行政院國家科學委員會專題研究計畫　成果報告

過敏原對樹突細胞活化及訊息傳導的影響(2/2)

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計畫名稱：過敏原對樹突細胞活化及訊息傳導的影響

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中文摘要

關鍵詞：樹突細胞、訊息傳導、過敏原

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。我們在這段期間分別建立了試管內模式，能夠利用樹突細胞來評估各種不同刺激對樹突細胞的影響。結果我們發現樹突細胞的試管內表現的確也可以表現出不同的細胞表面標記和細胞激素，如果 IL-12 的表現量較高則會讓免疫反應往第一型免疫反應的方向發展。我們也成功地利用由靈芝衍生來的多醣研究樹突細胞的訊息傳導途徑，得到相當不錯的研究成果，已經發表在 Journal of Leukocyte Biology。

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. 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. In this project, we have established an in vitro assay for the assessment of allergen, lipopolysaccharide and CpG motif on the function of dendritic cells. Further, we also like to correlate the phenotypes of dendritic cells and in vivo function of these molecules. Finally, we have also established the method for the assay of signal pathway of dendritic cell activation. We believe the information here will provide a very novel system in studying the mechanisms of allergic diseases, also other immunological diseases.

Key words: dendritic cells, signal pathway, mite allergens

前言

此一研究計畫主要是要建立一個模式來研究是否可以建立一個模式來評估過敏原對樹突細胞的影響，再了解過敏原引起特定的第二型 T 輔助細胞的機制。在過去一段時間，我們已經分別建立了樹突細胞的培養，並利用各種不同的刺激來了解是否可以來經由樹突細胞的表面分子表現和細胞激素的製造來達到評估免疫反應的目的。我們在過去一段時間利用了由細菌分離出來的多醣、靈芝和過敏原 mite 來進行這方面的研究，並進一步來研究樹突細胞的訊息傳導途徑，這部份一直是較少看到相關的研究。而樹突細胞被認為在整個免疫反應中扮演了一個最重要的角色，所以如果我們能夠有效地將樹突細胞培養出，再評估其細胞表面分子和細胞激素的分泌情形，便可以再進一步到體內來評估是否能夠誘發相關的免疫反應，就可以將試管內的樹突細胞表現與體內的免疫反應來加以分析。同時，有關樹突細胞的訊息傳導研究也是這幾年來才逐步有較多的研究，所以進一步研究這些不同刺激的訊息傳導，也將是一個有興趣的課題。

研究目的

此一研究計畫的主要目的是要建立一個試管內樹突細胞培養的方式來評估不同刺激對樹突細胞的影響，尤其是我們將同時評估在過敏反應中較重要的一些刺激，如過敏原、CpG motif 和相關的多醣。

文獻探討

而在抗原呈現細胞中，樹突細胞(dendritic cells, DC)又被稱為專業的抗原呈現細胞，且被認為是控制免疫反應極為關鍵的細胞。這幾年由於樹突細胞的培養愈來愈方便，所以有相當的研究及治療可以利用培養的樹突細胞來達到。近年來的研究顯示，樹突細胞在誘發與調控免疫反應上扮演重要角色。而且，來源不同的兩種樹突細胞 - myeloid 樹突細胞和 lymphoid 樹突細胞，對於 T 細胞的的發育會導致完全不同的影響。樹突細胞被認為是體內負責加工並呈獻抗原最重要的抗原呈獻細胞。目前已證明樹突細胞加入 GM-CSF、TNF- 和 IL-4 等細胞激素後便可成功的在活體外培養。有許多實驗已驗證活體外培養的樹突細胞在許多模式中都可以誘發第一類和第二類 MHC 分子限制的免疫反應。以往的觀念中，將 T 細胞的活化分成兩訊息傳導。最近的研究顯示樹突細胞在 T 細胞的活化上更扮演了一個主導 T 細胞發育的角色，所以抗原在與樹突細胞接觸時便已經決定了 T 細胞的功能。舉例來說，CpG motif 會刺激第一型 T 輔助細胞的發生，主要是因為 CpG motif 會刺激樹突細胞分泌較高的 IL-12 而幫助 T 細胞的發育。所以如果利用樹突細胞來進行這些過敏原的研究，便可以了解過敏原本身的特性。

所以我們利用此一樹突細胞的模式來進行研究，並進一步來探討是否過敏原和相關的環境因子，或是治療開發的製劑上會對樹突細胞有特定的影響，而達到我們研究的目的。同時，對樹突細胞的訊息傳導目前的研究也正是方興未瑧，所以如果建立相關的方法在未來的研究上應該會有相當大的助益。

也因此，我們能夠利用這些培養的樹突細胞來進行相關的研究。利用樹突細胞當成一個技術平台來評估各種抗原，能夠應用在促進免疫功能，進而可以用在腫瘤的免疫治療上。舉例而言，一個特定的製劑可刺激樹突細胞分泌細胞激素如跟第一型 T 輔助細胞相關的 IL-12，或是增加與免疫反應相關的輔助分子

(accessory molecules)，如第二型 MHC 分子和 B7.1 分子，也可能會將增進免疫反應，而達到治療過敏疾病的效果。如此，可以利用目前已經相當成熟的樹突細胞培養，來檢視一些相關的過敏原和分子在過敏疾病中所扮演的角色。

研究方法

動物在此一研究計畫中所使用的小鼠主要是雌性的Balb/c小鼠，是來自台大醫學院的動物中心。

骨髓-衍生的樹突細胞培養

為了要培養骨髓-衍生的樹突細胞，我們將小鼠的腿骨兩端剪開後以細胞培養液將其中的幹細胞沖洗出。將大約每 ml 培養液有 106 細胞的濃度加入細胞激素 GM-CSF (500 U/ml) and IL-4 (1000 U/ml) (Pepro Tech Inc., Rocky Hill, NJ)來一道培養。培養基為 RPMI-1640 medium supplemented with 5% heat-inactivated FCS, 4 mM L-glutamine, 25 mM Hepes (pH 7.2), 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin. 每隔一天，我們便會加入有 GM-CSF and IL-4 的新培養基. 在培養的第八天時將這些骨髓衍生的樹突細胞(BM-DC)收集後進一步測定其 MHC class II, B7-1, B7-2, CD11c and 33D1 的表現。這些 BM-DCs 也會被進一步測定其刺激 T 細胞的能力。

以不同刺激來刺激樹突細胞

為了進一步了解是否可以利用樹突細胞來評估不同刺激在試管內的情形，和這些表現與體內免疫反應的相關性，我們將分別利用過敏原、CpG motif 和與免疫反應相關的一些多醣。我們將前面培養出來的樹突細胞與

樹突細胞

為了進一步分析樹突細胞的表面標記，我們分別利用認識 MHC class II, Mac-1, CD11c, B7-1, B7-2, CD40 and OX40L 的抗體來進行細胞表面標記的染色。樹突細胞將置於 0.5 ml of PBS with 0.1% sodium azide 中來進行螢光流體計數儀的分析。我們將分別在螢光流體計數儀上分析 10,000 顆細胞，分別利用適當的軟體來加以分析 (FACScan, Becton Dickinson, Mountain View, CA). 對照組則是細胞不加入這些特定的抗體。

細胞激素的分泌情形

我們將樹突細胞與這些不同的刺激培養後，分別在 24, 48 and 72 小時後將培養的上清液取出， 將分別利用 Quantikine M ELISA Kit (R&D Systems, MN, USA)來測定如 IL-10 和 IL-12 的分泌情形。同時，在測定樹突細胞刺激 T 細胞進行細胞增殖的同時，我們也將進一步分析這些 T 細胞分泌細胞激素的情形，所以將分別測定這些細胞分泌如 IFN-g 和 IL-2 的濃度。

樹突細胞激媒的活性分析

樹突細胞受到刺激後，將分別收集細胞來分析其 ERK, JNK and MAP kinase 的活性。我們會將樹突細胞以不同刺激一道培養後，收取其 200μg 溶解物質加入 1μg of anti-ERK, anti-JNK1 or 1μg of anti-p38a 的抗體來處理，再利用 20μl of protein G-Sepharose 來處理。而這些酵素的受質分別為 myelin basic protein 用在 ERK assay, GST-c-Jun1-7 用在 JNK assay, GST-ATF-21-109 用在 p38 MAPK assay. 這些作用的免疫複合物將進一步加以跑膠，再利用 densitormeter 來測定其活性。

NF-kB 的活性分析

樹突細胞培養後將利用 Lipofectamine (Promega)將相關帶有reporter的質體送入細胞內。質體, 3xkB-L (kindly provided by Dr. Nakano, Juntendo University), 帶有 NF-kB 在minimal thymidine kinase promoter and a luciferase gene的上游 in pGL-2 vector (Promega)。在培養和刺激24小時後將相關的細胞溶解物取出後利用冷光分析儀來測定其冷光強度。

樹突細胞的 NF-kB 磷酸化分析

為了進一步了解樹突細胞活化時 NF-kB 的相關酵素的磷酸化情形，我們在樹突細胞活化後，利用抗體將相關的蛋捕捉後，這些免疫沈澱物將分別與 1μg of GST-I B (1-100), GST-I B (1-100AA), GST-I B (1-120), GST-I B (1-120AA), GST-I B (1-61), or GST-I B (1-61AA) and [-32P]ATP (10μCi)及激媒受質一道作用。此一反應再加入 Laemmli's sample buffer 來終止。這些產物則也是進一步跑電泳來分析其強度。而這些蛋白都會進一步利用西方墨點法確定其蛋白質的正確性。

TLR 在樹突細胞活化中所扮演的角色

為了進一步了解這些刺激影響樹突細胞的途徑究竟是來自哪一個受體，我們將分別利用抗 TLR-2、TLR-4 和 TLR-9 的抗體來加入培養中，以了解在何種抗體的阻斷下，樹突細胞的細胞激素製造會受到最明顯的影響。在這種情形下，我們便可以了解哪一個受體在樹突細胞的活化中扮演了一個最重要的角色。

結果與討論

一・我們利用樹突細胞的培養，已經成功地建立一個試管內模式，可以經由樹突細胞在試管內的表現來評估此一試劑進入體內的反應。我們在初期分別利用由靈芝衍生來的多醣和 CpG motif 來進行相關研究。我們發現樹突細胞在經由刺激後會表現較高量的輔助分子，同時其細胞激素的分泌也分別與引起的免疫反應有著密切的關係。

我們更進一步研究樹突細胞的訊息傳導途徑，結果能夠成功地分析其刺激後的與 NF-kB 和特定的酵素激媒有著密切的關係。此一研究成果已經發表在今年的 Journal of Leukocyte Biology (Lin, Y.-L. 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. J Leuko Biol 2005; 78:533-543)

二・我們也同時利用過敏原來進行對樹突細胞的分析，但是在目前的結果發現對樹突細胞的細胞激素分泌的影響並未與其他抗原有著明顯的差別。所以，我們目前還在研究其他的相關免疫調節分子，如 DcR3，目前有研究顯示 DcR3 分子可能在第二型 T 輔助細胞發育的調控上也扮演著重要的角色，在一段時間後應該會有更清楚的研究結果。

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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

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Abstract: ***Ganoderma lucidum*, a fungus native to China, has been widely used to promote health and longevity in the Chinese. The polysaccharide component with a branched (1→6)-β-D-glucan moiety of *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 resulted in the enhanced cell-surface expression of CD80, CD86, CD83, CD40, CD54, and human leukocyte antigen (HLA)-DR, as well as the enhanced production of interleukin (IL)-12p70, p40, and IL-10 and also IL-12p35, p40, and IL-10 mRNA expression, and the capacity for endocytosis was suppressed in DC. In addition, treatment of DC with PS-G resulted in enhanced T cell-stimulatory capacity and increased T cell secretion of interferon-γ and IL-10. Neutralization with antibodies against Toll-like receptor (TLR)-4 inhibited the PS-G-induced production of IL-12 p40 and IL-10, suggesting a vital role for TLR-4 in signaling DC upon incubation with PS-G. Further study showed that PS-G was able to augment inhibitor of κB (IκB) kinase and nuclear factor (NF)-κB activity and also IκBα and p38 mitogen-activated protein kinase (MAPK) phosphorylation. Further, inhibition of NF-κB by helenalin and p38 MAPK by SB98059 prevented the effects of PS-G in the expression of CD80, CD86, CD83, CD40, CD54, and HLA-DR and production of IL-12p70, p40, and IL-10 in various degrees. Taken together, our data demonstrate that PS-G can effectively promote the activation and maturation of immature DC, suggesting that PS-G may possess a potential in regulating immune responses. *J. Leukoc. Biol.* 78: 533–543; 2005.**

Key Words: *PS-G · signal transduction · T cells · IL-10 · IL-12 · IFN-γ*

INTRODUCTION

Ganoderma lucidum, a native fungus from China, has been widely used in China and other Asian countries. *G. lucidum* has been reported to be effective in modulating immune functions and inhibiting tumor growth and in the treatment of chronic hepatopathy, hypertension, and hyperglycemia [1]. 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 [2, 3]. PS-G has been reported to enhance the cytotoxic activity of natural killer (NK) cells and to increase tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) release from macrophages and lymphocytes, respectively [4, 5]. The polysaccharide component from *G. lucidum* also has been reported to elicit antiapoptotic effects on neutrophils, and this action primarily depends on the activation of Akt-regulated signaling pathways [6].

Dendritic cells (DC) are the most professional antigen-presenting cells (APCs), whose primary function is to capture, process, and present antigens to unprimed T cells [7]. Immature DC reside in nonlymphoid tissues, where they can capture and process antigens. Thereafter, DC migrate to the T cell areas of lymphoid organs, where they lose the antigen-processing activity and mature to become potent immunostimulatory cells [8]. The induction of DC maturation is critical for the induction of antigen-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 major histocompatibility complex (MHC) class II and costimulatory molecules (CD40, CD80, and CD86) but a decreased capacity to internalize antigens [9]. Up-regulation of CD83, a specific marker for DC maturation, also occurs [10]. Various stimuli, such as proinflammatory cytokines [e.g., TNF-α and interleukin (IL)-1], CD40 ligation, bacterial prod-

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ucts [e.g., lipopolysaccharide (LPS) and unmethylated DNA CpG motif], and contact sensitizers, can induce DC maturation in vivo and in vitro [11, 12]. Several reports have already indicated that the nuclear transcription factor (NF)-κB also plays an important role in DC maturation [13]. Another intracellular component involved in DC maturation, the three major mitogen-activated protein kinase (MAPK) signaling pathways in mammals, including p38 MAPK, extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinases (JNK), are activated in DC on maturation induced by LPS or TNF-α [14].

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 the activation and maturation of human monocyte-derived DC.

MATERIALS AND METHODS

Reagents

Escherichia coli LPS (L8274, *E. coli*) and lipoteichoic acid (LTA; L2515, from *Staphylococcus aureus*) were purchased from Sigma Chemical Co. (St. Louis, MO). Isotopes were obtained from Amersham Corp. (Arlington Heights, IL). Neutralization antibodies (without sodium azide) against Toll-like receptor (TLR)-2 and TLR-4 were purchased from eBiosciences (San Diego, CA), and helenalin, SB203580, PD98059, and JNK inhibitor II were purchased from Calbiochem (Germany). Treatment of immature DC with these inhibitors (helenalin, SB203580, PD98059, and JNK inhibitor II) before stimulation was performed for 60 min. These inhibitors were dissolved in dimethyl sulfoxide (DMSO), where a 0.1% (v/v) concentration of DMSO was used as a negative control whenever 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, Uppsala, 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 ~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.

Human DC generation

DC were generated from peripheral blood mononuclear cells (PBMC), as described previously [15, 16], with some modification. Briefly, PBMC were obtained from healthy donors by centrifugation with Ficoll-Hypaque (Pharmacia), 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 manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The $DC14^+$ cells were cultured at 1×10^6 cells per 1 ml RPMI 1640 containing 10% fetal calf serum in 24-well plates (Costar, Cambridge, MA) with granulocyte macrophage-colony stimulating factor (GM-CSF; 800 U/ml) and IL-4 (500 U/ml). Fresh medium containing GM-CSF and IL-4 was added every 2–3 days. Human monocyte-derived DC were used routinely at day 6 of culture.

Determination of cytokine levels

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

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from DC using TRIzol reagent (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. Total RNA was converted to cDNA with Moloney-murine leukemia virus RT (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 the forward primer 5′-GAGTCCGGGAAAGTCCTGCC-3′ and the reverse primer 5′-TCTGGCCTTCTGGAGCATGTT-3′. The IL-12 p40 primers used were the forward primer 5′-GGGGTGACGTGCGGAGCTGCT-3′ and the reverse primer 5′-TCTTGCCCTGGACCTGAACGC-3′. The IL-10 primers used were the forward primer 5′-TTTCTCTTGGAGCTTATTAAAG-3′ and the reverse primer 5′-AAGACTTTCTTTCAAATGAAGG-3′ (Invitrogen, Carlsbad, CA). The cDNA sequence of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified as a control using the following primers: 5′-CTCATGACCACAGTCCATGC-3′ and 5′-CCCTGTTGCTGTAGCCAAAT-3′. These primers produced a 450-base pair product. A thermal cycle of 30 s at 94°C, 30 s 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 s at 94°C, 30 s at 55°C, and 1 min at 72°C was used for 35 cycles for IL-10.

Intracellular staining of IL-10

Intracellular cytokine staining was performed by using the BD Cytofix/Cytoperm kit (BD Biosciences, San Diego, CA). Briefly, 1×10^5 $CD3^+$ T cells and DC were incubated at a ratio of 5:1 at 37°C for 2 days. After adding the transport inhibitor monesine, the culture was incubated at 37°C for 2.5 h. After washing with staining buffer, cells were labeled with CD3-fluorescein isothiocyanate (FITC) and permeabilized. Intracellular staining was performed with phycoerythrin-labeled IL-10 antibodies or isotype control.

Flow cytometric analysis

DC were harvested and washed with cold buffer [phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) and 0.1% sodium azide]. Cells were then incubated in cold buffer. Subsequent stainings with monoclonal antibodies (mAb) 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, San Jose, CA). 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 RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 25 mM HEPES. The cells were then incubated with FITC-labeled dextran (0.2 mg/ml) at 4°C or 37°C for 1 h. Finally, the 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 $CD3^+$ T cells were purified from PBMC using magnetic beads (Miltenyi Biotec). Theallogeneic $CD3^+$ 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.

Neutralization experiments

Human DC were preincubated for 1 h with 20 μg/ml antibody solution of TLR-2 and TLR-4. LPS, LTA, and PS-G were then added for 15 h. The cell culture supernatants were collected and were analyzed for IL-12 p70, IL-12 p40, and IL-10 by ELISA.

Inhibitor of κB (IκB) kinase (IKK) activity assay

The kinase activity assay was performed as described by Spiecker et al. [17] with some modifications. 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 phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin] for 30 min at 4°C. The cell lysate was clarified by centrifugation at 12,000 *g* 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, CA) and protein A/G-PLUS agarose for 12 h at 4°C. Kinase assay was carried out in 45 μl kinase buffer [40 mM Tris-NaOH, pH 7.5, 500 mM NaCl, 0.1% Nonidet P-40 (NP-40), 6 mM EDTA, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate, 300 μM sodium orthovanadate, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM dithiothreitol (DTT)] containing 5 μM cold adenosine 5′-triphosphate (ATP), 10 μCi [γ-^{32}P] ATP (5000Ci/mmol, Amersham), and 1 μg glutathione *S*-transferase (GST)-IκBα fusion protein (Santa Cruz Biotechnology) as substrate and incubated for 20 min at 25°C. Each sample was mixed with 8 μl 5× Laemmli's loading buffer to stop the reaction, heated for 10 min at 100°C, and subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. 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 polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated overnight at 4°C with 10% bovine serum albumin in PBS to block nonspecific immunoglobulins (Igs) and then incubated with anti-IκB-P polyclonal and anti-α-tubulin mAb (Santa Cruz Biotechnology) and anti-p38-P, anti-p42/44-P, anti-p46/54-P, and anti-total p38 polyclonal antibody (Cell Signaling Technology, Beverly, MA).

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

Nuclear and cytoplasmic extracts were prepared as described previously [18]. 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 s. Nuclei were pelleted by centrifugation at 12,000 *g* for 20 s. 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 PMSF) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12,000 *g* 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′-AGTTGA**GGGGACTTTCCC**AGGC-3′, and incubated at room temperature for 20 min. The incubation mixture included 1 μg poly (dI-dC) in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% NP-40, 5% glycerol, and 50 mM NaCl). The DNA-protein complex was electrophoresed on 4.5% nondenaturing polyacrylamide gels in 0.5× Tris-boric acid EDTA buffer (0.0445 M Tris, 0.0445 M borate, 0.001 M EDTA). A double-stranded, mutated oligonucleotide, 5′-AGTTGA**GGC-GACTTTCCC**AGGC-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 unlabeled oligonucleotide.

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

PS-G induces maturation of human monocyte-derived DC

LPS has been described as an inducer of DC activation and maturation [19]. 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, CD54, and MHC class II molecules on the cell membrane of human DC (**Fig. 1**).

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

To determine whether PS-G can affect the cytokine production in human DC, we compared cytokine concentrations in the supernatants of DC cultured with different doses of PS-G, which enhanced the production of IL-12 p70, IL-12 p40, and IL-10 (**Fig. 2A**). When human DC were treated with 10 μg/ml PS-G for 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 18, 24, and 48 h (Fig. 2B). 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

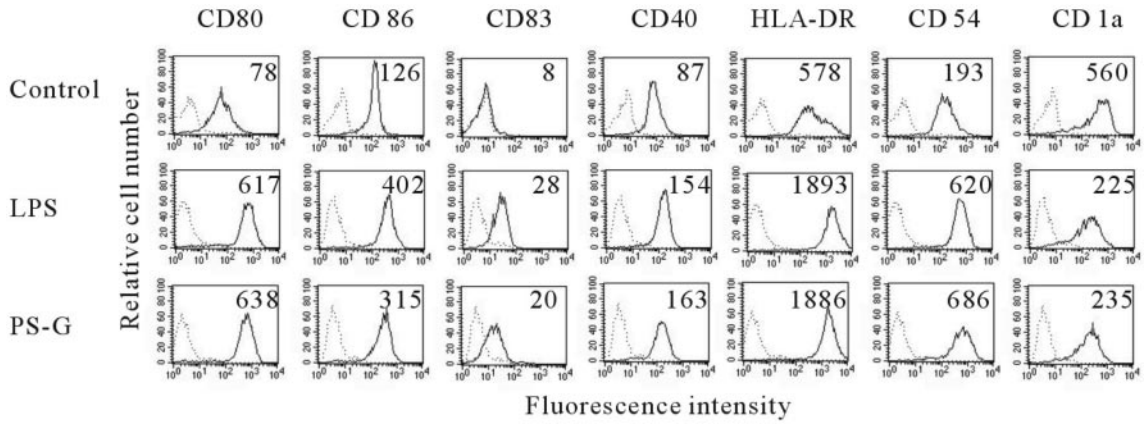

Fig. 1. The effect of PS-G and LPS on DC phenotype. Human DC were treated with PS-G (10 μg/ml), LPS (100 ng/ml), or medium alone for 24 h, and surface markers were analyzed by flow cytometry (dotted line, isotype control; solid line, specific mAb). The values shown in the flow cytometry profiles are the mean fluorescence intensity (MFI) indexes. HLA, Human leukocyte antigen.

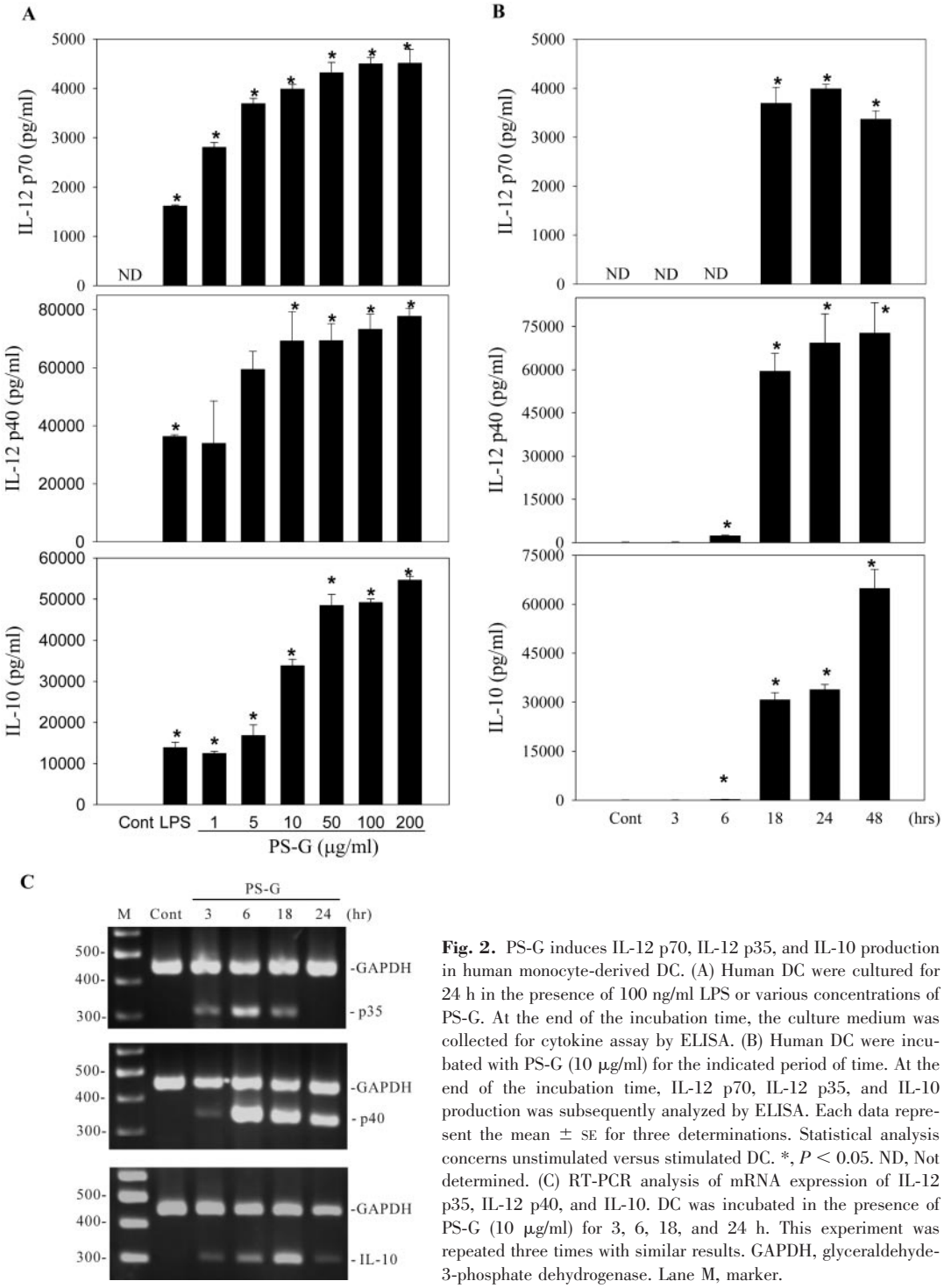

Fig. 2. PS-G induces IL-12 p70, IL-12 p35, and IL-10 production in human monocyte-derived DC. (A) Human DC were cultured for 24 h in the presence of 100 ng/ml LPS or various concentrations of PS-G. At the end of the incubation time, the culture medium was collected for cytokine assay by ELISA. (B) Human DC were incubated with PS-G (10 μg/ml) for the indicated period of time. At the end of the incubation time, IL-12 p70, IL-12 p35, and IL-10 production was subsequently analyzed by ELISA. Each data represent the mean ± SE for three determinations. Statistical analysis concerns unstimulated versus stimulated DC. *, $P < 0.05$. ND, Not determined. (C) RT-PCR analysis of mRNA expression of IL-12 p35, IL-12 p40, and IL-10. DC was 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. Lane M, marker.

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 h, 6 h, and 18 h, respectively, in human DC, especially highly expressed IL-12 p40 mRNA (Fig. 2C). In unstimulated DC, there was no detectable IL-12 p35, IL-12 p40, and IL-10 mRNA.

PS-G down-regulation of endocytotic activity of human DC

Immature DC capture and process antigens via their high endocytic capacity, and they lose their endocytic/processing activities of antigens and mature into potent immunostimulatory APCs during differentiation [7]. 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 is 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. 3**).

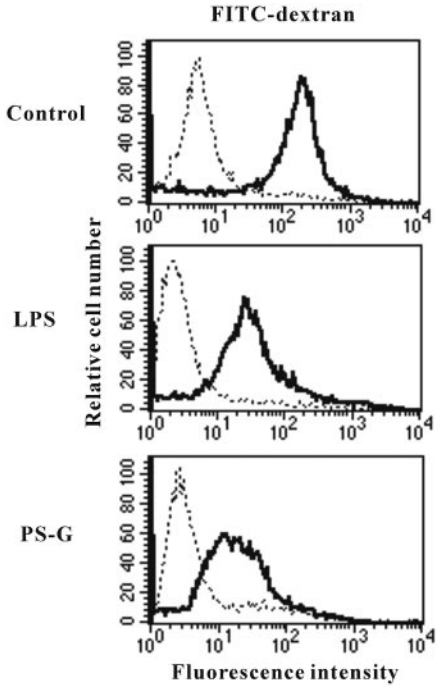

Fig. 3. PS-G on the endocytotic capacities of human DC. At day 6, immature DC were stimulated with medium alone, LPS (100 ng/ml), or PS-G (10 μg/ml) for 24 h, and cells were then incubated with FITC-dextran for 1 h at 4°C (dotted lines) or 37°C (solid lines). This experiment was repeated three times with similar results.

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

Mature DC have the capacity to induce proliferation in allogenic T cells at a much higher level than immature DC [8]. In human DC, we found that PS-G up-regulated cell-surface markers, increased IL-12 production, and induced the 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 allogenic, naive T cells. The results presented in **Figure 4A** show that PS-G-treated DC enhanced T cell activation, as evidenced by the secretion of IFN-γ in the culture supernatant (Fig. 4B). The IFN-γ production induced under these experimental conditions was far higher than that seen following LPS treatment of DC, especially at low DC/T cells ratios. It is interesting that we demonstrated that PS-G-treated DC enhanced T cell secretion of IL-10, except for LPS-treated DC, which were not able to enhance T cell secretion of IL-10 in the supernatant (Fig. 4C). To investigate if IL-10 is produced by the activated T cells in this experiment, we performed intracellular cytokine staining for IL-10 on $CD3^+$ T cells (>95% purity by flow), which were cocultured with autologous DC. Intracellular cytokine staining results showed that PS-G-treated DC enhanced the T cell production of IL-10, such that the percentage gated of double-positive CD3 and IL-10 is 6.04%. In DC alone and LPS-treated DC, the percentages gated of double-positive $CD3^+$ and IL-10 are 1.74% and 1.51%, respectively. Therefore, intracellular IL-10 staining of T cells in the PS-G-treated DC group is significantly higher than in DC alone or in the LPS-treated DC group. In the LPS group or the PS-G-treated group, DC could not induce T cell secretion of IL-4 cytokine (data not shown).

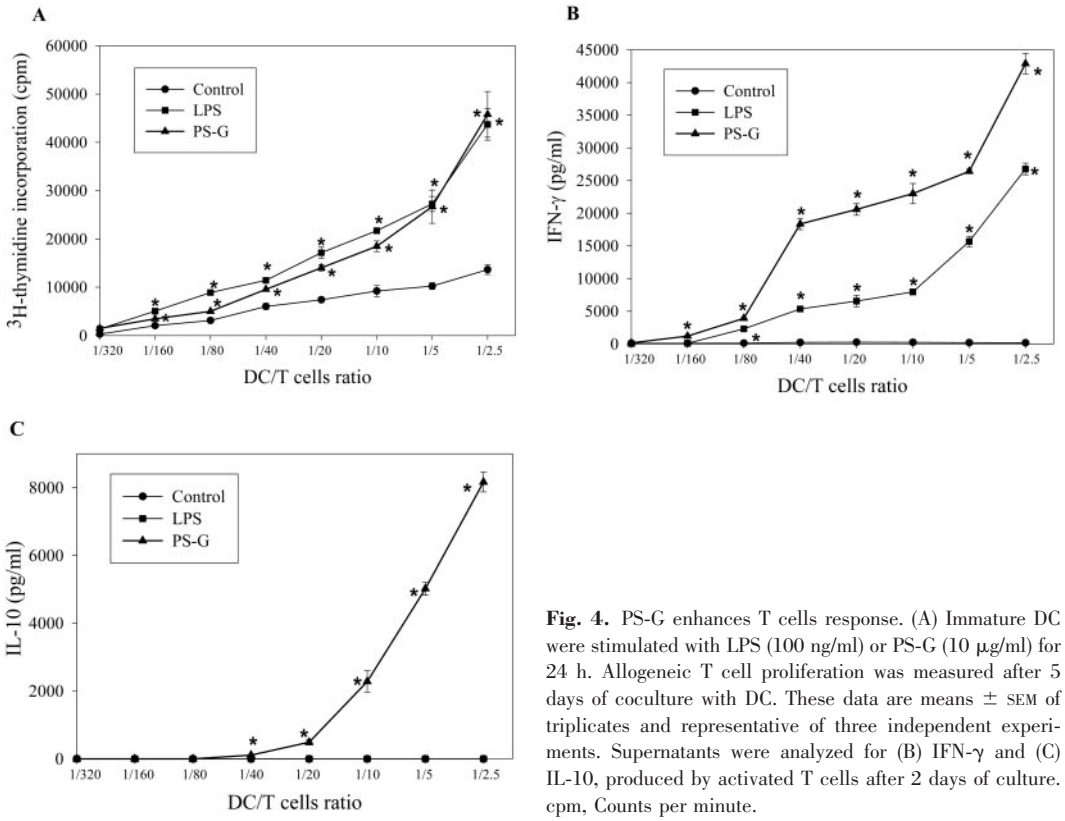

Fig. 4. PS-G enhances T cells response. (A) Immature DC were stimulated with LPS (100 ng/ml) or PS-G (10 μg/ml) for 24 h. Allogeneic T cell proliferation was measured after 5 days of coculture with DC. These data are means ± SEM of triplicates and representative of three independent experiments. Supernatants were analyzed for (B) IFN-γ and (C) IL-10, produced by activated T cells after 2 days of culture. cpm, Counts per minute.

PS-G induces IL-12 p40 and IL-10 synthesis through TLR-4

TLRs have been shown to be involved in the antifungal defense mechanism in Drosophila and the antibacterial defense in humans. To determine the involvement of these receptors in the interaction of DC with PS-G, neutralization experiments were performed. Cell-surface TLR-2 and TLR-4 receptors were blocked by neutralizing concentrations of their respective antibodes before DC were treated with LPS, LTA, or PS-G. We showed positive and negative controls for the neutralization of TLR-4 (by using LPS) and TLR-2 (by using LTA) in **Figure 5, A** and **B**. In Figure 5C, we demonstrated that the addition of an anti-TLR-4 mAb to human DC blocked PS-G (100 ng/ml, 1 μg/ml, and 10 μg/ml)-induced IL-12 p40 production ~70%, 31%, and 47%, respectively, and IL-10 production, ~55%, 39%, and 20%, respectively, but the addition of an anti-TLR-2 mAb failed to inhibit PS-G-induced IL-12 p40 and IL-10 production.

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

As the activation of IKK activity is necessary for IκBα phosphorylation, the effect of PS-G on IKK activity was likewise studied. Human DC were treated with PS-G (10 μg/ml) for the indicated periods of time. To measure IKK1 activity directly in human DC, IKK1 and IKK2 proteins were immunoprecipitated from cell extracts, and the kinase activity in the immunocomplex was assayed using recombinant GST-IκBα (1–317) as a substrate. **Figure 6A** illustrates the relative effect on IKK activity. After stimulation with PS-G, the GST-IκBα fusion

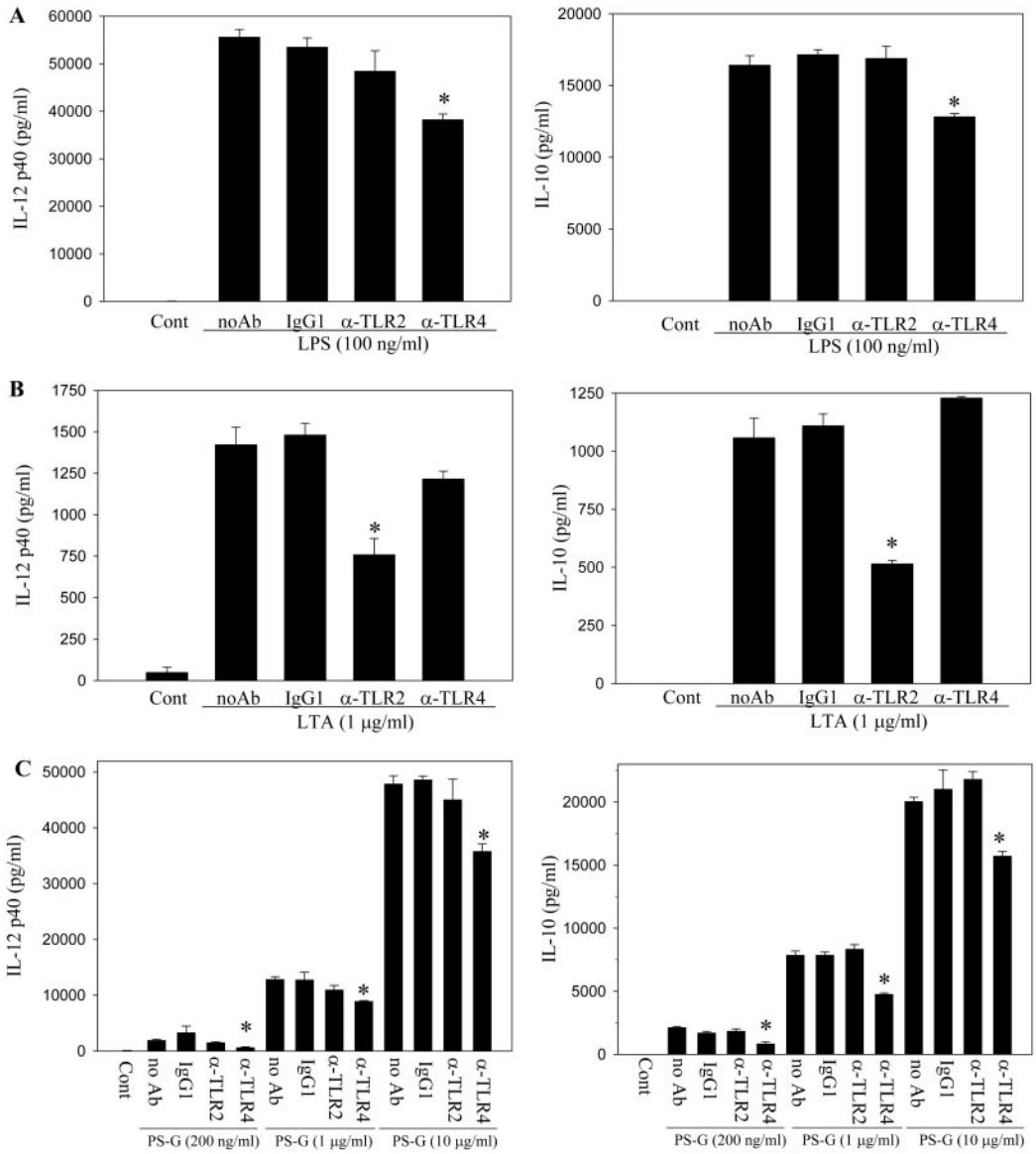

Fig. 5. Neutralization with TLR-4 mAb inhibits the synthesis of IL-12 p40 and IL-10 in PS-G-treated, human DC, which were preincubated with 20 μg/ml anti-TLR-2, TLR-4, and IgG1 antibodies separately for 1 h. DC were then challenged with LPS (A), LTA (B), or PS-G (C) for 15 h. The cell culture supernatants were collected for IL-12 p40 and IL-10 analysis. Data are represented as mean ± SE. Significant difference between DC treated with antibodies and no antibodies (noAb) is indicated by $P < 0.05$ (*).

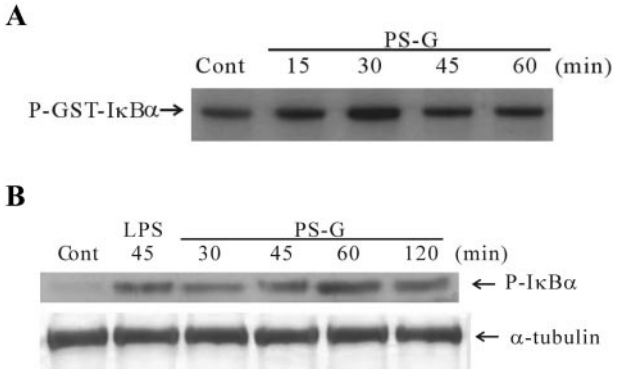

Fig. 6. PS-G induced IKK activity and IκBα phosphorylation in human DC. (A) Human DC were treated with PS-G (10 μg/ml) for the indicated time periods and collected the total cell lysates for IKK activity assay. Immunoprecipitated IKK was incubated with [γ-^{32}P] ATP and GST-IκB fusion protein, as substrates performed the kinase activity assay as described in Materials and Methods, and P-GST-IκBα is shown. (B) Human monocyte-derived DC were treated with LPS (100 ng/ml) for 45 min or PS-G (10 μg/ml) for the indicated time periods. Cytosolic fractions were prepared and analyzed for the phosphorylation level of IκB 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.

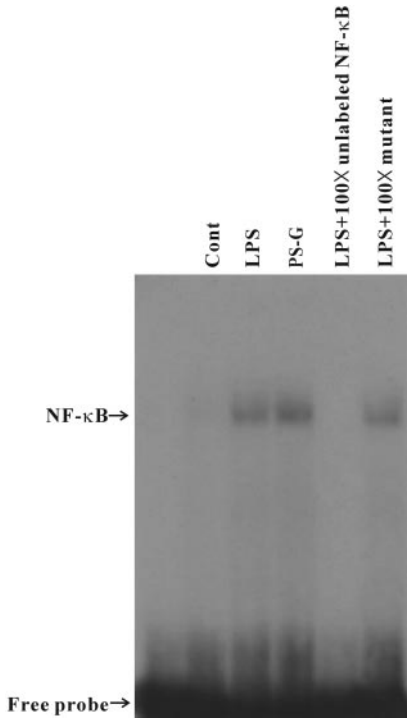

Fig. 7. PS-G induces NF-κB activation. Human monocyte-derived DC were treated with LPS (100 ng/ml) or PS-G (10 μg/ml) for 2 h or remained unstimulated, and nuclear fractions were prepared and analyzed for NF-κB binding activity by EMSA. To assess the specificity of the binding, 100-fold excess of cold NF-κB probe or mutant probe was added to the LPS condition. This experiment was repeated three times with similar results.

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 effect on IκBα phosphorylation. The cytoplasmic levels of IκBα-P protein were examined by Western blot analysis. LPS and PS-G induced the phosphorylation of IκBα. After 60 min from the activation of human DC with PS-G, the cytosolic IκBα protein was significantly phosphorylated (Fig. 6B).

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 a similar activation pathway, 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 EMSA. As shown in **Figure 7**, 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 unlabeled, competing NF-κB oligonucleotide.

PS-G induces phosphorylation of members of the three MAPK families 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 [20, 21]. This experiment focused on p38 MAPK, p42/44 ERK, and p46/54 JNK to further characterize the MAPK activation pathways involved in PS-G signaling. Human DC were stimulated with PS-G or none at all, and the level of MAPK phosphorylations was assessed by Western blotting with respective antityrosine-phosphorylated MAPK mAb. Total p38 mAb was used for internal control. Results presented in **Figure 8** show that PS-G induced the phosphorylation of all MAPK tested, especially in inducing a higher p38 phosphorylation at 30 min. The total amount of p38 was unchanged following stimulation.

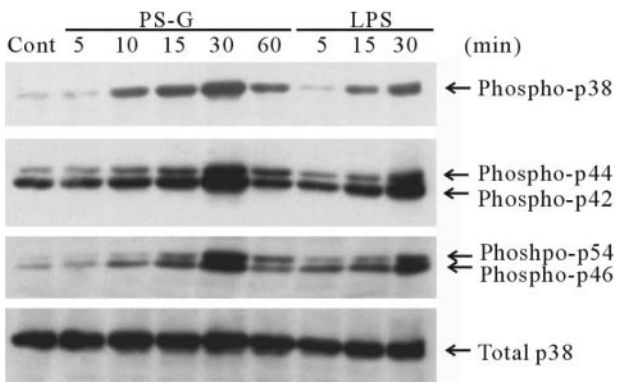

Fig. 8. PS-G induces the phosphorylation of p38 MAPK, p42/44 ERK, and p46/52 JNK kinase. Human monocyte-derived DC were treated with PS-G (10 μg/ml) inhibitors for the indicated time periods and then collected the cell lysate, and the level of MAPK phosphorylations was assessed by Western blotting with respective antityrosine-phosphorylated MAPK mAb, and total p38 mAb was for internal control.

Inhibition of NF-κB and MAPK 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. 2, A and B). We investigated whether the PS-G-induced secretions 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 was 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 SB203580 (**Fig. 9**). In contrast, PD98059 and JNK inhibitor II down-regulated IL-12 p70 and IL-10 production but only had 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, were investigated. Blocking the NF-κB pathway with helenalin significantly inhibited the PS-G and induced the up-regulation of CD80, CD86, CD83, CD40, CD54, and HLA-DR (**Fig. 10**). In contrast, blocking the p38 MAPK and JNK pathway with SB203580 and JNK inhibitor II, respectively, had little effect on CD80, CD86, CD83, CD40, CD54, and HLA-DR expression. 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.

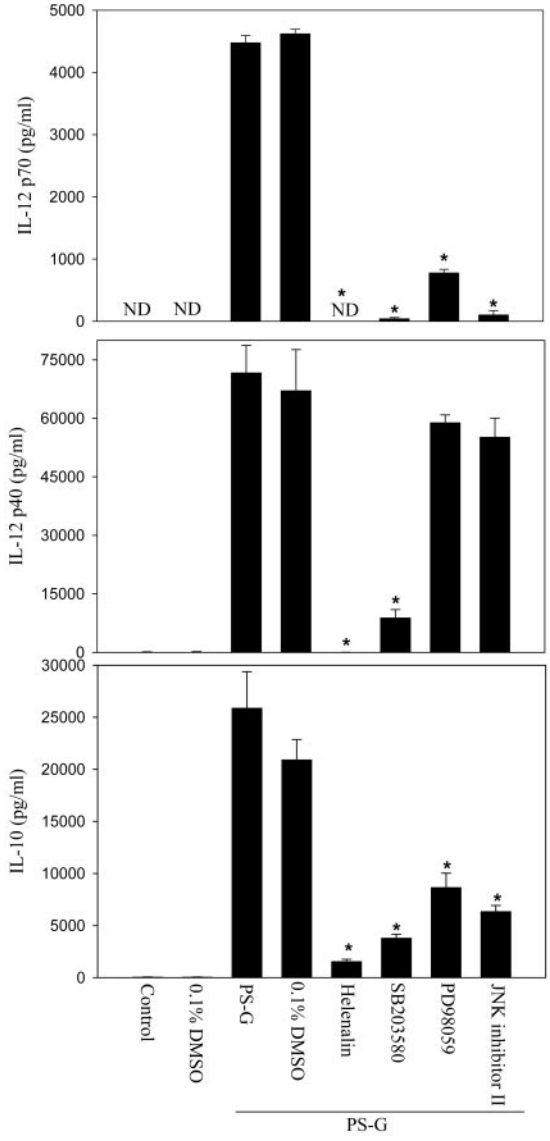

Fig. 9. The effect of inhibiting the NF-κB, p38 MAPK, ERK1/2, or JNK pathways on the PS-G-induced up-regulation of IL-12 p70, IL-12 p40, and IL-10 production in human monocyte-derived DC. Human DC were pretreated with 0.1% DMSO, 10 μM helenalin (a specific blocker of NF-κB), 20 μM SB203580 (a specific blocker of p38 MAPK), 50 μM PD98059 (an inhibitor of the ERK1/2 pathway), or 20 μM JNK inhibitor II (an inhibitor of the JNK pathway) for 1 h and then incubated with 10 μg/ml 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. Significant difference between DC treated with PS-G alone and pretreated with inhibitors is indicated by $P < 0.05$ (*). ND, Not determined.

DISCUSSION

The biological process of DC maturation represents a crucial step in the initiation of adaptive immune responses [22]. This process is regulated by various extracellular stimuli, including cytokines, bacterial products, and membrane-bound ligands [10, 23]. DC maturation is accompanied by changes in their morphological, phenotypic, and functional properties [24]. Recently, several researchers have demonstrated the immunomodulatory effects of polysaccharides purified from *G. lucidum* on T lymphocytes [25]. Cao and Lin [26] showed that *G. lucidum* polysaccharides could promote the maturation and function of murine DC. These results are similar to our finding in human DC. 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, and mature DC demonstrated characteristic morphology with enlarged size and numerous cytoplasmic processes, which gave rise to a stellate appearance (data not shown). Maturation of DC was characterized by a decreased antigen-processing capacity, an increased cell-surface expression of MHC class II molecule and costimulatory molecules CD80, CD86, and CD40, and the secretion of IL-12, which primed a strong stimulation of T lymphocyte growth and differentiation. The CD83 marker for mature human DC was also increased.

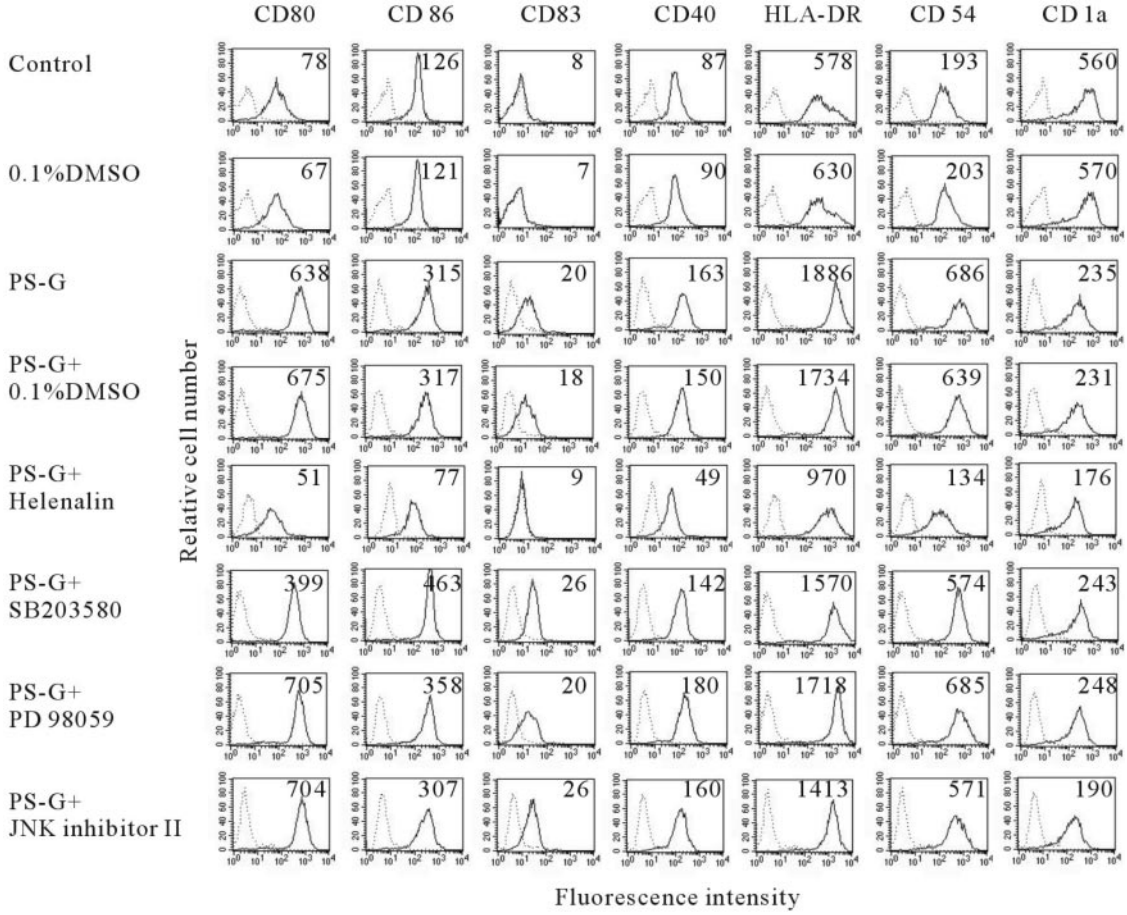

Fig. 10. The effect of inhibiting the NF-κB, p38 MAPK, ERK1/2, or JNK pathways on the PS-G-induced up-regulation of CD80, CD86, CD83, CD40, CD54, and HLA-DR in human monocyte-derived DC. Day 6 immature DC were pretreated with 0.1% DMSO, 10 μM helenalin (a specific blocker of NF-κB), 20 μM SB203580 (a specific blocker of p38 MAPK), 50 μM PD98059 (an inhibitor of the ERK1/2 pathway), or 20 μM 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 CD80, CD86, CD83, CD40, CD54, and HLA-DR was then measured using the flow cytometry (dotted line, isotype control; solid line, specific mAb). The values shown in the flow cytometry profiles are the MFI indexes. These results are representative of three independent experiments with similar results.

In immune responses, IL-12 plays a central role as a link between the innate and adaptive immune systems [27]. Thus, IL-12 induces and promotes NK and T cells to generate IFN-γ and lytic activity. In addition, IL-12 polarizes the immune system toward a primary T helper cell type 1 (Th1) response. In this study, we found out that LPS and 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 anti-inflammatory and immunosuppressive properties [24]. We suggested that when IL-12 p40 is overexpressed in PS-G-treated human DC, IL-10 could act as a feedback regulatory role, although LPS has the similar effect in human DC, but the cytotoxicity of LPS is higher than PS-G. Therefore, PS-G is a safe immune modulator for human DC. 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 for the secretion of IL-10. However, although DC are widely regarded as the most potent APCs, recent evidence also indicates that DC play an important role in inducing immune tolerance [28] and regulating Th1/Th2 immunity balance [29]. Furthermore, these immature DC could also be alternatively activated and induced to exert suppressive effects [30].

TLRs have been identified in humans as an important component of innate immunity against microbial pathogens. LPS is recognized by TLR-4 in DC. The effects of TNF-α are mediated by two distinct cell-surface receptors, TNF-R1 and TNR-R2, where TNF-R1 has been implicated in TNF-α-induced phenotypic and functional changes in DC [10]. Recent reports show that LPS and TNF-α, two potent DC maturation factors, induced the NF-κB activation and phosphorylation of p38, ERK1/2, and p46/54 JNK in monocyte-derived DC [31]. Our results demonstrated that PS-G activated NF-κB and all three MAPK pathways during maturation. Neutralization experiments with antibodies blocking TLR-2 and TLR-4 further demonstrated that TLR-4 played a critical role in the signal transduction cascade induced by PS-G. The blocking effect in high concentration of PS-G (10 μg/ml)-treated DC is not better than in low concentration (200 ng/ml). However, the percentage of inhibition by anti-TLR-4 antibody was similar between LPS and PS-G stimulation. This result is different from the

zymosan, as zymosan particles are recognized simultaneously by dectin-1 and TLR-2 [32–34]. Recent reports show that NF-κB is responsible for LPS-induced DC maturation in an in vitro murine model [35] and that cytokine-induced maturation of human DC results in increased NF-κB nuclear translocation [36]. Many proinflammatory cytokines displayed NF-κB-responsive elements in their promoters, conferring a major role on immune responses [37]. Moreover, the p38 MAPK pathway has been shown to contribute to NF-κB-mediated transactivation [38, 39]. 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, p38 MAPK, ERK1/2, and p46/54 JNK pathways are activated when immature human DC are exposed to PS-G, suggesting a role of these pathways in the maturation process. The promoters of human (h)IL-12 p35 and hIL-12 p40 gene contain κB-binding sites [40]. It likely that NF-κB is also involved in the 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 [41]. Recently, it has been suggested that p38 MAPK is involved in the regulation of IL-10 production [42].

Early phosphorylation of p38 MAPK, ERK1/2, and p46/54 JNK was investigated in PS-G-treated, human monocyte-derived DC. Our results corroborate recent reports using the murine models, as well as human DC in vitro models showing activation of all three MAPK pathways during maturation [35, 43]. 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 MAPK in DC maturation. In cytokines analysis, pretreatment of helenalin and SB203580 significantly inhibited the IL-12 p70, IL-12 p40, and IL-10 productions 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 costimulatory 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 p38 MAPK and p46/54 JNK by SB203580 and JNK inhibitor II, respectively, before PS-G stimulation had a weak effect on the CD80, CD86, CD83, CD40, CD54, and MHC class II expression induced during DC maturation. PD98059 had no effect on the 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 a result of nonspecific toxicity, as these inhibitors did not modify the viability of DC (data not shown). Collectively, these results show that the NF-κB and p38 MAPK pathways play critical roles in the initiation of DC maturation. The human CD86 promoter has been cloned recently, and two canonical NF-κB binding sites have been revealed [44]. One of them is essential for the Th-induced CD86 gene transcription. Moreover, NF-κB activation has been shown previously to drive CD 80 transcription [45]. A recent report describes the generation of MAPK kinase 3-deficient mice to study the role of the p38 MAPK pathway in vivo [46]. 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 signal transduction pathways regulate the different aspects of DC maturation.

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

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