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

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ABBREVIATION: NF-κB, nuclear factor-κB; DC, dendritic cell; Th1, T helper cell type-1; Th2, T helper cell type-2; OVA, ovalbumin; IFN-γ, interferon-γ.

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ABSTRACT

Ganoderma Lucidum is a medicinal mushroom in China and other Asian countries. The polysaccharide from *Ganoderma Lucidum* (PS-G) is a branched (1 → 6)-β-D-glucan moiety. In this study, we examined the effects of PS-G on human monocyte-derived dendritic cells (DC) with microarray analysis by Human Genome U133 Plus 2.0 GeneChip. In comparing mean signal values between PS-G-treated DC with untreated DC, 3,477 (17%) probe sets were up-regulated, and 4,418 (19%) probe sets were down-regulated after PS-G treatment. These results demonstrate that genes associated with phagocytosis (CD36, CD206, and CD209) are decreased and 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) are increased. To confirm the microarray data, we further investigated the effect of PS-G on antigen-specific antibody and cytokine production in BALB/c mice. Immunization with ovalbumin (OVA)/PS-G showed that the anti-OVA IgG2a levels were significantly increased compared with OVA alone in BALB/c mice. Taken together, our data demonstrates that PS-G could effectively promote the activation and maturation of immature DC, preferring a Th1 response. Further, the results also demonstrate that the data from microarray analysis could be correlated with the *in vivo* effect of the immune-enhancing compound.

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Introduction

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

Dendritic cells (DC) are the most potent antigen presenting cells, whose primary function is to capture, process, and present antigens to naïve T cells (Banchereau and Steinman, 1998). 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 antigen-processing activity and mature to become potent

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immunostimulatory cells (Cella et al., 1997). 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 cell immunity. Fully mature DCs show high surface expression of MHC class II and costimulatory molecules, and the secretion of cytokines and chemokines, but a decreased capacity to internalize antigens (Cella et al., 1997). Up-regulation of CD83, a specific marker for DC maturation, also occurs (Sallusto et al., 1995). Various stimuli, such as pro-inflammatory cytokines (e.g. TNF-α and IL-1), CD40 ligation, bacterial products (e.g. LPS and unmethylated DNA CpG motif), and contact sensitizers, can induce DC maturation *in vivo* and *in vitro* (Caux et al., 1998; Jakob et al., 1998). Several studies have already indicated that nuclear transcription factor NF-κB also plays an important role in DC maturation (Yoshimura et al., 2001).

Specific immune responses that can be differentiated into Th1 and Th2 responses have distinct roles in the immune system (Mossmann and Coffman, 1989). Th1 cells modulate cellular immunity by producing IL-2 and IFN-γ whereas Th2 cells are implicated in humoral response by secreting IL-4, IL-5, and IL-6. In addition, IFN-γ suppresses Th2 immune responses (Dickensheets et al., 1999), while IL-4 and IL-10 downregulate Th1 responses (Yin et al, 1997). Antigen-presenting cells such as dendritic cells also play a major role in T helper cell differentiation. IL-12 derived

from antigen-presenting cells stimulates IFN-γ production from T cells, thereby favoring a Th1-pattern of response (Romagnani, 1994). There is clear evidence that IL-12 can suppress IL-4 mRNA induction, both directly and indirectly, through induction of IFN-γ (Finkelman et al., 1994). In the antibody response, IgG2a responses are induced by IFN-γ and suppressed by IL-4 (Snapper and Paul, 1987). IFN-γ promotes isotype switching to IgG2a. IL-12 also stimulates the production of IgG2a, presumably through induction of IFN-γ from T cells and NK cells (McKnight et al, 1994). Switching to IgG1 antibody is regulated by IL-4 and inhibited by IFN-γ (Schmitt et al., 1994). Thus, Th1 cells are involved in the differentiation of B lymphocytes and production of the IgG2a isotype (Schmitt et al., 1994). Th2 cells help antibody-producing cells to induce class-switching of IgG1.

We have reported that PS-G can induce important changes in the phenotype and function of DC (Lin et al., 2005) To date, there are no published reports describing genomic-scale analysis of these changes. Therefore, to explore further the concept of PS-G-induced DC maturation and perhaps identify novel genes that are regulated 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 immune-enhancing compound.

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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. Since IFN-γ increase and Th1 polarization is usually the consequence of IL-12 production by DC, we used anti-IL-12 antibody in MLR to clarify this point. We also investigated its *in vivo* effect on antigen-specific IgG2a/IgG1 antibodies production and *ex vivo* effect on Th1/Th2 cytokine production by cultured splenocytes derived from OVA-immunized BALB/c mice.

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Materials and Methods

PS-G Purification from G. Lucidum. As in our previous study (Wang et al, 1997), fruiting bodies of *G. lucidum* were washed, disintegrated, and extracted with boiling water for 8-12 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 (Miyazaki and Nishijima, 1981). 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 (Lin et al., 2005; Zhou and Tedder, 1996), with some modification. Briefly, PBMCs were obtained from healthy donors by centrifugation with the Ficoll-Hypaque method (PharMacia, Uppsala, Sweden). PBMCs were incubated with anti-$CD14^+$ microbeads in conjunction with the MiniMACS system by following the manufacturer

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instructions (Miltenyi Biotech, Auborn, CA, USA). The $CD14^+$ cells were 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 a Trizole kit (Invitrogen, Valencia, CA) according to the manufacturer 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

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denatured at 94°C before hybridization.

Microarray Hybridization and Data Analysis. Labeled cRNA was hybridized to the Affymetrix U133 Plus 2.0 Genechip while rotating at 60 rpm for 16 h at 45°C. After hybridization, the microarray was washed using the Affymetrix Fluidics Station in buffer containing biotinylated anti-streptavidin 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 Affymetrix software.

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

Autologous mixed leukocyte reaction (MLR). PBMCs were obtained as described above, and naïve $CD4^+$ T cells were purified by a naïve $CD4^+$ T cell

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isolation kit (Miltenyi Biotec, Auburn, CA). The autologous $CD4^+$ T cells obtained were distributed at 10^5 cells per well and incubated for 3 or 5 days in the presence of 10^4 cells per well of PS-G-treated or non-treated DCs plus or non-plus anti-IL-12 monoclonal antibody (20 μg/ml). On day 3, the culture supernatants were harvested for IL-5 and IFN-γ analysis. On day 5, tritiated thymidine (1 μCi/well; New England Nuclear, Boston, MA) was added and the cells were incubated for another 16 h. The cells were harvested on a cell harvester (Packard Instrument Co., Meriden, CT, USA), and the incorporated radioactivity was measured using a beta counter (Packard Instrument Co., Meriden, CT, USA).

Mice and Immunizations. Female BALB/c mice were obtained from the Animal Center of the College of Medicine, National Taiwan University. 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 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

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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 additional 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 plates 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

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culture supernatants were assayed with an ELISA kit (R&D) according to the manufacturer's instructions.

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

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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, the human DC were cultured with PS-G for 16 h. Changes in gene expression were analyzed using the Human Genome U133 Plus 2.0 GeneChip, which contains ~38,500 genes and more than 54,000 probe sets. Comparing mean signal values between PS-G-treated with untreated control DC, 3,477 (17%) probe sets were up-regulated, and 4,418 (19%) probe sets were down-regulated after PS-G treatment. The entire control and PS-G raw databases can be found on the web site (http://backup.mc.ntu.edu.tw/ntu2net/20060105/).

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).

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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). Of especial significance, the transcript level of CCL20 (MIP-3α) was 1,363–fold higher than untreated DC.

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 a two-fold increase) in PS-G-treated DC, while the levels for CD1A, CD1B, and CD1C were lower (more than a two-fold decrease). Of particular note was 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. 4).

Transcripts for Signal Transduction. 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

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transcript levels for NFKB1, NFKB2, RELA, RELB, and MAPK11 were increased in PS-G-treated DC (Fig. 5). The results also show a marked effect of PS-G on the expression of numerous IFN-regulated genes (IFI27, IFI35, IFI44, IFIT1, IFIT2, IFIT4, and IFITM3) in human DC. In this set of microarray data, ISG20 (interferon stimulated gene 20kDA) was the most highly expressed (1,522-fold) gene in PS-G-treated DC.

PS-G-stimulated DCs polarizes naïve T cells for Th1 response, and IL-12 neutralization reduces IFN-γ production. In the current study, we demonstrated that treatment of DC with PS-G resulted in the enhanced expression of IL-12 p35 and IL-12 p40 mRNA by RT-PCR, and enhanced production of IL-12 p70 and p40 by the ELISA method (Lin et al., 2005). Since IFN-γ increase and Th1 polarization is usually the consequence of IL-12 production by dendritic cells. To clarify whether PS-G-stimulated DCs can induce Th1 polarization and its reversal induced by anti-IL-12 antibody were studied. DCs were treated with PS-G, these cells were then used to activate $CD4^+$ naïve T cells. The results presented in Figure 6A show that PS-G-treated DCs significantly enhanced T cell activation compared with untreated DCs ($p=0.00029$). The IFN-γ production induced under these experiment conditions was far higher than untreated-DCs ($p=0.01489$) (Fig. 6B) and the IL-5 production was no effect (Fig. 6C). When the addition of an anti-IL-12 antibody to the

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co-culture of PS-G-treated DCs with T cells, we demonstrated that the secretion of IFN-γ was markedly decreased compared with no addition of an anti-IL-12 antibody ($p=0.016291$), thus indicating that IL-12 production by PS-G-treated DCs is critical for the Th1 polarization.

Effect of PS-G on Serum Anti-ovalbumin Antibody Levels. To confirm the microarray data, we studied the effect of PS-G on antigen-specific IgG1 and IgG2a in OVA-immunized BALB/c mice. We obtained serum from OVA-immunized 38 and 49 days after first immunization. In PS-G admixed with OVA-immunized mice, antigen-specific IgG2a production was significantly increased at the time point ($p=0.000045$, day 38; $p=0.000145$, day 49) examined after immunization 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

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of IFN-γ significantly increased in PS-G admixed with OVA-immunized mice compared with OVA-immunized mice ($p=0.026$) (Fig. 8A). The production of IL-5 significantly decreased in PS-G admixed with OVA-immunized mice compared with OVA-immunized mice ($p=0.036$) (Fig. 8B). These results indicate that PS-G changed the balance of Th1/Th2 cell immune responses from Th2-dominant to Th1-dominant in OVA-immunized mice.

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Discussion

In the current study, we demonstrated that PS-G induced morphological, phenotypical, and functional changes in human monocyte-derived DC (Lin et al, 2005). 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 examined changes in gene expression in human monocyte-derived DC that were treated by exposure to PS-G using Affymetrix GeneChip microarrays. In comparison of mean signal values between PS-G-treated DC 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. This process is regulated by various extracellular stimuli, including cytokines, bacterial products, and membrane-bound ligands (O'Sullivan and Thomas, 2002). DC maturation is accompanied by changes in their morphological, phenotypic, and functional properties (Sallusto et al., 1995). PS-G promoted the maturation of DC, while mature DC demonstrated characteristic morphology, with enlarged size and numerous cytoplasmic processes that gave rise to

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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 molecules 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 (data not shown) and that the association 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 were correlated with our current report, and 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 enhanced production of IL-12p70 and p40 by the ELISA method (Lin et al., 2005). 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 (Trinchieri, 1998). 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, IL-12 has been shown to prevent development of the Th2 immune response in several mice models of

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immune activation and infection (Lee et al., 2001). Some studies indicate that IL-23 and IL-27, two cytokines that are closely related to IL-12, also regulate Th1-cell responses (Hunter, 2005). IL-23, comprised of a p19 subunit and IL-12p40, is produced by activated macrophages and dendritic cells (Langrish et al., 2004). IL-27 is a heterodimeric cytokine composed of p28, a newly identified IL-12p35-related protein, and Epstein-Barr virus-induced gene 3 (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 (Chiyo et al., 2005). A number of studies have demonstrated that secretion of Th1-type cytokines from tumors activated host defense mechanisms have consequently produced anti-tumor effects. Transfer of cytokine genes is thereby a possible strategy for cancer treatment and is currently being investigated for its clinical feasibility (Chiyo et al., 2004; Triozzi et al., 2005).

Chemokines cause recruitment and polarization of T cells (Wong and Fish, 2003). 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. Of particular importance, PS-G induced the highest transcript of CCL20 about 1,363-fold

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compared with 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 in skin and mucosal surfaces under homeostatic and inflammatory conditions, and in pathology, including cancer and rheumatoid arthritis (Schutyser et al., 2003). Adenovirus-mediated gene transfer of human CCL20 cDNA by injection in a variety of preformed mice subcutaneous tumors, led to intra-tumor expression of CCL20 (Fushimi et al., 2000). 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 (Wang et al., 1997; Furusawa et al., 1992; Lee et al., 1995). Recent clinical studies have demonstrated that the polysaccharide fractions of *Ganoderma Lucidum* polysaccharides have potential antitumor activity and enhance host immune functions (Gao et al., 2005). Therefore, we suggest that PS-G from *Ganoderma Lucidum* could induce transcripts of CCL20, IL-27, IL-23A, IL-12A, and IL-12B in human DC. As such, these genes might play an important role in the treatment of cancer.

In this study, we demonstrated that TRIF-dependent genes including IRF-1 (8-fold), IRF-7 (11-fold), Mx1 (12-fold), Mx2 (23-fold), and ISG-20 (1,522-fold)

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were significantly up-regulated in PS-G-treated DC, which have been implicated in the generation of antiviral immune responses or viral replication (Sato et al., 2000; Horisberger, 1995). IRF-1 has also been reported to be required for Th1 responses (Lohoff et al., 1997). ISG-20 is a 3'→5' exonuclease whose gene is transcriptionally induced by both type I and type II IFN (Espert et al., 2003). Its induction by IFN is strictly dependent upon the activation and binding of 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 (Espert et al., 2004). *Ganoderma Lucidum* has been reported to have antiviral activity (Li et al., 2005). From our results, we found that ISG20 is the most highly expressed in PS-G-treated 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 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 (Gao et al., 2003). However, 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

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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 increase in Th1 response including anti-ovalbumin IgG2a and IFN-γ production. Moreover, PS-G had no effect on the anti-OVA IgG1 levels. These findings demonstrate that PS-G could be used as adjuvant to induce Th1 immunity in BALB/c mice. 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 the potential capacity in regulating immune responses. The precise mechanism by which PS-G induced Th1 responses *in vivo* might be that the PS-G can induce DC activation and maturation, and thus induce Th1 related cytokines and chemokines production.

In conclusion, we demonstrated that PS-G effectively and rapidly induced the significant activation and maturation of human 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 study may provide information for the further design of PS-G-treated-DC-based immunotherapies for many diseases. Additionally, it is hoped that some of the

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transcript changes identified with microarray analysis will be shown to be suitable for use in the development of an *in vitro* predictive assay for Chinese herbs.

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Footnotes:

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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 the Materials and Methods section.

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 the Materials and Methods section.

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 the Materials

and Methods section.

Fig. 4. 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 the Materials and Methods section.

Fig. 5. 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 the Materials and Methods section.

Fig. 6. T cells primed with DCs treated with anti-IL-12 release lower amount of IFN-γ. (A) iDCs (10^6 cells/ml) were treated with or without PS-G (10 μg/ml) for 24 h and then washed and cocultured with naïve $CD4^+$ autologous T cells (10^5 cells/well) plus or non-plus anti-IL-12 monoclonal antibody (20 μg/ml) at DC-T ratio of 1:10. After 5

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days, proliferation of autologous T cells was measured by [^{3}H] thymidine incorporation. Supernatants were analyzed for (B) IFN-γ and (C) IL-5, produced by activated T cells after 2 days of culture. Data represents the mean ± SE of triplicates.

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.

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 determine IFN-γ and IL-5 production. The data shown are means ± SE.

Figure 1

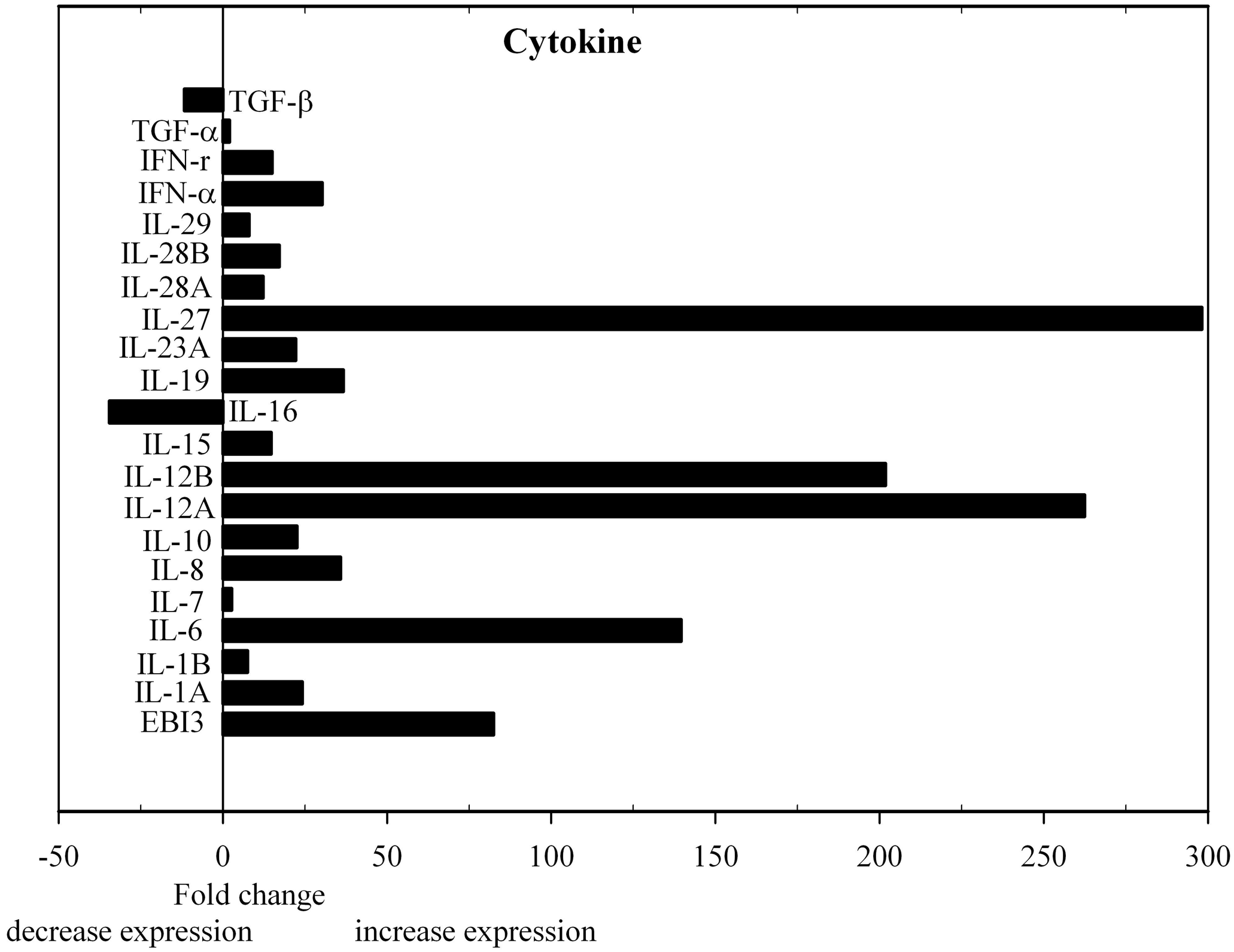

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Figure 2

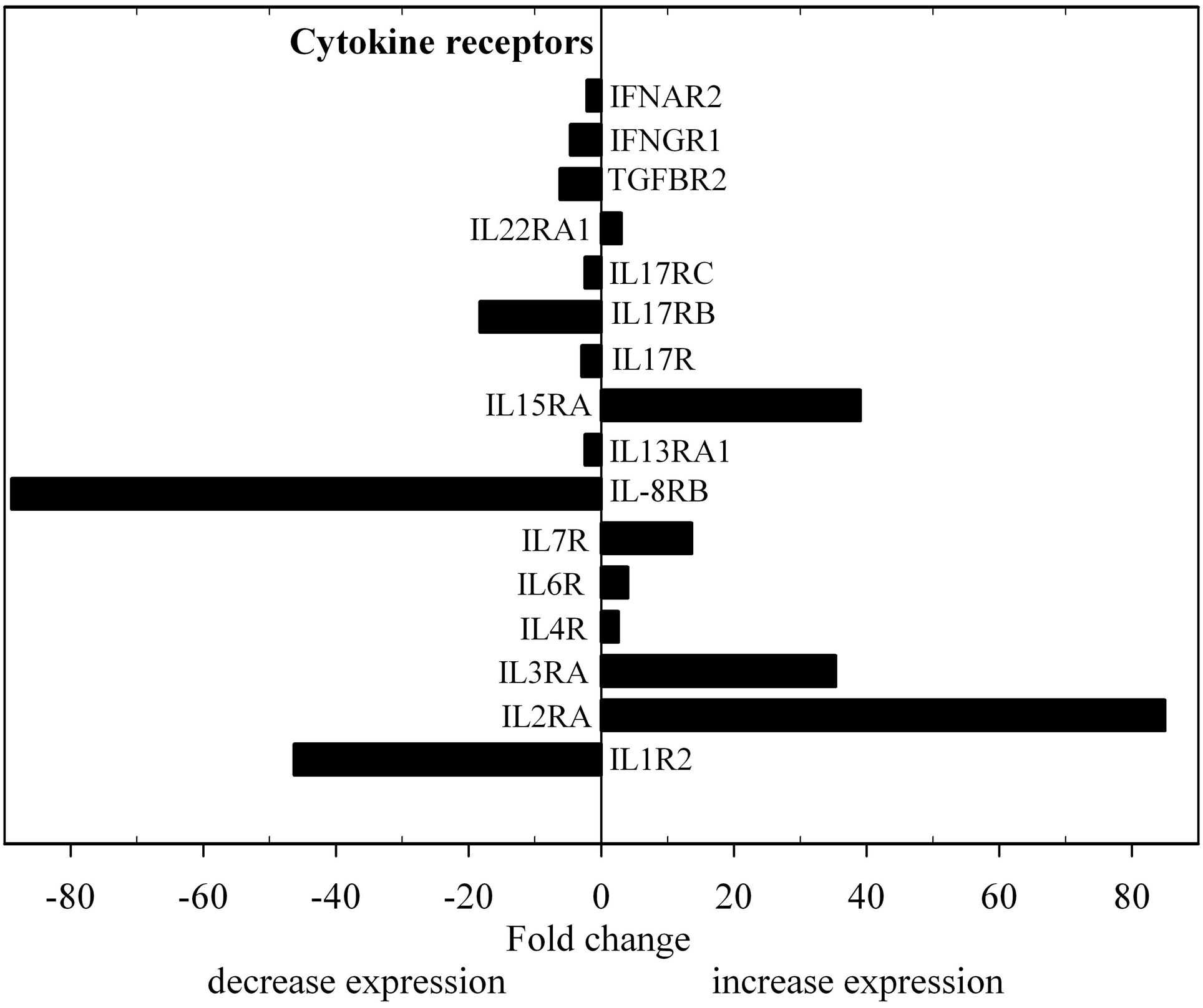

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Figure 3

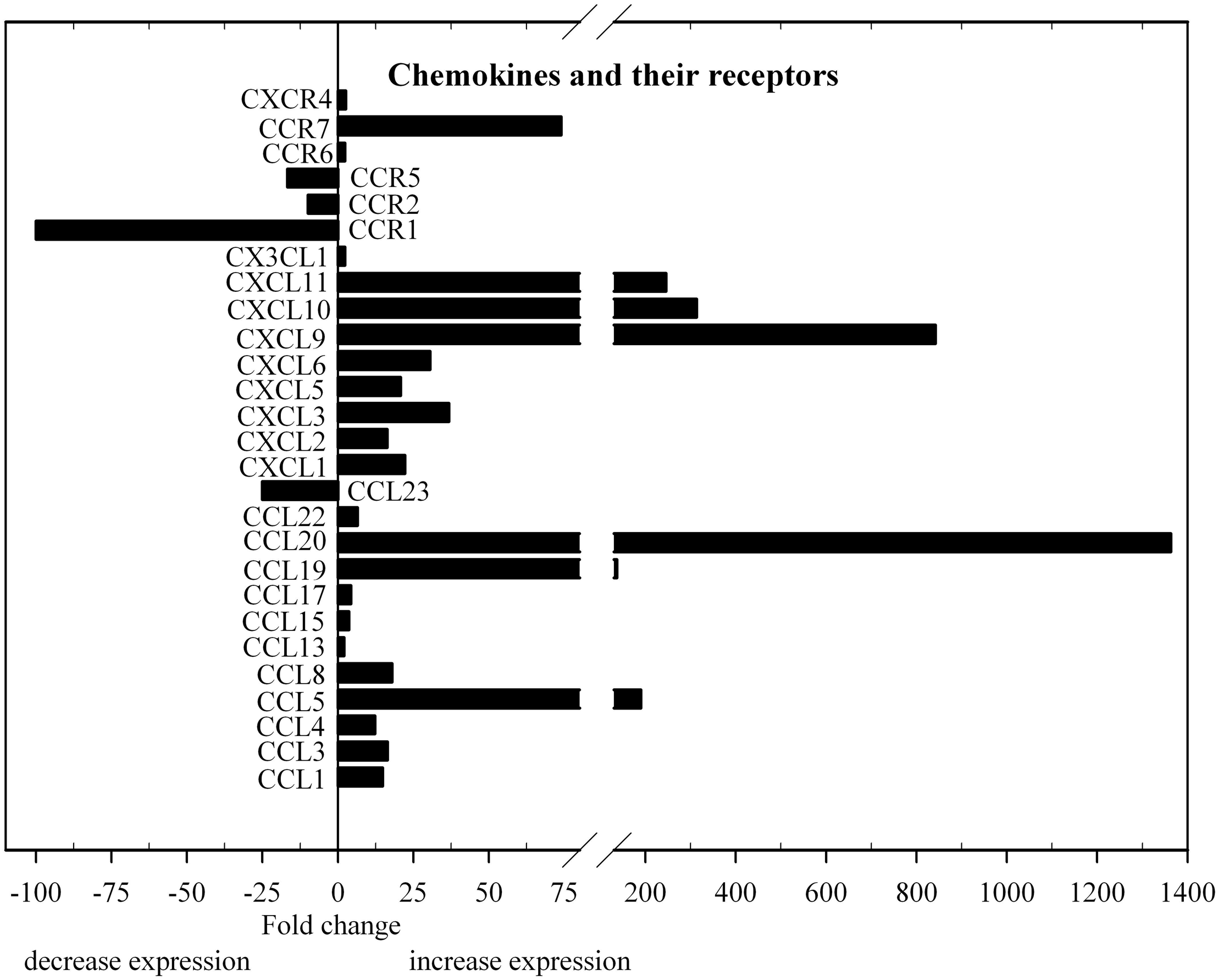

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Figure 4

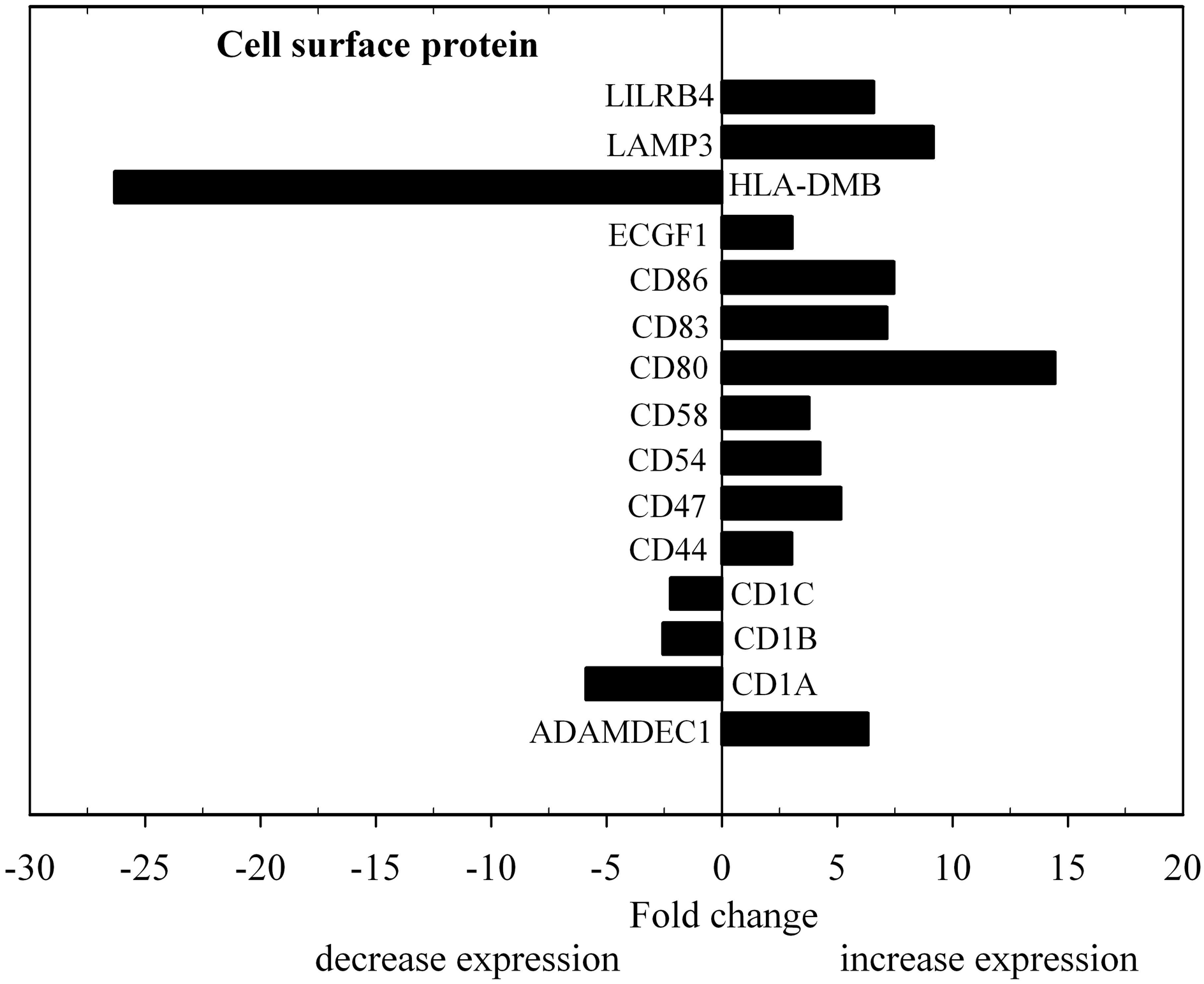

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Figure 5

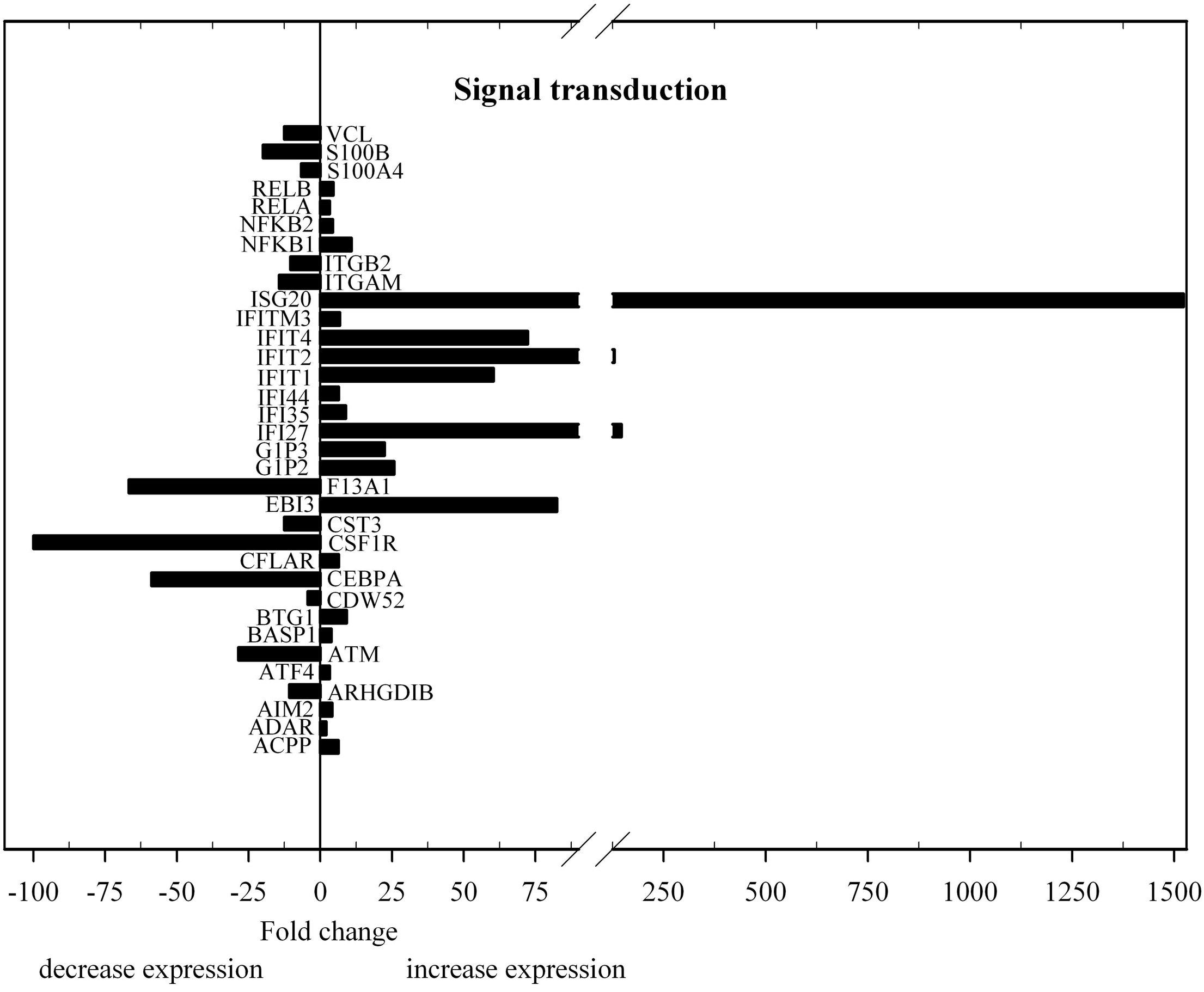

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Figure 6

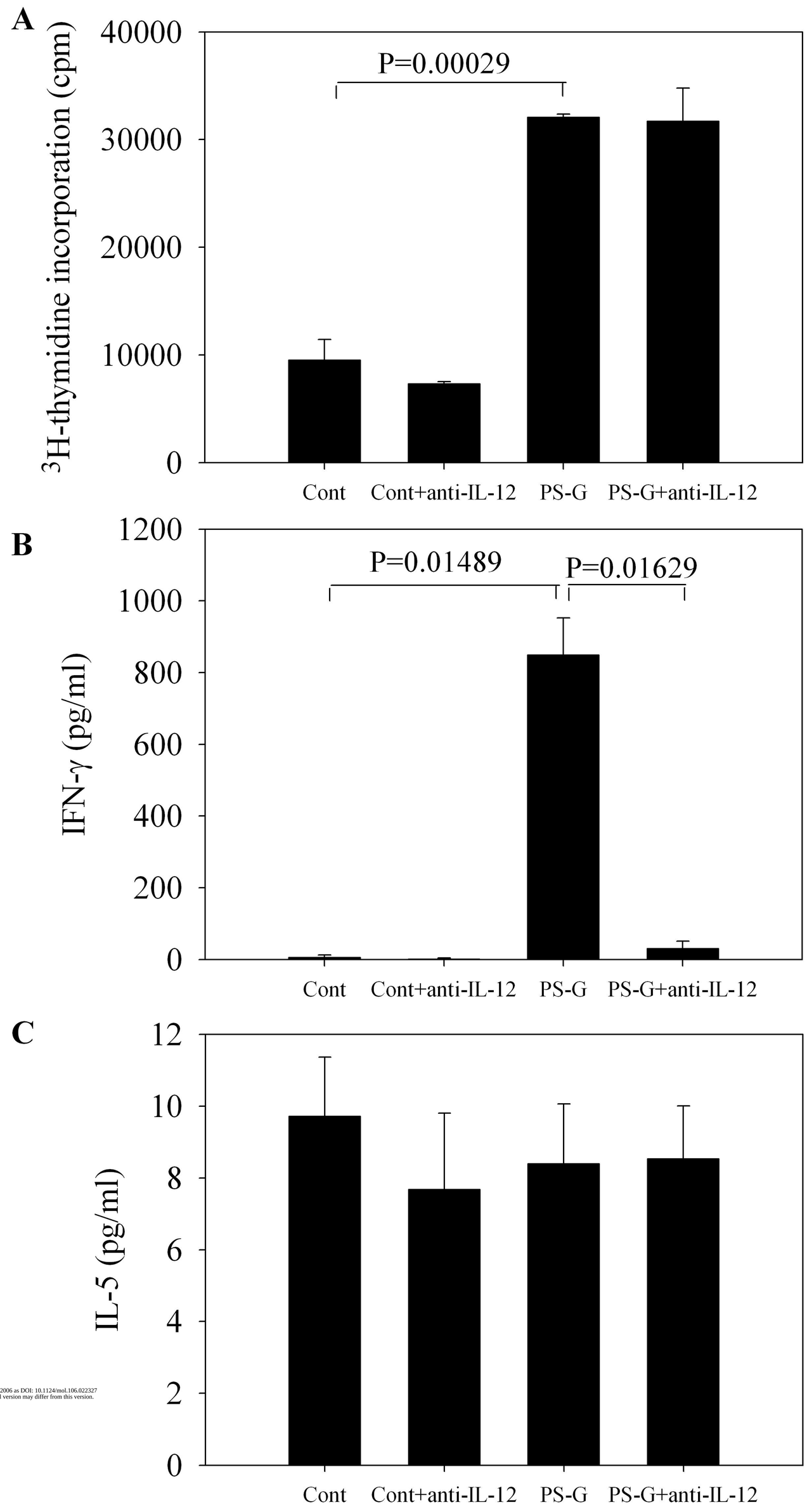

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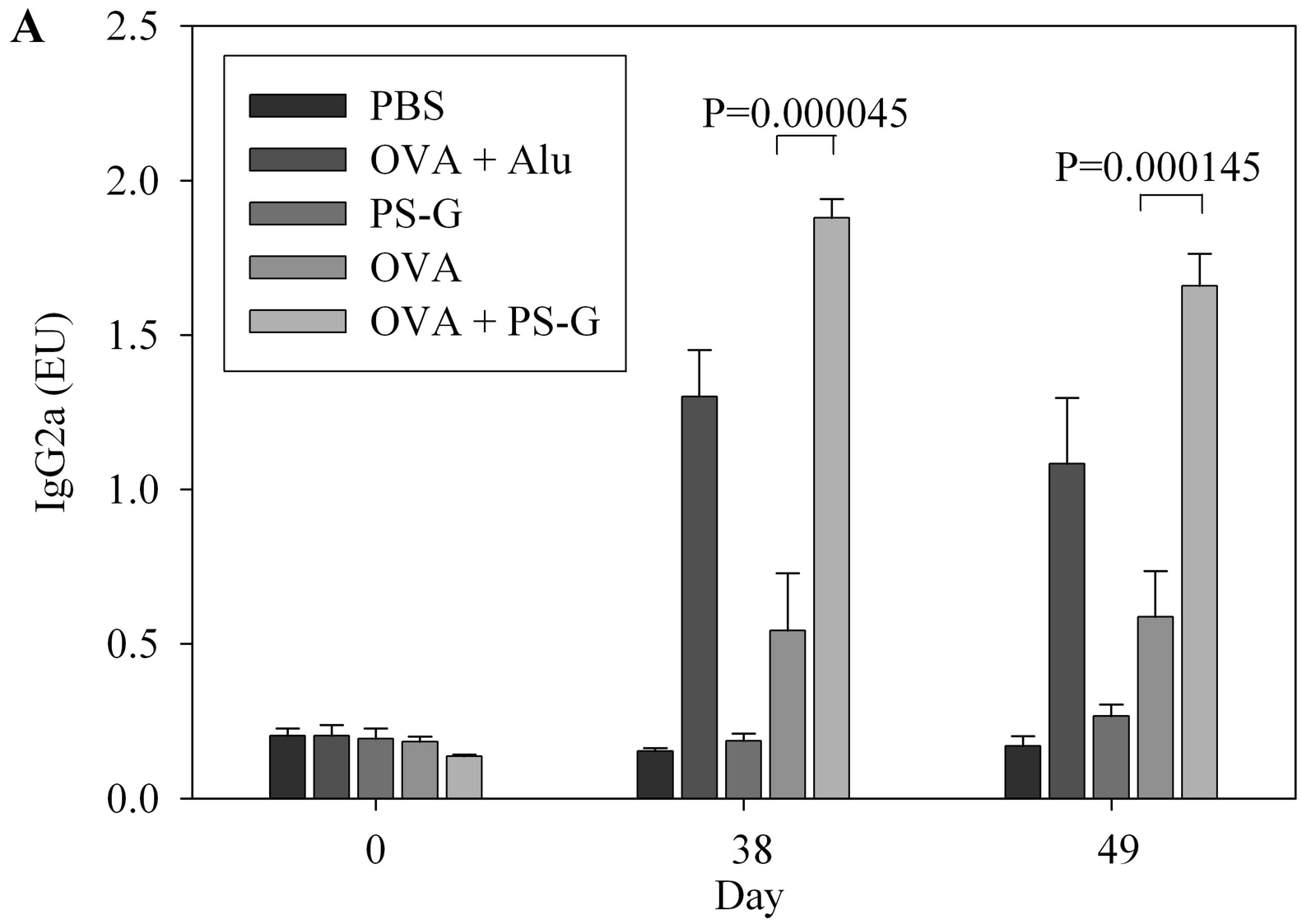
Figure 7



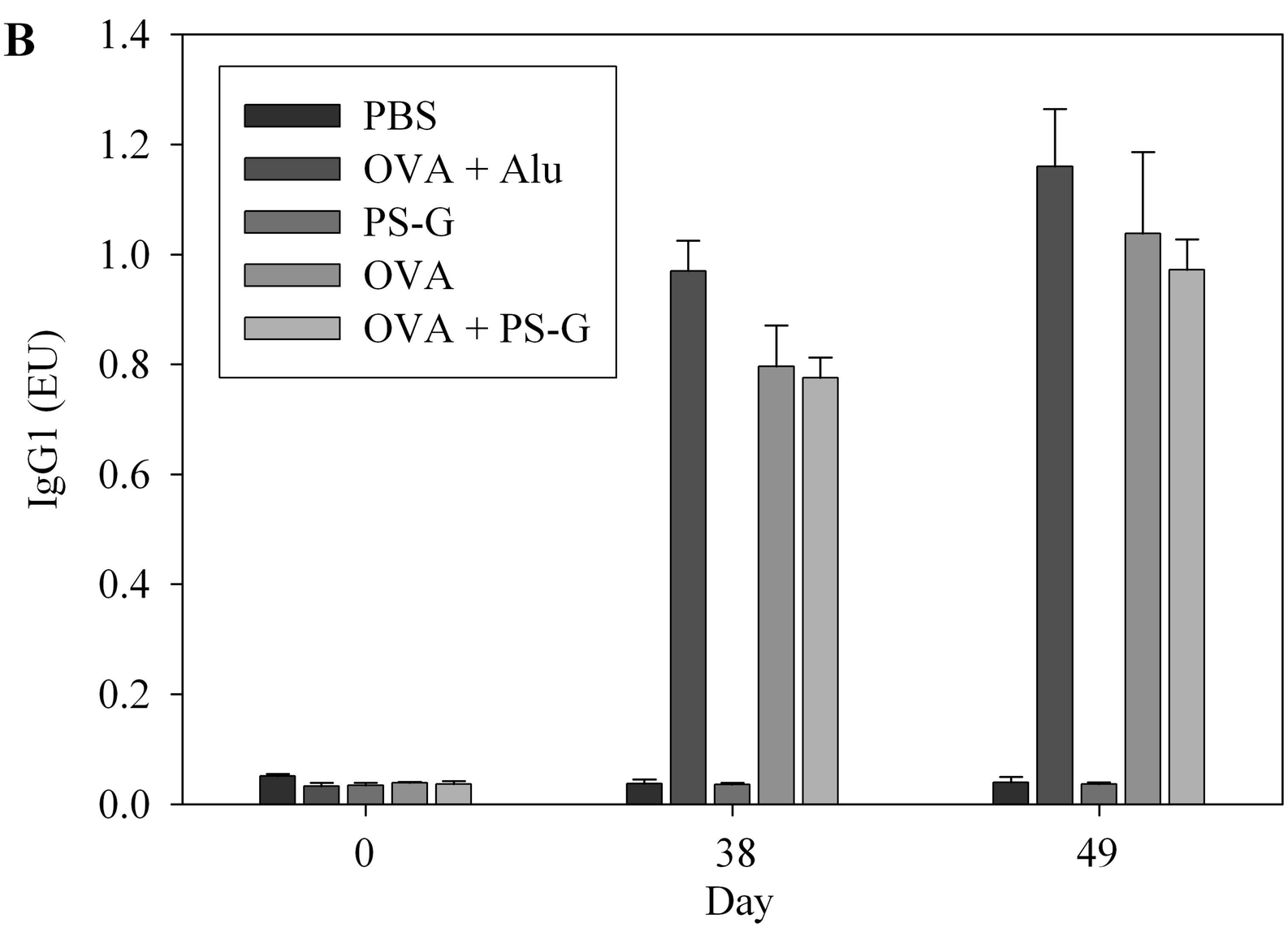


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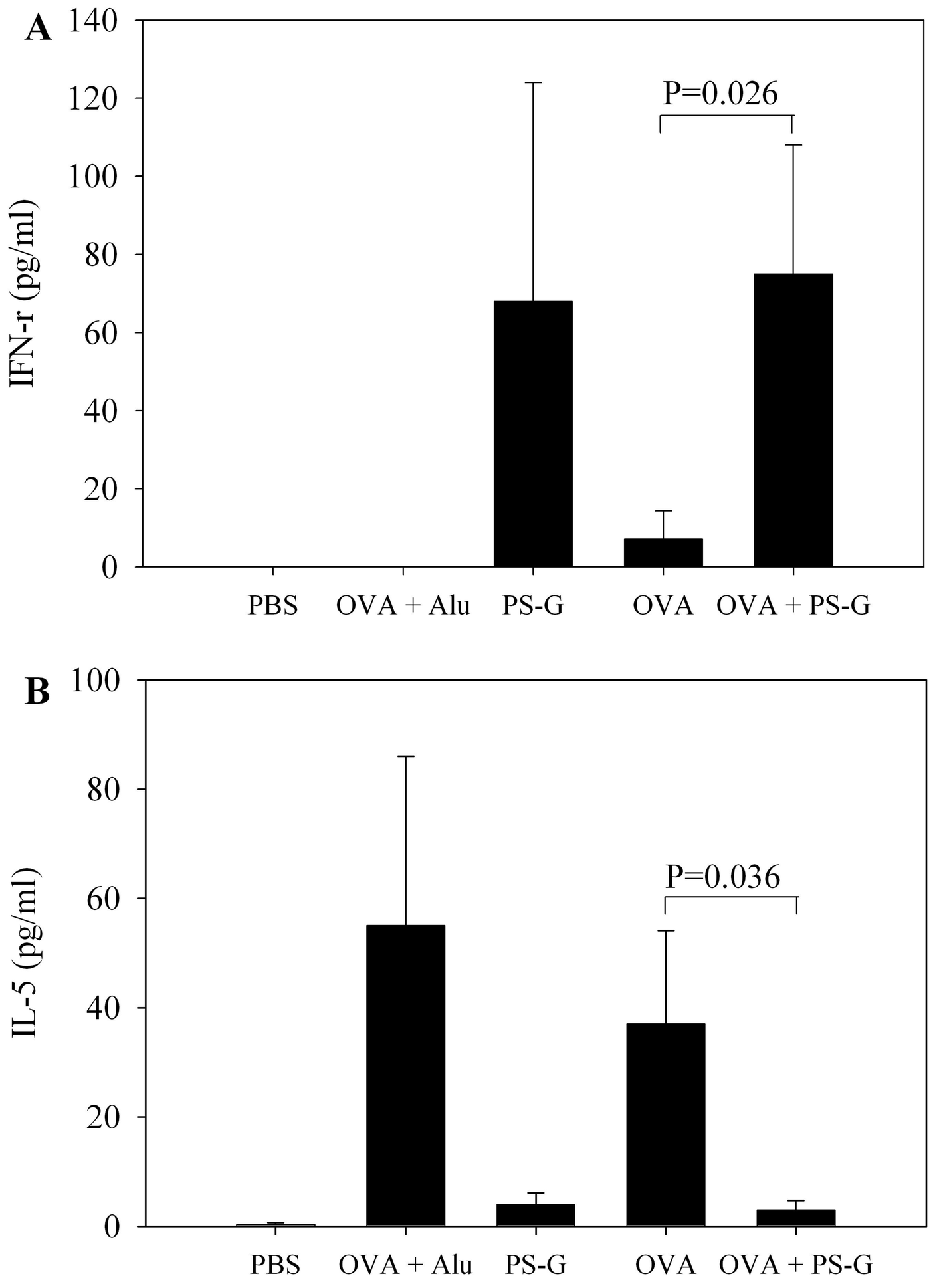
Figure 8



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