II expression but no effect on the CD40 expression induced during DC maturation. Using PD98059 had no effect on costimulatory molecules and MHC class II expression in the process of DC maturation triggered by PS-G. Moreover, the inhibitory effects of these inhibitors were not due to nonspecific toxicity, because the viability of DC were not modified by these inhibitors (data not shown). Collectively, these results show that the NF-κB and p38 MAPK pathways play the critical roles in the initiation of DC maturation. The human CD86 promoter has been recently cloned and two canonical NF-κB binding sites have been revealed (50). One of them is essential for Th-induced CD86 gene transcription. Moreover, NF-κB activation has been previously shown to drive CD 80 transcription (51, 52). A recent report describes the generation of MKK3-deficient mice to study the role of the p38 MAPK pathway *in vivo* (53). Using this animal model, the authors showed that p38 MAPK is required for the production of IL-12 in macrophages and DC. It appears that different aspects of DC maturation are regulated by different signal transduction pathways.

In conclusion, we demonstrated that PS-G can effectively and rapidly induce activation and maturation of human DC significantly by the NF-κB and p38 MAPK pathways. Therefore, PS-G is a good remedy to regulate host immune responses.

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Footnotes:

[1] This study was supported by the National Science Council NSC 92-2314-B-002-201.

[2] Abbreviations used in this paper: DC, dendritic cells; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; RT-PCR, reverse transcriptase-polymerase chain reaction.

Figure legends:

FIGURE 1. PS-G induces IL-12 p70, IL-12 p 35, and IL-10 production in human monocyte-derived DC. (A) Human DC were cultured for 24 h in the presence of 100 ng/ml of LPS or various concentrations of PS-G. At the end of the incubation time, the culture medium was collected for cytokine assay by ELISA. (B) Human DC were incubated with PS-G (10 μg/ml) for the indicated time of period. At the end of the incubation time, IL-12 p70, IL-12 p 35, and IL-10 production was subsequently analyzed by ELISA. Each data represents the mean ± SE for three determinations. (C) RT-PCR analysis of mRNA expression of IL-12 p35, IL-12 p40, and IL-10. DC were incubated in the presence of PS-G (10 μg/ml) for 3, 6, 18, and 24 h. This experiment was repeated three times with similar results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIGURE 2. PS-G on the endocytotic capacities of human DC. At day 6, immature DC were stimulated with medium alone, LPS (100 ng/ml) or PS-G (10 μg/ml) for 24 h, and cells were then incubated with FITC-dextran for 1 h at 4°C (dotted lines) or 37°C (solid lines). This experiment was repeated three times with similar results.

FIGURE 3. PS-G enhances T cells response. (A) Immature DC were stimulated with LPS or PS-G for 24 h. Allogeneic T-cells proliferation was measured after 5 days of coculture with DC. These data are means ± SEM of triplicates and representative of three independent experiments. Supernatants were analyzed for (B) IFN-γ and (C) IL-10 produced by activated T cells after 2 days of culture.

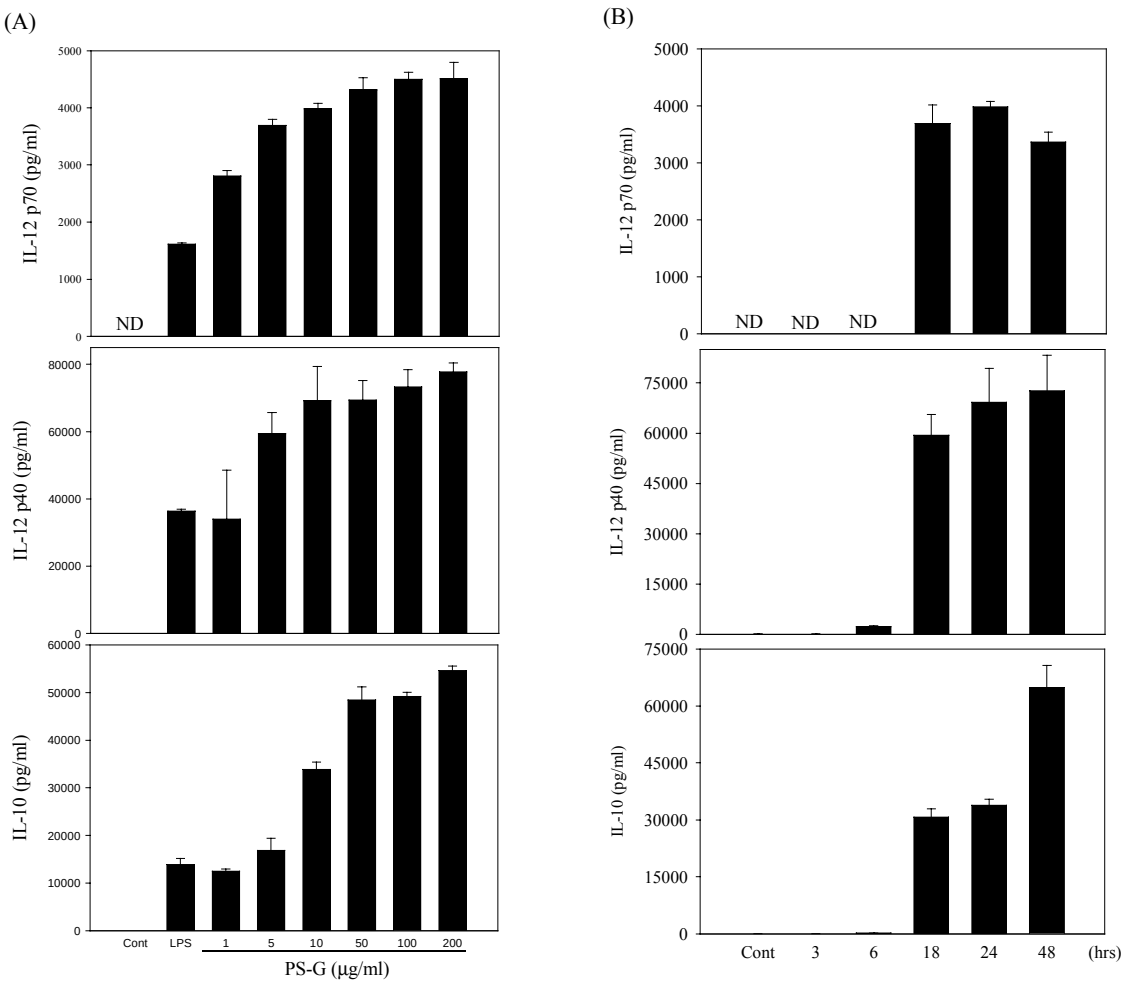
FIGURE 4. PS-G induced IKK activity and IκBα phosphorylation in human DC. (A) Human DC were treated with PS-G (10 μg/ml) for the indicated time periods. IKK was immunoprecipitated using the anti-IKK1 and IKK2 antibody, and the activity in the immune complexes were assayed using GST-IκBα as a substrate. (B) Human monocyte-derived DC were treated with LPS (100 μg/ml) for 45 min or PS-G (10 μg/ml) for the indicated time periods. Cytosolic fractions were prepared and analyzed for the content of IκB-P protein by Western blotting. The lower panel shows the blot probed for α–tubulin to demonstrate equal loading of samples. This experiment was repeated three times with similar results.

FIGURE 5. PS-G induces NF-κB activation. Human monocyte-derived DC were treated with LPS (100 ng/ml) or PS-G (10 μg/ml) for 2 h or remained unstimulated, and. nuclear fractions were prepared and analyzed for NF-κB binding activity by electrophoretic mobility shift assay. To assess the specificity of the binding, 100-fold excess of cold NF-κB probe or irrelevant probe was added to the LPS condition. This experiment was repeated three times with similar results.

FIGURE 6. PS-G induces the phosphorylation of p38 MAPK, p42/44 ERK, and p46/52 JNK kinase. Human monocyte-derived DC were treated with PS-G (10 μg/ml) inhibitors for the indicated time periods, then collected the cell lysate, and the level of MAPKs phosphorylations were assessed by Western blotting with respective anti-tyrosine phosphorylated MAPKs mAb, and total-p38 mAb was for internal control.

FIGURE 7. The effect of inhibiting the NF-κB, p38 MAPK, ERK1/2, or JNK pathways on the PS-G-induced up-regulation of IL-12 p70, IL-12 p40, and IL-10 production in human monocyte-derived DC. Human DC were pretreated with 0.1% DMSO, 10 μM of helenalin (a specific blocker of NF-κB), 20 μM of SB203580 (a specific blocker of p38 MAPK), 50 μM of PD98059 (an inhibitor of the ERK1/2 pathway), or 20 μM of JNK inhibitor II (an inhibitor of the JNK pathway) for 1 h, and then incubated with 10 μg/ml of PS-G for 24 h. At the end of the incubation time, the supernatant was collected for IL-12 p70, IL-12 p 35, and IL-10 production by ELISA.

FIGURE 8. The effect of inhibiting the NF-κB, p38 MAPK, ERK1/2, or JNK pathways on the PS-G-induced up-regulation of CD 80, CD 86, CD 83, CD 40, and HLA-DR in human monocyte-derived DC. Day 6 immature DC were pretreated with 0.1% DMSO, 10 μM of helenalin (a specific blocker of NF-κB), 20 μM of SB203580 (a specific blocker of p38 MAPK), 50 μM of PD98059 (an inhibitor of the ERK1/2 pathway), or 20 μM of JNK inhibitor II (an inhibitor of the JNK pathway) for 1 h before the addition of PS-G (10 μg/ml) for 24 h. The cell surface expression of CD 80, CD 86, CD 83, CD 40, and HLA-DR was then measured using the flow cytometry (dotted line, isotype control; solid line, specific mAbs). The values shown in the flow cytometry prolifes are the mean fluorescence intensity indexes. These results are representative of three independent experiments with similar results.



(C)

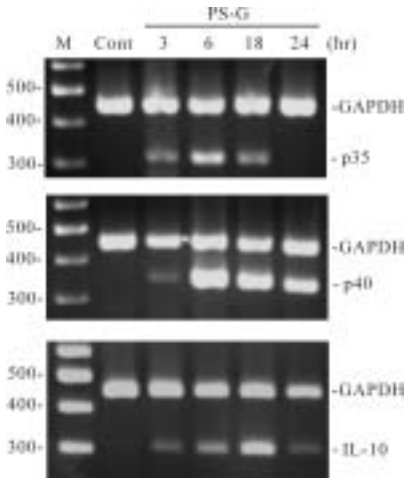


FIGURE 1. (A), (B), and (C)

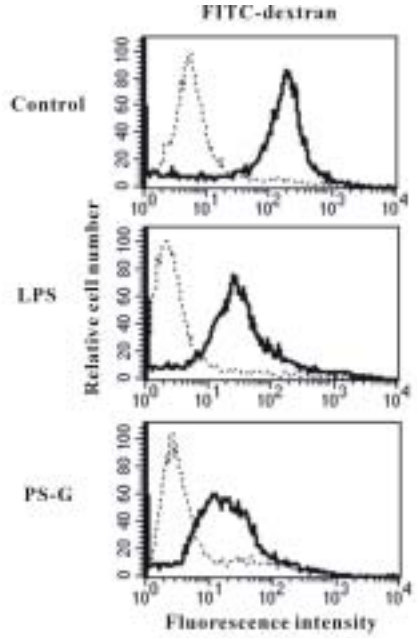
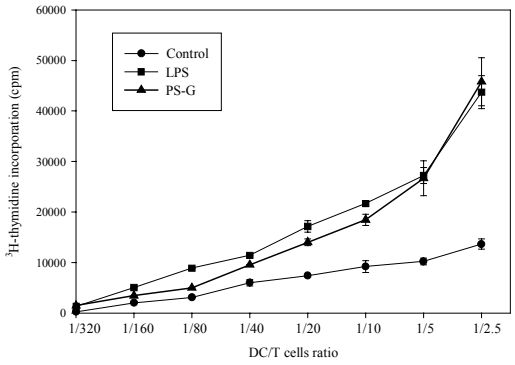


FIGURE 2.

(A)



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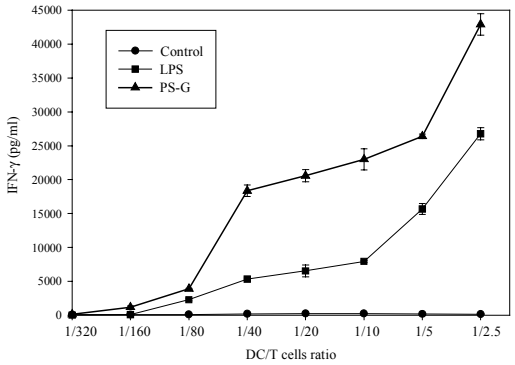

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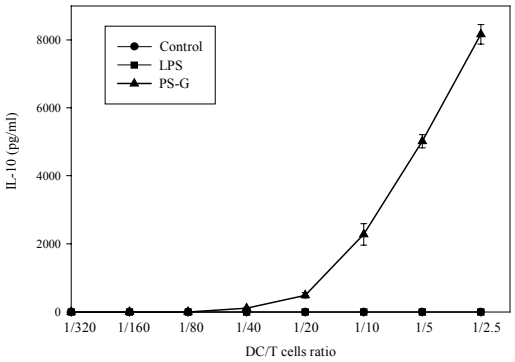
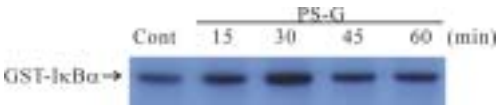


FIGURE 3. (A), (B), and (C)

(A)



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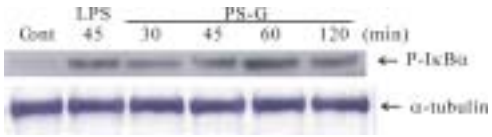

FIGURE 4 (A) and (B).

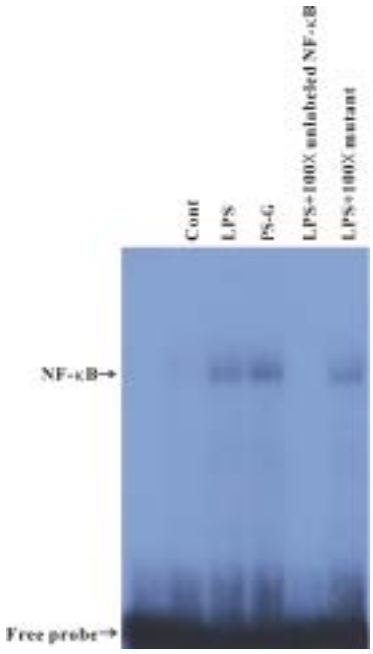

FIGURE 5.

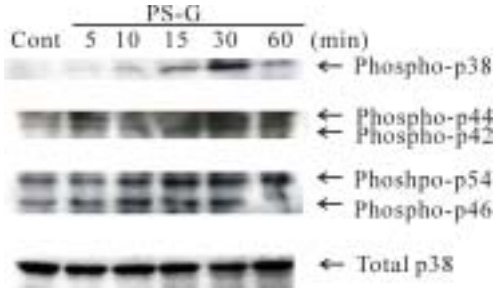

FIGURE 6.

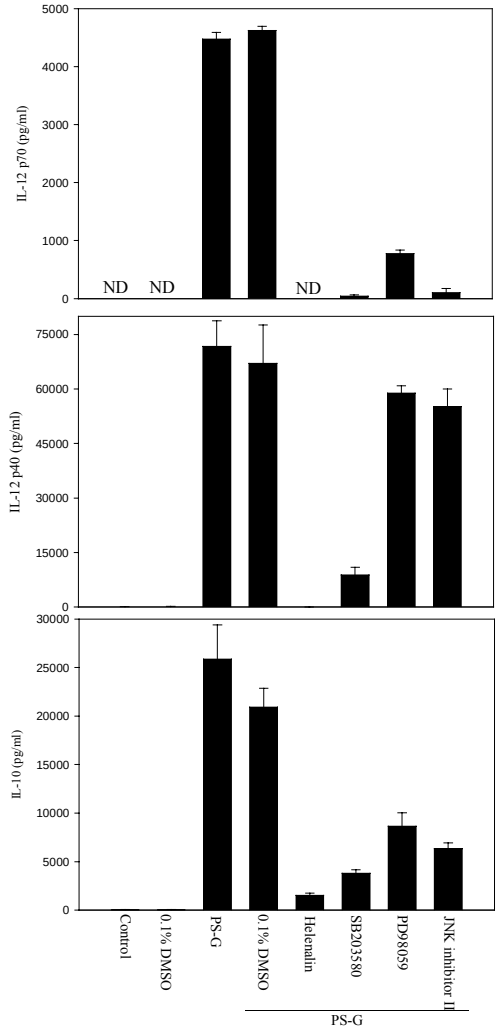

FIGURE 7.

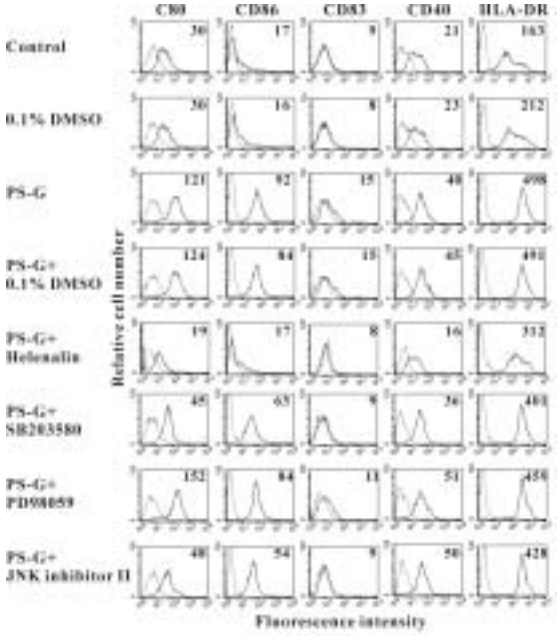
CD80
CD86
CD83
CD40
HLA-DR
Control
0.1% DMSO
PS-G
PS-G+ 0.1% DMSO
PS-G+ Helenalin
PS-G+ SB203580
PS-G+ PD98059
PS-G+ JNK inhibitor II
Relative cell number
Fluorescence intensity

FIGURE 8.