行政院國家科學委員會專題研究計畫　成果報告

氣喘及免疫調節的導致評估及開發

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計畫主持人：江伯倫 教授

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中文摘要

關鍵詞：這幾年過敏疾病有逐年增加的趨勢，所以如何來開發過敏性氣喘的新藥物已經是個刻不容緩的問題。這幾年來生物藥劑的開發逐漸受到重視，所以如何來開發包括如中藥、民間藥或是海洋生物在內的天然藥物，甚或者目前已經使用中的藥物，能夠應用在過敏性氣喘的治療上，對整個藥物及生物製劑的開發其實應該具有舉足輕重的地位。目前在台灣地區單是過敏性氣喘的流行率可能便高達 15-19%左右，而如果加上過敏性鼻炎的患者便可能會高達 40%，所以如果能夠開發出治療過敏性氣喘的藥物將是對本地的藥物研發有非常重要的意義。在本研究計畫的主要目的便是要利用樹突細胞建立一個試管內的藥物活性評估的平台，再加上體內的氣喘功能改善的功能測定，來開發能夠應用在過敏性氣喘治療的藥物。為了能夠建立一個體內和體外的系統來評估和分析藥物的活性，我們計畫建立樹突細胞的培養系統來達到此一目的。在此計畫中我們想利用試管內的樹突細胞當成平台來進行初步的篩選，一但找出具有這些功能的藥物，我們將進一步利用建立好的氣喘動物模式來確定這些藥物的功能。所以我們進行的方法如下：

1．分離出骨髓的幹細胞加上細胞激素來加以培養，一但培養出樹突細胞後將進一步進行分析，包括細胞表面標記的變化和細胞激素分泌的情形，以確定樹突細胞的純度和功能。

2．利用不同的藥物或是中藥的成份與樹突細胞一道培養，再取得樹突細胞來加以分析，以了解是否能夠促進輔助分子的表現和與第一型 T 輔助細胞活性有關的細胞激素 IL-12。

3．利用氣喘的小鼠模式來評估這些藥物的體內活性，所以我們利用這些藥物經由注射、吸入或是口服的方式來給予，再分別追蹤抗原特異性 IgE、呼吸道的阻力和氣管內沖洗液內的嗜酸性白血球數目。

4．建立生物晶片來評估樹突細胞的活性，經由這樣的方法來大量篩檢能夠影響免疫活性的藥物。如果有其他研究室想要評估這類的藥物，便可以透過生物晶片的方式迅速地篩選。

我們相信如果能夠建立一個試管內平台的方式來篩選能夠改善過敏性氣喘的藥物，將對未來國內生技和藥物開發有相當重要的影響。

Abstract

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. In this study, we like to establish both in vitro and in vivo system to evaluate the drugs with the possible immunomudulatory function for allergic diseases. Atopic diseases were mediated predominantly by type 2 T helper (Th2)-mediated activity including allergen-specific IgE antibody and eosinophils. It has been documented that cytokines such as IFN-g, IL-12 or IL-18 can alleviate the disease severity of allergic diseases. Furthermore, BCG or CpG motif has been found to prevent allergen-specific IgE antibody production and airway inflammation. In this study, we like to establish both in vitro and in vivo systems to screen functional herb drugs or bioactive substances for the treatment of bronchial asthma. Most recently, dendritic cells have been to play a critical role in induction and modulation of immune responses. In addition, the studies also suggested that myeloid dendritic cells and lymphoid dendritic cells might exert totally different effect on T helper cells development. Dendritic cells have been suggested to be a professional antigen presenting cells which can process and present antigen in vivo. Recently, it has been demonstrated that dendritic cells can be grown in vitro with the addition of cytokines such as GM-CSF, TNF- and IL-4. Dendritic cells have been applied in enhancing immune response to tumor. However, the results suggested that dendritic cell itself might play a critical role in the initiation of airway inflammation. It has been documented that CpG motif could affect the function of the dendritic cells to secrete large amount of IL-12 and drive the T cells to Th1 direction. Most recently, several research groups and companies have applied CpG motif for the treatment of asthma. The pathogenic mechanisms of allergic diseases have been found to be caused by the imbalance between Th1 and Th2 regulation. Dendritic cells become the most critical cells in linking innate and acquired immunity, which makes the interaction between antigens and dendritic cells extremely important. We like to apply the dendritic cells as the in vitro assay system to screen the effective drugs or components which can increase accessory molecules expression and Th1-related cytokine production. Further, these drugs will be evaluated for their therapeutic effect on asthma with the establishment of asthma model. Allergic diseases still tend to increase in these years, it will be very important to develop more effective drugs for the treatment of the diseases.

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

這幾年過敏疾病有逐年增加的趨勢，所以如何來開發過敏性氣喘的新藥物已經是個刻不容緩的問題。這幾年來生物藥劑的開發逐漸受到重視，所以如何來開發包括如中藥、民間藥或是海洋生物在內的天然藥物，甚或者目前已經使用中的藥物，能夠應用在過敏性氣喘的治療上，對整個藥物及生物製劑的開發其實應該具有舉足輕重的地位。本研究計畫的主要目的便是要利用樹突細胞建立一個試管內的藥物活性評估的平台，再加上體內的氣喘功能改善的功能測定，來開發能夠應用在過敏性氣喘治療的藥物。

過敏性氣喘主要是由第二型 T 輔助細胞(Th2)及相關的細胞激素如介白質-4、介白質-5 和介白質-13 等，會幫助 B 細胞製造 IgE 過敏抗體，遇到過敏原時便會刺激肥大細胞而釋放出來發炎物質。這些發炎性物質會造成氣管的收縮、黏液的分泌和血管的擴張，而導致症狀的發生。目前的研究證據顯示如果藥物能夠有效地將過敏免疫反應調節成第一型 T 輔助細胞，這些的藥物或是生物製劑便可能應用到氣喘疾病的治療上。最近幾年最明顯的例子便是 BCG 和 CpG motif，這兩類跟結核桿菌(mycobacteria)相關的生物製劑，便發現可以誘發良好的第一型 T 輔助細胞的活性，所以已經被許多生物科技公司認為是用在氣喘治療上的下一波藥物。

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。所以如果能夠在試管內將生物製劑或是天然藥材與樹突細胞一道培養，來測定樹突細胞的表面標記和細胞激素的分泌，能夠來找出更有效的藥物來誘發較高的第一型 T 輔助細胞活性。

而我們在最近的研究也顯示我們利用一個已經應用在治療肝炎的藥物 ribavirin，與樹突細胞一道培養，結果發現可以有效地促進 IL-12 的分泌，我們更進一步將以 ribavirin 處理過的樹突細胞再注射入小鼠體內，可以有效地抑制故敏原特異性 IgE 抗體的製造，同時也可以降低呼吸道的阻力和嗜伊紅性白血球的數目 (Chiang et al 2003)。而由此一研究結果，我們在生技製藥計畫中提出一個建立篩選可能應用在過敏性氣喘治療的藥物平台，先利用一個試管內樹突細胞篩選的方法來大量篩選藥物，再進一步找出一些特定的藥物來進行動物模式的研究。未來甚至可以利用這些找出來的基因和分子來更快地來找出能夠應用在過敏疾病治療上的藥物。

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篩選可能的佐劑，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，來檢視一些特定的製劑是否在未來可能應用在過敏疾病的免疫療法上的應用。

研究方法

樹突細胞(dendritic cells, DCs)的培養方式的建立

(A). 用小鼠骨髓來做樹突細胞的培養

1. 分離並培養源自骨髓之樹突細胞

老鼠犧牲後，將骨髓細胞加入 GM-CSF(500U/ml) 和 IL-4 (1000U/ml) 培養便可得樹突細胞以供後續實驗使用。

2. 分析所培養的樹突細胞之細胞表面標記

為了進一步確定樹突細胞的表面標記，我們利用可辨識第二類 MHC 分子、B7-1 和 B7-2 的各種抗體來分析我們所培養的樹突細胞表面上所帶的表面標記分子。染色後的細胞清洗後將其置於含有 0.1% sodium azide 的 PBS 中成為 0.5ml 的細胞懸浮液，再以 FMF 分析。以適當的分析軟體 (FACScan, Becton Dickinson, Mountain View, CA) 計算 10,000 顆細胞中，每顆細胞所帶細胞表面分子的頻率與密度。以沒有染色的細胞置於培養基中當作對照組。

3. 同源異個體混合淋巴球反應

為了確定所培養的樹突細胞可以刺激同源異個體之 T 細胞，我們將來自 Balb/c 品系老鼠之樹突細胞與來自 B6 品系老鼠之 T 細胞一起培養。細胞在 37°C、5% CO2 的狀態下培養 5 天後，在收細胞前 18 小時加入 1μCi 的 [3H] TdR。 再以液體閃爍計數器計算嵌入細胞內的 TdR 含量，並且用刺激指數 (stimulation index；S.I.)來表示實驗結果。

4．建立生物晶片來評估樹突細胞的功能

在未來如果有更多的藥物需要篩選，可能需要更多而且更快地篩選方法。為了能夠更大規模地來篩選能夠調節免疫功能的新藥物，如果能將一些主要的相關基因或蛋白利用生物晶片的方式建立起來。未來可以利用更少數的樹突細胞和藥物來進行更大規模的篩檢，來找出有效的成份。所以我們考慮將一些相關的基因或是蛋白如 IL-12、IL-18 或是 IFN-a 等利用生物晶片的方式來大量篩選藥物。

結果與討論

一．本計畫主要是利用試管內評估的方式，我們建立利用樹突細胞來篩選能夠調節或是促進免疫功能的藥物。在細胞模式篩選藥物部份，自 Balb/c 母鼠的大腿骨 (femur 腓骨)及小腿骨 (tibia，脛骨) 內，取得骨髓細胞，以 GM-CSF 及 IL-4 培養 6 天，可得到 dendritic cells，給予藥物十八小時後，收集培養液上清液，分析 dendritic cwlls 分泌 IL-12p40 的情形。使用之純化合物藥物濃度為 50 μg/ml，部分萃取物藥物濃度為 150 μg/ml。我們利用此一方法一共篩選了 523 種不同的藥物，其中包括純化的藥物和粗萃藥物。而在這些藥物中，有 33 種藥物在試驗中為優(其試管內免疫促進的反應為 300%)，而另外有 57 種藥物為良(其免疫促進效果為 150-300%)。

在動物氣喘模式部份，以雞卵蛋白致敏 Balb/c 母鼠三次後，連續六日腹腔注射欲篩選藥物。以雞卵蛋白對 Balb/c 母鼠進行鼻腔內刺激，測量其呼吸道過度反應後，取其肺沖洗液分析細胞比例及細胞激素分泌情形。目前正在進行第一批動物實驗，篩選 4 支藥物為 YCH2-12-1、CYM6-252-1、PCY1-20-4 (PI-06) 及 03331 (PI-14)，各種藥物施以兩種劑量：每隻小鼠 1 μg 或 100 μg。

二．我們也建立一個試管內樹突細胞培養的模式，再利用免疫促進劑刺激後進一步利用 microarray 的方法分析其基因的表現。此一部份完成後，我們再將此一免疫促進劑注射入動物體內觀察其免疫反應的發展是否與這些表現的基因有著密切的關係，結果的確發現樹突細胞的基因表現與體內免疫反應的發展有著密切的關連性。我們在這方面的研究也已經完成論文的寫作，附上論文供參考。

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Polysaccharide Purified from *Ganoderma Lucidum* Induce Gene Expression Changes in Human Dendritic Cells and Promote Th1 Immune Response in BALB/c Mice

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ABSTRACT

Ganoderma Lucidum, is a medicinal mushroom in China and other Asian. The polysaccharide from *Ganoderma Lucidum* (PS-G) is a branched (1→6)-β-D-glucan moiety. PS-G has been reported to exert anti-tumor activity and activation of natural killer calls. In this study, we examined the effects of PS-G on human monocyte-derived dendritic cells (DC) with the method of microarray analysis. Human DCs were cultured with PS-G for 16 h. Changes in gene expression were analyzed using Human Genome U133 Plus 2.0 GeneChip. Comparison of mean signal values between PS-G-treated with untreated control DC, 3,477 (17%) probe sets were up-regulated and 4,418 (19%) probe sets were down-regulated after PS-G treatment. These results demonstrated that genes associated with phagocytosis (CD36, CD206, and CD209) were decreased and associated with pro-inflammatory chemokines (CCL20, CCL5, and CCL19), cytokines (IL-27, IL-23A, IL-12A, and IL-12B), and costimulatory molecules (CD40, CD54, CD80, and CD86) were increased. To confirm the microarray data, we further investigated the effect of PS-G on antigen-specific antibody and cytokine production in BALB/c mice. Immunization with ovalbumin (OVA)/PS-G showed that the anti-OVA IgG2a levels were significantly increased compared with OVA alone. Although PS-G had no effect on the anti-OVA IgG1 levels, it induced the secretion of interferon-γ (IFN-γ) and absence of interleukin (IL)-5. The findings demonstrate that PS-G could be used as adjuvant to induce a T helper cell type-1 (Th1) immunity in BALB/c mice. Taken together, our data demonstrated that PS-G could effectively promote the activation and maturation of immature DC and prefer a Th1 response, 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, anti-tumor activity, antiviral, and in the treatment of asthma, 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 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 *Ganoderma 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 potent antigen presenting cells (APCs), whose primary function is to capture, process, and present antigens to naïve T cells (7). Immature DC reside in non-lymphoid 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 cells responses and may be essential for the development of human vaccines relying on T cells immunity. Fully mature DC show a high surface expression of MHC class II and costimulatory molecules, and the secretion of cytokines and chemokines, 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 pro-inflammatory cytokines (e.g. 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, 12). Several studies have already indicated that the nuclear transcription factor NF-κB also plays an important role in DC maturation (13).

Specific immune responses could be differentiated into Th1 and Th2 responses (14), have distinct roles in the immune system (15). Th1 cells modulate cellular immunity by producing IL-2 and IFN-γ whereas Th2 cells are implicated in humoral response by secreting IL-4, IL-5, and IL-6. In addition, IFN-γ suppresses Th2 immune responses (16), while IL-4 (17) and IL-10 downregulate Th1 responses (18). Antigen-presenting cells such as dendritic cells also play a major role in T helper cell differentiation. IL-12 derived from antigen-presenting cells stimulates IFN-γ production from T cells, thereby favoring a Th1-pattern of response (19). There is clear evidence that IL-12 can suppress IL-4 mRNA induction, both directly and

indirectly, through induction of IFN-γ (20). In the antibody response, IgG2a responses are induced by IFN-γ and suppressed by IL-4 (21). IFN-γ promotes isotype switching to IgG2a (22). IL-12 also stimulates the production of IgG2a, presumably through induction of IFN-γ from T cells and NK cells (23). Switching to IgG1 antibody is regulated by IL-4 and inhibited by IFN-γ (24). Thus, Th1 cells are involved in the differentiation of B lymphocytes and production of the IgG2a isotype (24). Th2 cells help antibody-producing cells to induce class-switching of IgG1.

We have reported that PS-G can induce important changes in the phenotype and function of DC (25), to date, there are no published reports describing genomic-scale analysis of these changes. Therefore, to explore further the concept of PS-G-induced DC maturation and perhaps identify novel genes that are regulated following the interaction of PS-G with DC, we examined at the transcriptional level the effects of PS-G exposure to DC. Additionally, information gained by transcript profiling may prove useful in the development of endpoint measures that can serve as the basis for an *in vitro* method to identify potential China herb.

In the present study, to evaluate the ability of PS-G to modulate the Th1/Th2 balance, we analyzed the gene expression changes in immature human monocyte-derived DC with PS-G, using the Human Genome U133 Plus 2.0 GeneChip (Affymetrix) oligonucleotide microarrays. We also investigated its *in vivo* effect on antigen-specific IgG2a/IgG1 antibodies production and *ex vivo* effect on Th1/Th2 cytokine production by cultured splenocytes derived from OVA-immunized BALB/c mice.

Materials and Methods

PS-G Purification from G. Lucidum. As in our previous study (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 the PS-G samples.

Generation of Human DC. DC were generated from PBMC, as described previously (25,26), with some modification. Briefly, PBMCs were obtained from healthy donors by centrifugation with Ficoll-Hypaque method (Pharmacia, Uppsala, Sweden). PBMCs were incubated with anti-$CD14^+$ microbeads in conjunction with the MiniMACS system by following the manufacturers instructions (Miltenyi Biotech, Auborn, CA, USA). The $CD14^+$ cells at 95-99% purity, as assessed by flow cytometry. The $CD14^+$ cells were cultured at 1×10^6 cells per 1 ml RPMI-1640, 2 mM L-glutamine, streptomycin/penicillin, and 10% FCS supplemented with GM-CSF (800 U/ml) and IL-4 (500 U/ml) in 24-well plates (Costar, Cambridge, MA) to obtain immature dendritic cells (iDC). 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.

RNA Preparation. Cells were harvested 16 h after stimulation. Total RNA from DC was immediately isolated with the Trizole kit (Invitrogen, Valencia, CA) according to the manfacture's instructions and was used to generate cRNA probes. Preparation of cRNA, hybridization, and scanning of the microarrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Briefly, 10 μg/ml of RNA was converted into double-stranded cDNA by RT using a cDNA synthesis kit (SuperScript Choice, Life Technologies, Gaithersburg, MD) with an $oligo(dT)_{24}$ primer containing a T7 RNA polymerase promoter site-added 3' of poly(T) (Genset, La Jolla, CA). After second-strand synthesis, labeled cRNA was generated from the cDNA sample by an *in vitro* transcription reaction supplemented with the Bioarray High Yield RNA transcription labeling kit (Enzo, Farmingdale, NY). The labeled cRNA was purified using RNeasy spin columns (Qiagen, Valencia, CA) and

denatured at 94°C before hybridization.

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

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

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

OVA-Specific Antibody Assay. Sera anti-OVA IgG1 and IgG2a antibody titers were determined by ELISA. Briefly, 96-well flat-bottom plates were coated with 10 μg/ml OVA. After overnight incubation at 4°C, plates were washed and blocked with 3% bovine serum albumin (BSA) in PBS for 2 h at 37°C. Serum samples were diluted and added to each well overnight at 4°C. Then the plates were washed and biotin-conjugated anti-mouse IgG1 (1:5000, PharMingen) or IgG2a (1:1000, PharMingen) was added for 1 h at 37°C. Streptavidin-conjugated HRP (1:10000) was added for an addition 2 h at room temperature. Finally, the reaction was developed by H_2O_2 and tetramethylbenzidine, followed by 50 μl/well of H_2SO_4 stop solution. A_{450} was measured using a microplate reader (Anthos reader 2010; Anthos Labtec Inc,

Salzburg, Austria). The results were expressed in ELISA units (EU): EU = (A_{sample} – A_{blank})/($A_{positive}$ – A_{blank}).

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

Statistical Analysis. The Student *t* test was used to analyze the results, and a *P* value of less than 0.05 was considered to be statistically significant.

Results

Global Characteristics of Gene Expression in PS-G Treated-Human Monoctye-Derived DC. To obtain monocytes with high purity, we preferred magnetic sorting of $CD14^+$ cells rather than enrichment by adherence, as the latter often results in inhomogeneous cell populations. After differentiation to immature DC, then the human DC were cultured with PS-G for 16 h. Changes in gene expression were analyzed using Human Genome U133 Plus 2.0 GeneChip, which contain ~38,500 genes and more than 54,000 probe sets. Comparison of mean signal values from revealed between PS-G-treated with untreated control DC, 3,477 (17%) probe sets were up-regulated and 4,418 (19%) probe sets were down-regulated after PS-G treatment. The entire database can be found on the web site (**http://www**.).

Cytokines and Cytokine Receptors. We demonstrated that transcript levels for cytokines IL-27, IL-12A, IL-12B, IL-23A, and EBI3 were significantly increased after PS-G-treated human dendritic cells, while only transcripts for IL-16 and TGF-β were reduced (Fig. 1). Transcript levels for cytokine receptors IL-2RA, IL-3RA, IL-4R, IL-6R, IL-7R, IL-15R, and IL-22RA1 were increased after PS-G treated, while transcripts for Type II IL-1 receptor (IL1R2) were reduced (Fig. 2).

Chemokines and Chemokines Receptors. Chemokines are a diverse superfamily of small secreted proteins, the regulated expression of chemokines and chemokine receptors is an important component of an integrated immune response. Dendritic cells secrete chemokines and express chemokine receptors. We found the levels of transcripts for CCL20, CCL19, CCL5, CXCL9, CXCL10, CXCL11 and the levels for CCR7 were higher in PS-G-treated DC than in immature DC (Fig. 3). Especial the transcript level of CCL20 (MIP-3α) was 1,363–fold higher than untreated DC.

Transcripts for Cell Surface Receptors. We demonstrated that transcript levels for Toll-like receptor 7 (TLR7) and TLR8 were increased after PS-G-treated human dendritic cells, while transcripts for TLR1, TLR2, and TLR4 were reduced (Fig. 4). When DC maturation leads to the cessation of antigen uptake for antigen presentation, therefore genes for phagocytosis were decreased. We found that transcript levels for CD36, CD206 (MRC1, mannose receptor C type 1), MRC2 (mannose receptor C type 2), CD209 (DC-SIGN), CR3, CLECSF6 (C type lectin, superfamily member 6) and CLECSF12 were significantly decreased (Fig. 4).

Transcripts for Cell Surface Proteins. DC maturation induced high levels of costimulatory molecules. Transcript levels for MHC Class I and Class II molecules were largely affected by maturation. We demonstrated that transcript levels for CD80, CD83, and CD86 were higher (more than twofold increase) in PS-G-treated DC, while the levels for CD1A, CD1B, and CD1C were lower (more than twofold decrease). A notable is maturation associated with reduction of transcript levels for HLA-DM, a molecule that mediates loading of antigenic peptides into MHC Class II molecules, a process more active in immature DC (Fig. 5).

Transcripts for Signal Transduction. In recently, we demonstrated that PS-G go through the TLR4 and rapidly induce the significant activation and maturation of human DC by the NF-κB and p38 MAPK pathways. In this study, we found that transcript levels for NFKB1, NFKB2, RELA, RELB, and MAPK11 were increased in PS-G-treated DC (Fig. 6). The results also show a marked effect of the PS-G on the expression of numerous IFN-regulated genes (IFI27, IFI35, IFI44, IFIT1, IFIT2, IFIT4, and IFITM3) in human DC. In this microarray data, ISG20 (interferon stimulated gene 20kDA) is the most highly expressed (1,522-fold) gene in PS-G-treated DC.

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

Regulatory Effect of PS-G on the Balance of Th1/Th2 Cell Responses in OVA-Immunized Mice. It has been reported that cytokines play an important role in the antibody response. Therefore, we examined the regulatory effect of PS-G on Th1/Th2 cell responses in OVA-immunized mice, mice were immunized with OVA

plus PS-G on day 0, 14, and 28. IFN-γ (Th1 cytokine) and IL-5 (Th2 cytokine) production in splenocytes stimulated with OVA was assayed (Fig. 8). The production of IFN-γ significant increased in PS-G admixed with OVA-immunized mice compared with OVA-immunized mice ($p=0.026$) (Fig. 8A). The production of IL-5 significant decreased in PS-G admixed with OVA-immunized mice compared with OVA-immunized mice ($p=0.036$) (Fig. 8B). These results indicated that the PS-G changed the balance of Th1/Th2 cell immune responses from Th2-dominant to Th1-dominant in OVA-immunized mice.

Discussion

In the current studies, we demonstrated that PS-G induced morphological, phenotypical, and functional changes in human monocyte-derived DC (25). However, there are no published reports describing genomic-scale analysis of the changes induced in human DC resulting from PS-G treatment. In this study, we examine changes in gene expression in human monocyte-derived DC that are treated by exposure to PS-G using Affymetrix GeneChip microarrays. Comparison of mean signal values between PS-G-treated with untreated control DC, 3,477 (17%) probe sets were two-fold up-regulated and 4,418 (19%) probe sets were two-fold down-regulated after PS-G treatment, we focused our attention only on those genes for which expression was found to be significantly different from control DC.

The biological process of DC maturation represents a crucial step in the initiation of adaptive immune responses (27). This process is regulated by various extracellular stimuli, including cytokines, bacterial products, and membrane-bound ligands (28). DC maturation is accompanied by changes in their morphological, phenotypic, and functional properties (10). PS-G promoted the maturation of DC, while mature DC demonstrated characteristic morphology, with enlarged size and numerous cytoplasmic processes that gave rise to a stellate appearance (data not shown). Maturation of DC was characterized by a decreased antigen processing capacity and an increased cell surface expression of MHC class II molecule and co-stimulatory molecules, and the secretion of IL-12, which primed a strong stimulation of T lymphocytes growth and differentiation. The CD83 marker for mature human DC was also increased. In this study we demonstrated that genes associated with phagocytosis (CD36, CD206, and CD209) were down-regulated and associated with pro-inflammatory chemokines (CCL20, CCL5, and CCL19) and cytokines (IL-27, IL-23A, IL-12A, and IL-12B), and costimulatory molecules (CD40, CD80, and CD86) were increased. These results are correlated with our current report, we found that treatment of DC with PS-G resulted in the enhanced cell-surface expression of CD80, CD86, CD83, CD40, CD54, and HLA-DR by flow cytometry, as well as the enhanced

production of IL-12p70 and p40 by ELISA method (25), and the significant activation and maturation of human DC is by the NF-κB and p38MAPK pathways.

In immune responses, IL-12 plays a central role as a link between the innate and adaptive immune systems (29). Thus, IL-12 induces and promotes natural killer (NK) and T cells to generate IFN-γ and lytic activity. In addition, IL-12 polarizes the immune system towards a Th1 response. Moreover, since IL-12 has been shown to prevent development of the Th2 immune response in several mice models of immune activation and infection (30). Some studies indicated that IL-23 and IL-27, two cytokines that are closely related to IL-12, also regulate Th1-cell responses (31). IL-23, comprises of a p19 subunit and IL-12p40, is produced by activated macrophages and dendritic cells (32). IL-27 is a heterodimeric cytokine composed of p28, a newly identified IL-12p35-related protein, and Epstein-Barr virus-induced gene 3 (EBI3), an IL-12p40-related protein. IL-27 is produced primarily from activated dendritic cells and induces an early phase of T-helper type I differentiation. Chiyo et al. suggested that expressed IL-27 in tumors produces T cell-dependent and-independent anti-tumor effects and is a possible therapeutic strategy for cancer (33). A number of studies demonstrated that secretion of Th1-type cytokines from tumors activated host defense mechanisms and consequently produced anti-tumor effects. Transfer of cytokine genes is thereby a possible strategy for cancer treatment and is currently investigated for its clinical feasibility (34,35).

Chemokines cause recruitment and polarization of T cells (36). Furthermore certain chemokines such as CCL5 (RANTES) are able to costimulate T cell proliferation. In this study, we report that PS-G can induce the transcripts of CCL20, CCL19, CCL5, CXCL9, CXCL10, and CXCL11 in human DC. Especial PS-G induced the highly transcript of CCL20 about 1,363-fold than in untreated DC. CCL20 (macrophage inflammatory protein-3α, MIP-3α) is the only chemokine known to interact with CC chemokine receptor 6 (CCR6), a property shared with the antimicrobial β-defensins. The CCL20-CCR6 is responsible for the chemoattraction of immature DC, effect/memory T cells and B cells and plays a role at skin and mucosal surfaces under homeostatic and inflammatory conditions, and in pathology, including cancer and rheumatoid arthritis (37). Adenovirus-mediated gene transfer of human CCL20 cDNA by injection in a variety of preformed mice subcutaneous tumors, led to intra-tumor expression of CCL20 (38). This strategy induced a local accumulation of immature DC, resulting in tumor-specific cellular immunity and significant growth suppression of established tumors. *Ganoderma Lucidum* has been reported to have anti-tumor activity (2-4). Recent clinical studies have demonstrated that the polysaccharide fractions of *Ganoderma Lucidum* polysaccharides have potential antitumor activity and enhance host immune functions (39). Therefore, we

suggested that PS-G from *Ganoderma Lucidum* could induce transcripts of CCL20, IL-27, IL-23A, IL-12A, and IL-12B in human DC, these genes might play an important role in the treatment of cancer.

In this study, we demonstrated that TRIF-dependent genes include IRF-1 (8-fold), IRF-7 (11-fold), Mx1 (12-fold), Mx2 (23-fold), and ISG-20 (1,522-fold) were significantly up-regulated in PS-G-treated DC, which have been implicated in the generation of antiviral immune responses or viral replication (40,41). IRF-1 was also reported to be required for Th1 responses (42). ISG-20 is a 3'→5' exonuclease whose gene is transcriptionally induced by both type I and type II IFN (43). Its induction by IFN is strictly dependent upon the activation and binding of IRF1 to a specific ISRE on the Isg20 promoter. Moreover, the TATA-less Isg20 promoter contains one E-box and putative NF-κB and Sp1 binding sites suggesting that it could be induced by other stimuli. ISG20 has an antiviral activity, supporting the idea that it might represent a novel antiviral pathway (44). *Ganoderma Lucidum* has been reported to have antiviral activity (45). From our result, we found that ISG20 is the most highly expressed in PS-G-treated DC. While PS-G also can induce transcripts of type I and type II IFN. Therefore, we inferred that one reason for PS-G could involve in the antiviral function by type I, type II IFN, and ISG20 production.

Recently, several researchers have demonstrated the immunomodulatory effects of polysaccharides purified from *Ganoderma lucidum* on T lymphocytes (46). But there are only a limited number of studies on the adjuvant effects of polysaccharides purified from *Ganoderma lucidum* on antibody production. Therefore, we further investigated the adjuvant effects of PS-G on antigen-specific antibody and cytokine production using BALB/c mice immunized with OVA antigen. This study demonstrates that PS-G appears to have marked induction effects on Th1 responses since treatment of mice with PS-G was followed by an increased in Th1 response including anti-ovalbumin IgG2a and IFN-γ production. Moreover, PS-G had no effect on the anti-OVA IgG1 levels. These findings demonstrate that PS-G could be used as adjuvant to induce a Th1 immunity in BALB/c mice. Taken together, our data demonstrated that PS-G could effectively promote the activation and maturation of immature DC and prefer a Th1 response, suggesting that PS-G may possess a potential capacity in regulating immune responses. The precise mechanism by which PS-G induced Th1 responses *in vivo* might be the PS-G can induce DC activation and maturation, and induce Th1 related cytokines and chemokines production.

In conclusion, we demonstrated that PS-G effectively and rapidly induced the significant activation and maturation of human DC. PS-G also is an adjuvant-active molecule that stimulates Th1 response, as a non-toxic and very stable compound, it could find its application as an adjuvant for vaccines. Therefore, PS-G is a good and

potential part of the treatment regimen to regulate host immune responses, and this may provide information for further designing PS-G-treated-DC-based immunotherapies for many diseases. Additionally, it is hoped that some of the transcript changes identified with microarray analysis will be shown to be suitable for use in the development of an *in vitro* predictive assay for China herbs.

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Figure legends:

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

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

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

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

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

Fig. 6. List of signal transduction genes that were significantly changed (more than two-fold) in PS-G-treated compared with untreated human monocyte-derived dendritic cells. Human dendritic cells were treated with PS-G or untreated for 16 h. RNA was prepared and processed for hybridization to Affymetrix Human Genome U133 Plus 2.0 GeneChip and analyzed as described in Materials and Methods.

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

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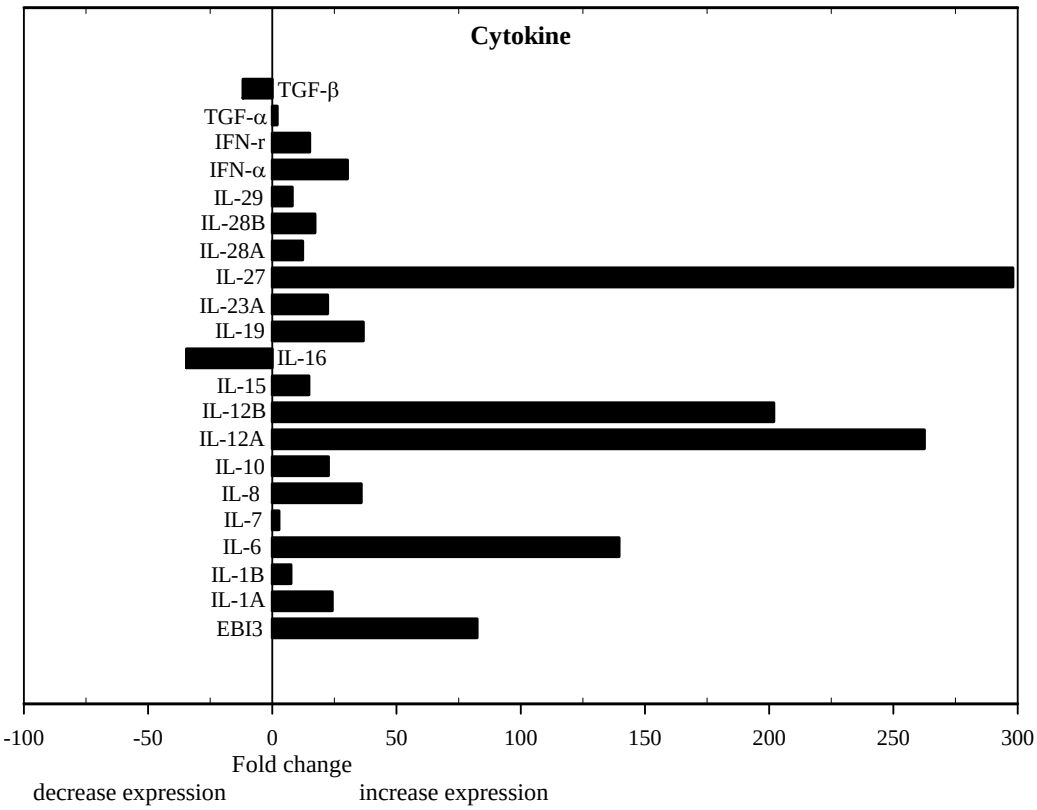
Fig. 8. IFN-γ and IL-5 production by murine splenocytes after immunization of Balb/c mice with OVA plus PS-G. Mice were immunized as described in Fig. 7. The splenocytes were prepared on day 49, and were incubated at 37 °C with OVA (10 μg/ml) for 48 h. The culture supernatants were collected and used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


Cytokine
TGF-β
TGF-α
IFN-r
IFN-α
IL-29
IL-28B
IL-28A
IL-27
IL-23A
IL-19
IL-16
IL-15
IL-12B
IL-12A
IL-10
IL-8
IL-7
IL-6
IL-1B
IL-1A
EBI3
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-50
0
50
100
150
200
250
300
Fold change
decrease expression
increase expression

Fig. 1.

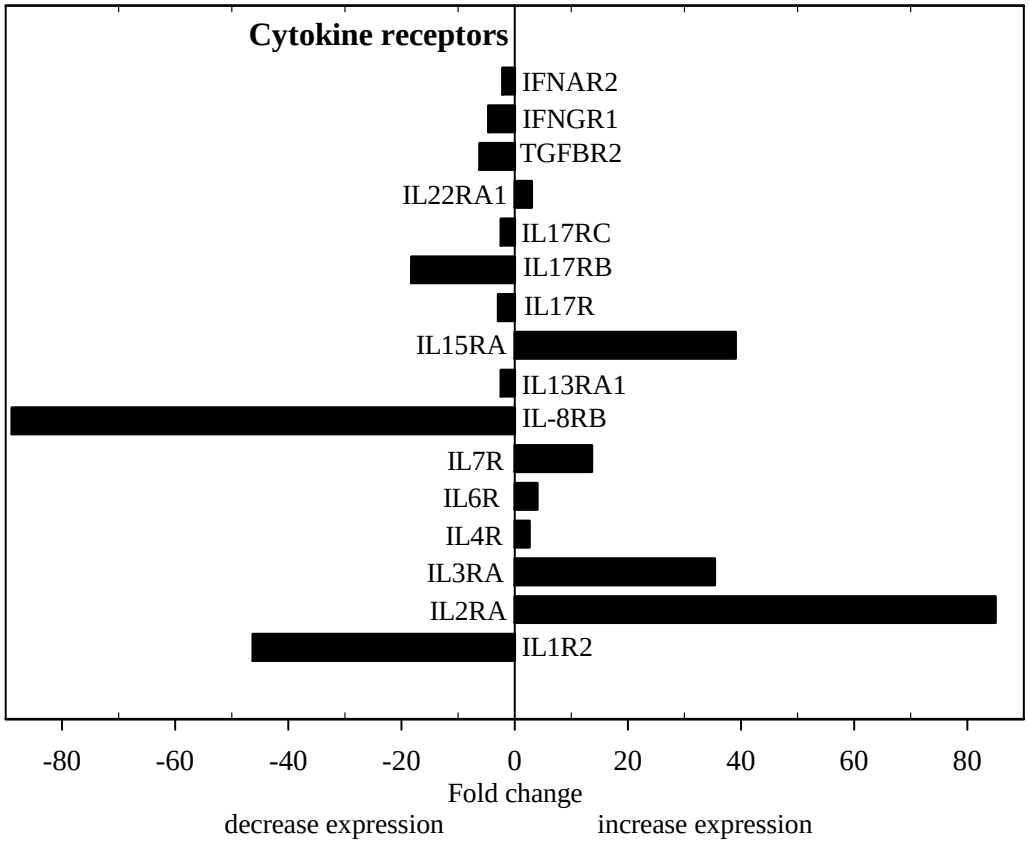
Cytokine receptors
IFNAR2
IFNGR1
TGFBR2
IL22RA1
IL17RC
IL17RB
IL17R
IL15RA
IL13RA1
IL-8RB
IL7R
IL6R
IL4R
IL3RA
IL2RA
IL1R2
-80
-60
-40
-20
0
20
40
60
80
Fold change
decrease expression
increase expression

Fig. 2

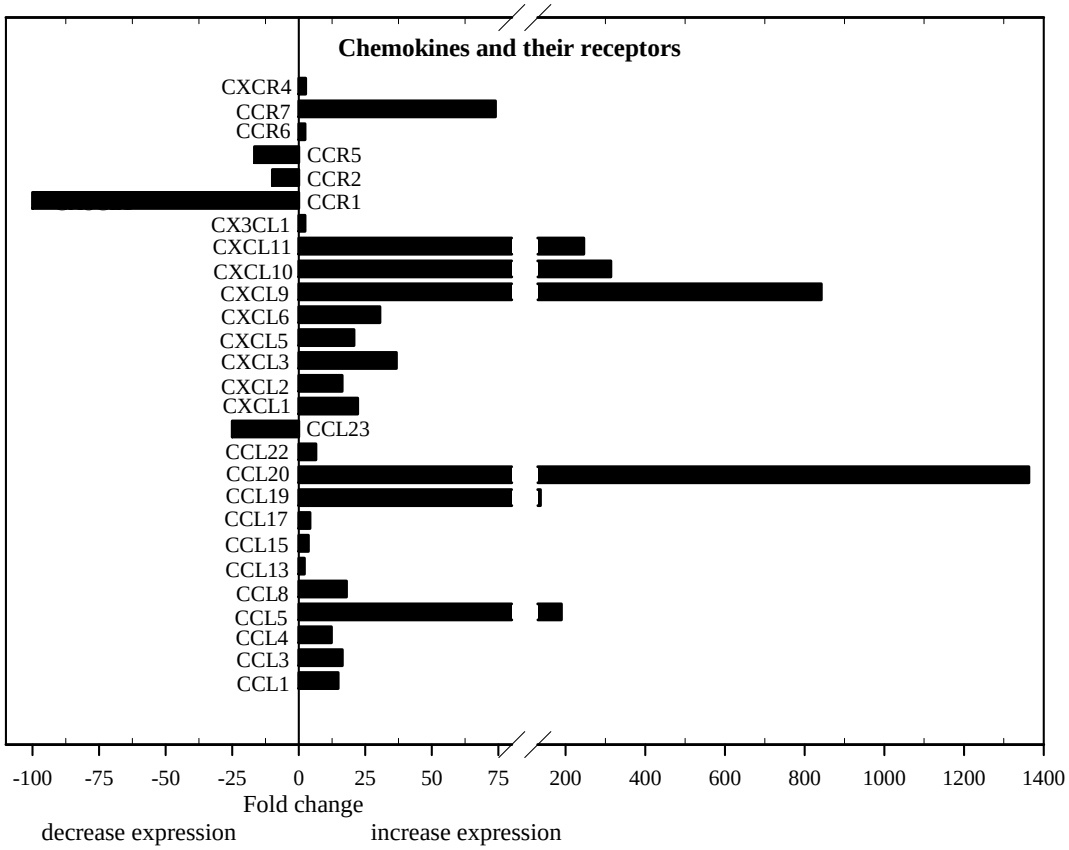
Chemokines and their receptors
CXCR4
CCR7
CCR6
CCR5
CCR2
CCR1
CX3CL1
CXCL11
CXCL10
CXCL9
CXCL6
CXCL5
CXCL3
CXCL2
CXCL1
CCL23
CCL22
CCL20
CCL19
CCL17
CCL15
CCL13
CCL8
CCL5
CCL4
CCL3
CCL1
-100 -75 -50 -25 0 25 50 75 200 400 600 800 1000 1200 1400
Fold change
decrease expression
increase expression

Fig. 3.

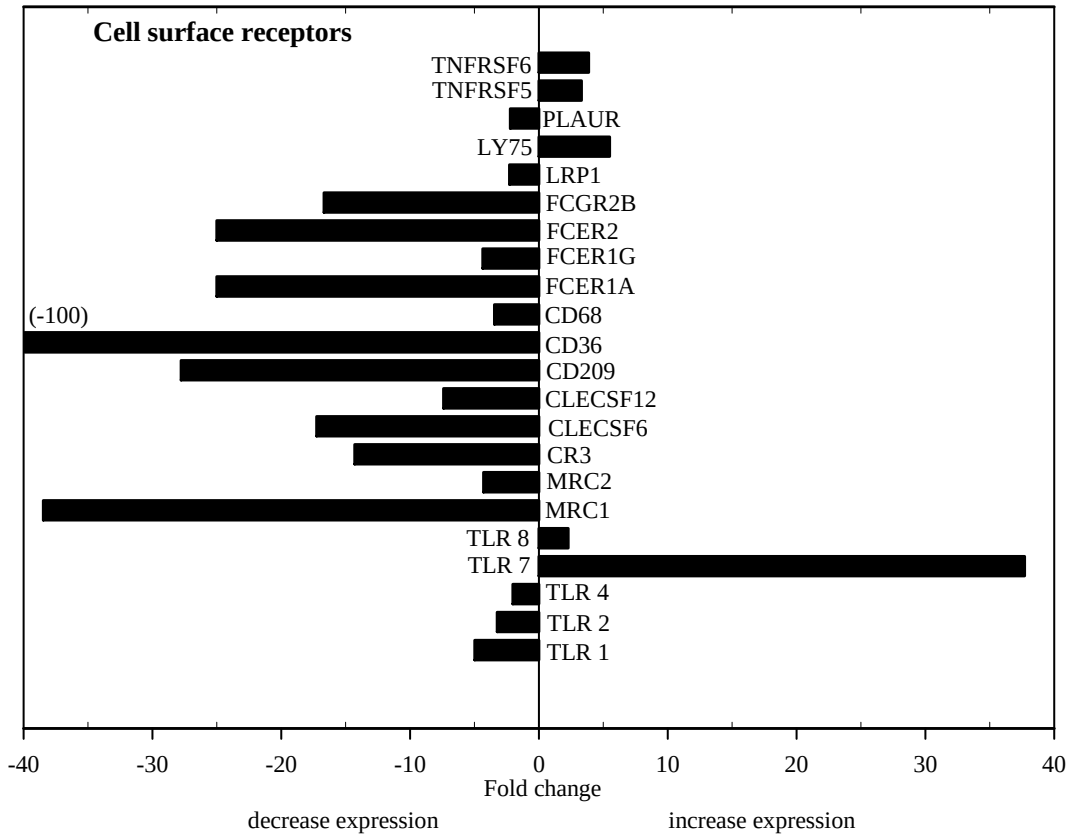
Cell surface receptors
TNFRSF6
TNFRSF5
PLAUR
LY75
LRP1
FCGR2B
FCER2
FCER1G
FCER1A
CD68
CD36
CD209
CLECSF12
CLECSF6
CR3
MRC2
MRC1
TLR 8
TLR 7
TLR 4
TLR 2
TLR 1
(-100)
-40
-30
-20
-10
0
10
20
30
40
Fold change
decrease expression
increase expression

Fig. 4.

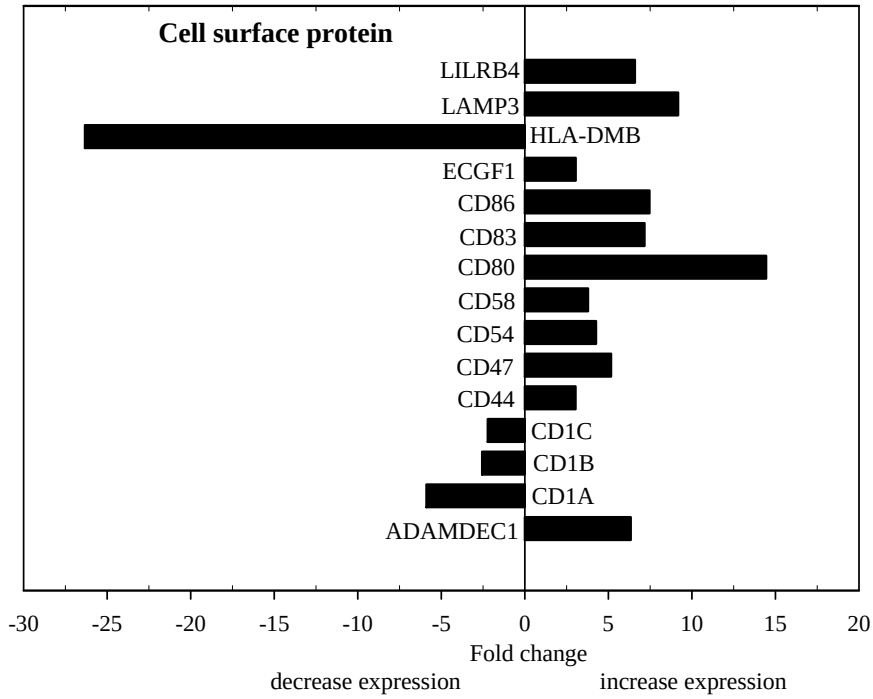
Cell surface protein
LILRB4
LAMP3
HLA-DMB
ECGF1
CD86
CD83
CD80
CD58
CD54
CD47
CD44
CD1C
CD1B
CD1A
ADAMDEC1
-30
-25
-20
-15
-10
-5
0
5
10
15
20
Fold change
decrease expression
increase expression

Fig. 5.

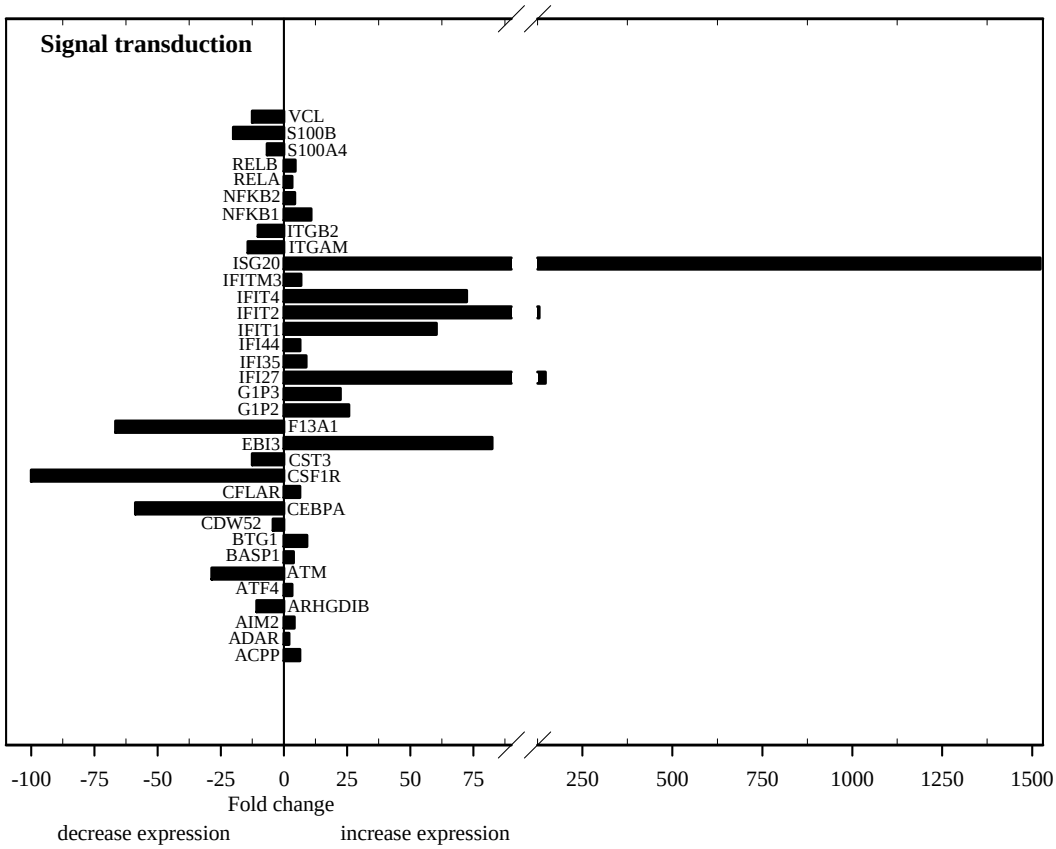
Signal transduction
VCL
S100B
S100A4
RELB
RELA
NFKB2
NFKB1
ITGB2
ITGAM
ISG20
IFITM3
IFIT4
IFIT2
IFIT1
IFI44
IFI35
IFI27
G1P3
G1P2
F13A1
EBI3
CST3
CSF1R
CFLAR
CEBPA
CDW52
BTG1
BASP1
ATM
ATF4
ARHGDIB
AIM2
ADAR
ACPP
-100 -75 -50 -25 0 25 50 75 250 500 750 1000 1250 1500
Fold change
decrease expression
increase expression

Fig. 6.

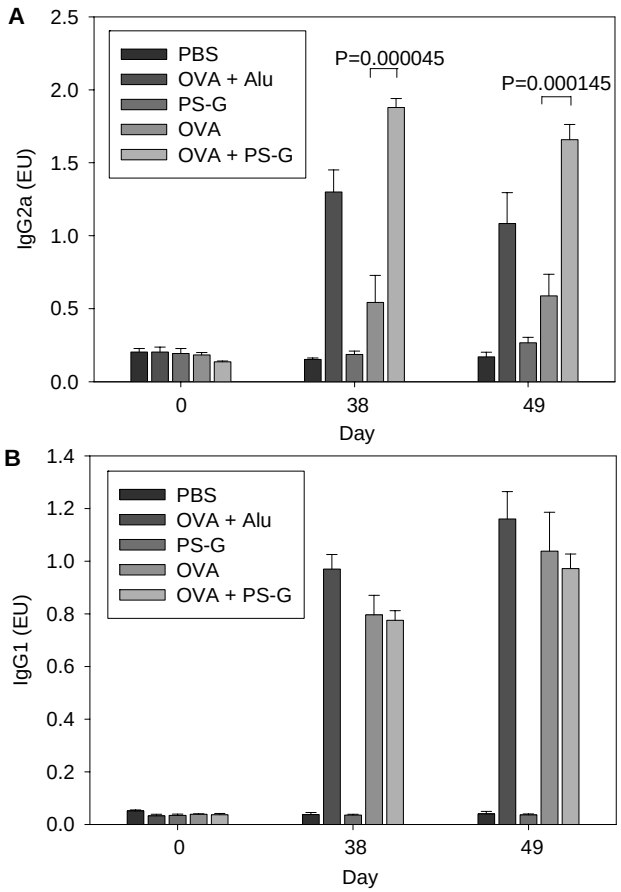
A
IgG2a (EU)
2.5
2.0
1.5
1.0
0.5
0.0
PBS
OVA + Alu
PS-G
OVA
OVA + PS-G
P=0.000045
P=0.000145
0
38
49
Day
B
IgG1 (EU)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PBS
OVA + Alu
PS-G
OVA
OVA + PS-G
0
38
49
Day

Fig. 7.

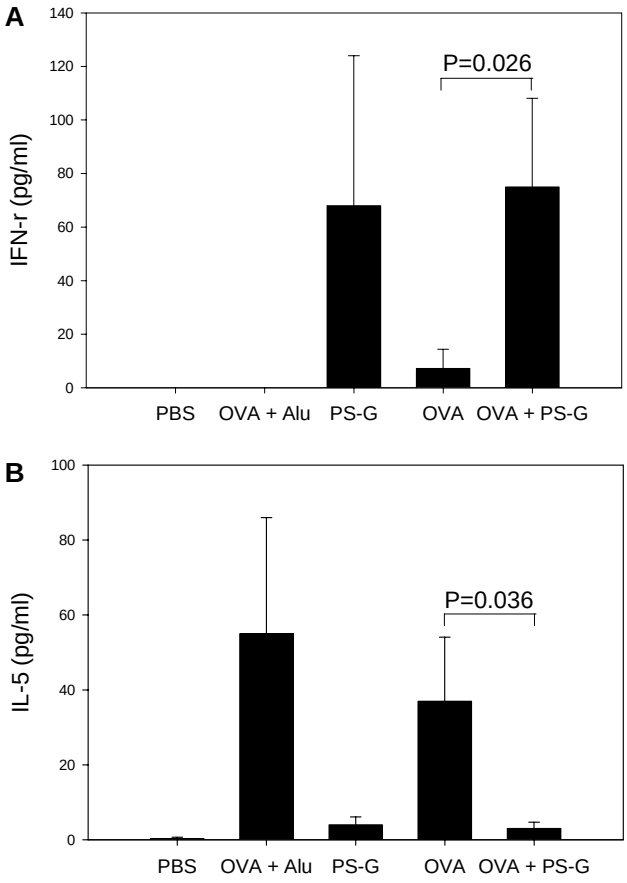
A
IFN-r (pg/ml)
140
120
100
80
60
40
20
0
P=0.026
PBS
OVA + Alu
PS-G
OVA
OVA + PS-G
B
IL-5 (pg/ml)
100
80
60
40
20
0
P=0.036
PBS
OVA + Alu
PS-G
OVA
OVA + PS-G

Fig. 8.