The American Journal of Chinese Medicine, Vol. 40, No. 4, 833–844

Institute for Advanced Research in Asian Science and Medicine
DOI: 10.1142/S0192415X12500620

Immunomodulatory Effects of Aqueous and Organic Fractions from *Petiveria alliacea* on Human Dendritic Cells

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Abstract: *Petiveria alliacea* is a plant traditionally known for its anti-inflammatory and anti-tumor activities; however, the molecular and cellular mechanisms of its immunomodulatory properties are still unknown. Dendritic cells (DC) promote adaptive immune response by activating T lymphocytes, inducing an effector response or tolerance depending on the DC differentiation level. Herein, we evaluated the immunomodulatory activity of aqueous and organic plant fractions from *P. alliacea* using human monocyte-derived dendritic cells. The phenotype, cytokine secretion and gene expression were estimated after treatment with the plant fractions. We found that *P. alliacea* aqueous fraction induced morphological changes and co-stimulatory expression of CD86, indicating partial DC maturation. In addition, pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α were secreted. The fraction also increased NF-κB gene expression while down-regulating TGFβ gene expression. These results suggest that the aqueous fraction can induce partial DC activation, a situation that can be relevant in tolerance induction. It is important to state that the organic fraction by itself does not show any immunomodulatory activity. This study provides evidence for possible immunomodulatory activity of *P. alliacea* extracts which has been used in traditional medicine in Colombia.

Keywords: *Petiveria alliacea*; Immunomodulation; Dendritic Cells; Cytokines; Gene Expression.

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Introduction

Throughout history, plants have been used as a major source of new medicines because of their antitumor and immunomodulatory properties. The plant *Petiveria alliacea* or anamu grows in the Caribbean, South America, and West Africa (Rosner *et al.*, 2001) and has been used in traditional medicine since the Aztecs for treating several diseases (Queiroz *et al.*, 2000). The plant roots and leaves have been claimed to possess anti-inflammatory, anti-tumor, antispasmodic, antibacterial, and analgesic activities (Lopes-Martins *et al.*, 2002). However, the types of immunomodulation and plant metabolites involved in such activities are not yet fully defined.

It has been reported that a hydro-alcoholic root extract from *P. alliacea* can stimulate the phagocytic activity of mice injected intraperitoneally with *Escherichia coli* (Delavueau *et al.*, 1980). In addition, *P. alliacea* root extracts may also act as chemoprotectors as reported in *Listeria monocytogenes* infected mice, inducing natural killer cell activity, IL-2 and IFN-γ secretion and an increase in granulocyte and monocyte colony numbers (Quadros *et al.*, 1999; Queiroz *et al.*, 2000). In contrast, anti-inflammatory activities have been seen in other experimental models with a decrease in granuloma formation and dermatitis in rodents previously treated with pro-inflammatory agents (Germano *et al.*, 1993). β-sitosterol, a compound present in *P. alliacea* extracts inhibits the activity of prostaglandin synthetase, and may therefore contribute to the anti-inflammatory activity (Awad *et al.*, 1996). Dibenzyl trisulfide (DTS) a compound present in *P. alliacea* displays antitumor, anti-inflammatory, antibacterial, and antifungal activities (Webster *et al.*, 2008), along with the decrease of pro-inflammatory cytokines secretion.

Dendritic cells (DCs) can promote immune responses by activating T lymphocytes (Banchereau *et al.*, 2000). DC activation and maturation result in either tolerogenic or effector immune response, depending upon the surroundings signals in which antigen presentation occurs (Banchereau *et al.*, 2000; Liu, 2001; Théry and Amigorena, 2001; Steinman, 2007; Blancoa *et al.*, 2008). Immature DCs are highly efficient in capturing antigens, but not so efficient in presenting antigens, due to the low expression of co-stimulatory molecules like CD86, CD83, CD80 and major histocompatibility complex class II (MHC-II). In addition, they are unable to secrete polarizing cytokines (Lutz, 2002). In contrast, mature DCs display low endocytosis activities, but they are efficient antigen presenting cells and secrete pro-inflammatory cytokines (Steinman, 2002). However, Lutz (2002) identified an intermediate differentiation stage after TNF-α activation, in which DCs express co-stimulatory molecules, along with high levels of MHC-II, but are unable to secrete proinflammatory cytokines such as IL-1β, IL-6, IL-12p70 or TNF-α.

We examined the potential immunomodulatory activities of two *P. alliacea* fractions during the maturation process of monocyte-derived DCs (MDDC). The aqueous fraction (AF), characterized by its high contents of primary metabolites and the organic fraction (OF), rich in secondary metabolites. Our results show that AF was able to induce partial activation of MDDC with an intermediate maturation profile and preferential secretion of IL-1β, which may therefore modulate subsequent immune responses.

Materials and Methods

Plant Material

The plant material was collected in Viotá (Cundinamarca, Colombia), authenticated by Dr. Antonio Luis Mejía Piñeros from Fundación Universitaria Juan N. Corpas as *Petiveria alliacea* L. by comparing the plant material with a sample of the Colombian National Herbarium, registry number 333406 of August 12, 1991.

Organic Fraction Extraction

P. alliacea dry ground leaves and stems (300 g) were extracted in Soxhlet (60°C) with 96% ethanol (1.5l) for 3 h. The extract was filtered, concentrated under vacuum (175 mbar) until half the volume and then flocculated by adding distilled water and heating to 65°C for 20 min with constant shaking. The precipitate was discarded and the supernatant recovered by filtration. In a separation funnel, the supernatant was exhaustively extracted (seven times) with ethyl acetate. The ethyl acetate soluble fraction was named the organic fraction (OF). The OF was characterized using HPLC/MS/MS and the identified compounds by dereplication were leridal/petiveral, 7-demethyl leridal and DTS (Santander *et al.*, 2009).

Aqueous Fraction Extraction

P. alliacea dry ground leaves and stems (500 g) previously sterilized with ethylene oxide were extracted with 96% ethanol (2.5l) for 3 days. The residue ethanol-insoluble was extracted with distilled water (1:l) at 60 ± 1°C for 2 h. The aqueous solution was concentrated under reduced pressure and ethanol (2000 ml, 75%) was added to selectively precipitate polysaccharides and glycoproteins (Ebringerová *et al.*, 2003), corresponding to the fraction enriched in primary metabolites (FAO, 1998). The precipitate was recovered by centrifugation at 3500 rpm for 5 min (22°C), lyophilized and stored at 4°C. A stock solution was prepared (50 mg/ml) and named aqueous fraction (AF). A preliminary fraction characterization was carried out with colorimetric assays to determine the presence of sugars, tannins, and phenolic compounds. Anthrone and phenol-sulfuric acid assays were used to confirm sugar presence in the sample (Dubois *et al.*, 1956). The protein content was determined by Bradford assay giving a concentration of 927 μg/ml. The presence of glycoproteins was determined with periodic acid-Schiff staining after SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) separation (Masahiro *et al.*, 1999).

Preparation of DCs

Peripheral blood mononuclear cells (PBMCs) were obtained from fresh buffy coats (60 ml) of healthy volunteers, who signed informed consent. Mononuclear cell purification was carried out by Ficoll density gradient centrifugation (Amersham, GE Health Care Europe

GmbH). Monocytes were isolated by positive selection using anti-CD14+ micro-beads with MiniMACS Systems according to the manufacturers instructions (MiltenyiBiotec, BergischGladbach, Germany). The cells used in the assays had more than 98% purity in accordance to flow cytometry estimations. Monocytes were cultured for 5 days in RPMI 1640 medium, 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, streptomycin (Eurobio, Paris, France), granulocyte-macrophage colony-stimulating factor (GM-CSF) (800 IU/ml) and IL-4 (1000 IU/ml) (R&D Systems, Minneapolis, MN, USA). On day 3, half of the medium was replaced with fresh media containing GM-CSF and IL-4. On day 5, the MDDCs were stimulated for 2 days with increasing amounts of AF (6, 12.5 and 25 μg/ml) or OF (7, 25, and 63 μg/ml). The assayed concentrations were chosen above and below the IC50 previously calculated on tumor cells K562 (81.58 μg/ml, Santander *et al.*, 2009). Stimulation with lipopolysaccharide (LPS) was used as a positive control of MDDC stimulation (1 μg/ml). Cells culture with fraction eluents were considered as the negative controls. Plant fractions AF and OF were treated with polymyxin B coated agarose beads (Sigma, St Louis, MO, USA) to eliminate any possible LPS contamination (Sigma). All reagents were tested for LPS presence using Lymulus spp assay (Bio Whittaker Inc., Walkersville, MD, USA). Normal human mesenchymal cells and fibroblasts were cultured in different concentrations of AF and OF (31.2, 62.5, 125, 250 and 500 μg/ml) to estimate cell cytotoxicity by trypan blue exclusion test.

Assessment of Phenotype Changes

MDDC phenotypes were analyzed using the following monoclonal antibodies (mAbs): CD1a- Pacific Blue™, CD86-PE, CD83-FITC, HLA-DR-APC-H7, and CD209-PercP-Cy5 (BD Biosciences, San Jose, CA). Cells were collected and washed in PBS containing 2% FCS and 0.1% sodium azide. For immunostaining treated or untreated MDDCs, cells were incubated with mAbs for 20 min at 4°C. After washing, data acquisition was performed on a FACSARIA™ (Becton-Dickinson, Colombia) and analyzed using Flowjo 8.7 software (Tree Star, Ashland, OR, USA).

Measurement of Cytokine Levels

The production of IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α was determined on culture supernatants after 24 or 48 h stimulation with either LPS, AF or OF using the Cytometric Bead Array kit of human inflammation (BD Biosciences), according to the manufacturers instructions, followed by acquisition data on a FACSCanto II™ flow cytometer with the program Cytometric Bead Array kit (CBA) and FCAP array software (BD Biosciences).

Real-Time PCR Expression Profiling

Total RNA was extracted from unstimulated MDDCs or stimulated with LPS, AF and OF for 24 and 48 h using TRIzol reagent (GIBCO, USA) according to the manufacturers

Table 1. Real-Time Primer Sequences

	Genes	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Size
Housekeeping Gene	GAPDH	AGCCGAGCCACATCGCT	CCCTGGTGACCAGGC	87
Cytokines	IL1-β	ACAGACCTTCCAGGAGAATG	GCAGTTCAGTGATCGTACAG	127
	IL-6	AATTCGGTACATCCTCGACGG	GGTTGTTTTCTGCCAGTGCC	100
	IL-10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTGTTTT	138
	IL-12p35	TGGAGTGCCAGGAGGACAGT	TCTTGGGTGGGTCAGGTTTG	147
	TNFα	TCTTCTCGAACCCCGAGTGA	CCTCTGATGGCACCACCAG	151
	TGFβ	CAGCAACAATTCCTGGCGATA	AAGGCGAAAGCCCTCAATTT	136
Transcription Factors	NFκB	ATTTGAAACACTGGAAGCACGA	GCGGATTAGCTCTTTTTCCCG	145

instructions (Invitrogen, USA). Total RNA was quantified in a NanoDrop spectrophotometer (NanoDrop Technologies), and the quality was confirmed by migration in a 1% agarose gel stained with ethidium bromide. cDNA was synthesized with RNA PCR Core using random hexamers following the manufacturers instructions (Applied Biosystems, Foster City, CA, USA). 3 μl of a 1:20 dilution was used for reverse transcription reaction in a real-time PCR quantitative (qPCR) reaction, using 12.5 μl of 2X SYBR Green PCR Master Mix (Applied Biosystems), 50-400 nM of forward and reverse primers (Table 1) in 25 μl volume. Reactions were performed in 96-well optical plates using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and in duplicates. The thermal cycling conditions were previously described (Santander *et al.*, 2011).

Results

Determination of the Cytotoxicity of the Fraction

The potential cytotoxicity of AF evaluated with human mesenchymal cells using the concentrations detailed above, did not show any deleterious effects on cell viability. Based on these findings, MDDC viability was confirmed from 6 to 50 μg/ml AF concentrations. However, variations in cell shapes were observed at concentrations below 25 μg/ml, similar to those reported during DC maturation.

The OF cytotoxicity was evaluated on human fibroblasts at 24, 48 and 72 h. Neither morphological alterations nor cytotoxicity was observed at any of the tested concentrations on normal cells. Based on these results, we chose to use fractions at 25 μg/ml.

P. alliacea AF Increases CD86 (Co-Stimulatory Molecule) Expression on MDDC

The expression of cell surface molecules on MDDCs was evaluated after either AF or OF stimulation and compared to the results obtained with LPS. Results showed that AF increased CD86 expression with no effect on the cell markers (Fig. 1). In addition, the

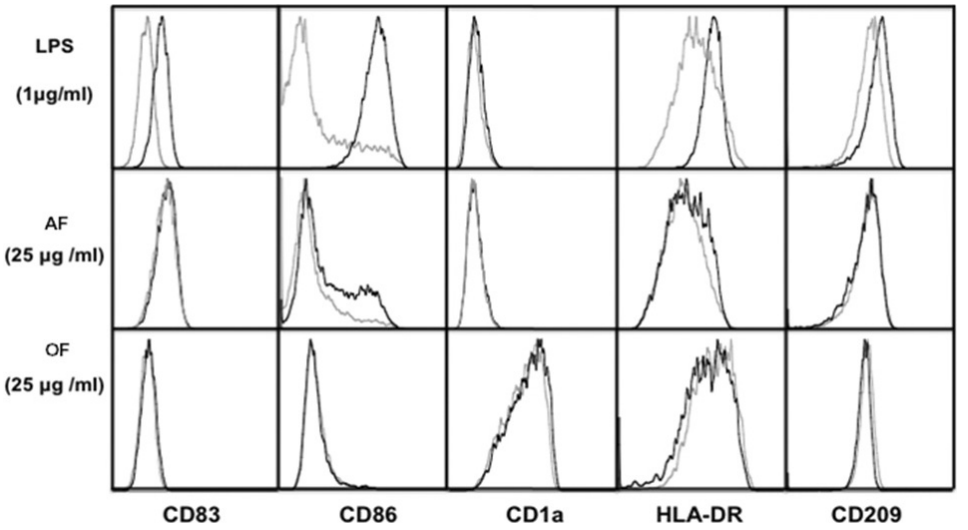

Figure 1. *P. alliacea* AF increases CD86 expression in MDDC. MDDCs were stimulated with LPS (1 μg/ml), AF (25 μg/ml) or OF (25 μg/ml). Histograms represent one of five independent experiments (gray for controls and black for treated samples). The phenotype profiles show an increase in CD86 expression by AF while OF shows no visible effect.

fraction induced morphological changes such as dendrite elongation, a well-known cytoskeletal characteristic of mature DC phenotype (Mellman, 2001; West *et al.*, 2004, Fig. 2).

OF did not induce the expression of any of the molecules tested and, as expected, no morphological changes were observed, exhibiting a very similar behavior as with ethanol (vehicle) treatment.

P. alliacea Aqueous Fraction Increases Gene and Protein Expressions of Pro-Inflammatory Cytokines

To further characterize MDDC activation state (immunostimulatory or regulatory), we evaluated the cytokines produced by MDDCs matured with AF or OF. Gene expression and/or protein secretion of IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, TGFβ, and NF-κB transcription factors were quantified after 24 or 48 h. Our results showed that AF was able to induce IL-1β, IL-6, and TNF-α both at the transcription and the translation levels, with a slight delay compared to the cytokine production in the presence of LPS. In addition, IL-10 was secreted and detected in the supernatants, while the mRNA levels were decreased by 1.7-fold as compared to the control (Fig. 3 and Table 2). The fraction also decreased TGFβ by 7.8-fold and increased NF-κB by 2.9-fold (Table 2). OF showed only minor and non-significant changes.

To evaluate whether AF induced activity lasts, MDDC cytokine gene expression and protein secretion were estimated after stimulation for 48 h. The expressions of IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 were increased, which coincided with an increase of IL-12 mRNA levels after 24 h of treatment (Table 2).

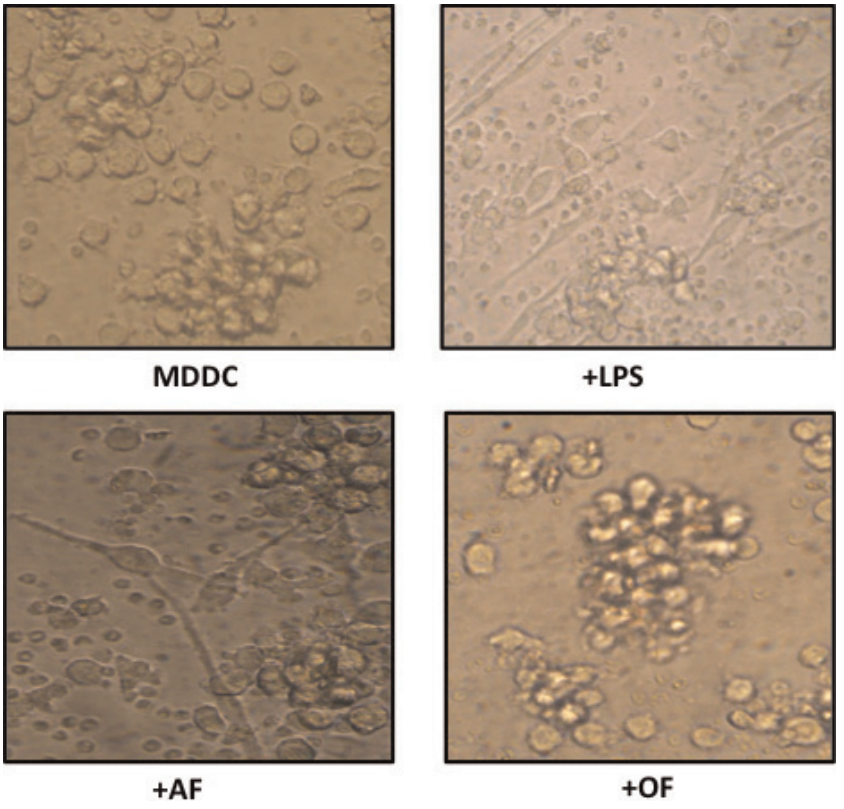

Figure 2. Effect of *P. alliacea* fractions on MDDC morphology. Differences in the morphology of non-stimulated MDDCs or MDDCs treated with LPS (1 μg/ml), AF (25 μg/ml) or OF (25 μg/ml) for 48 h. AF induces morphological changes similar to mature MDDC stimulated with LPS.

There was no difference in cytokine secretion after treatment for 24 or 48 h with OF (Fig. 3). IL-8 protein secretion levels were evaluated with both fractions and only AF induced a sustained production during the assessed time (Fig. 3). The selected time frame of 24 and 48 h was chosen based on previous reports about LPS and some polysaccharide activity over DCs (Ishii *et al.*, 2005; Lin *et al.*, 2006; Kim *et al.*, 2007; Santander *et al.*, 2007; 2011).

Discussion

Water plant infusion is the traditional usage of *Petiveria alliacea*. This type of preparation has been suggested to exhibit immunomodulatory activity (Lopes-Martins *et al.*, 2002; Ruffa *et al.*, 2002). However, the cell target and metabolites involved in this activity have not been clearly identified. Previous studies by our group and others have shown that the plant organic fractions, rich in secondary metabolites are active against tumor cells (Rosner *et al.*, 2001; Urueña *et al.*, 2008; Cifuentes *et al.*, 2009; Santander *et al.*, 2009). In addition,

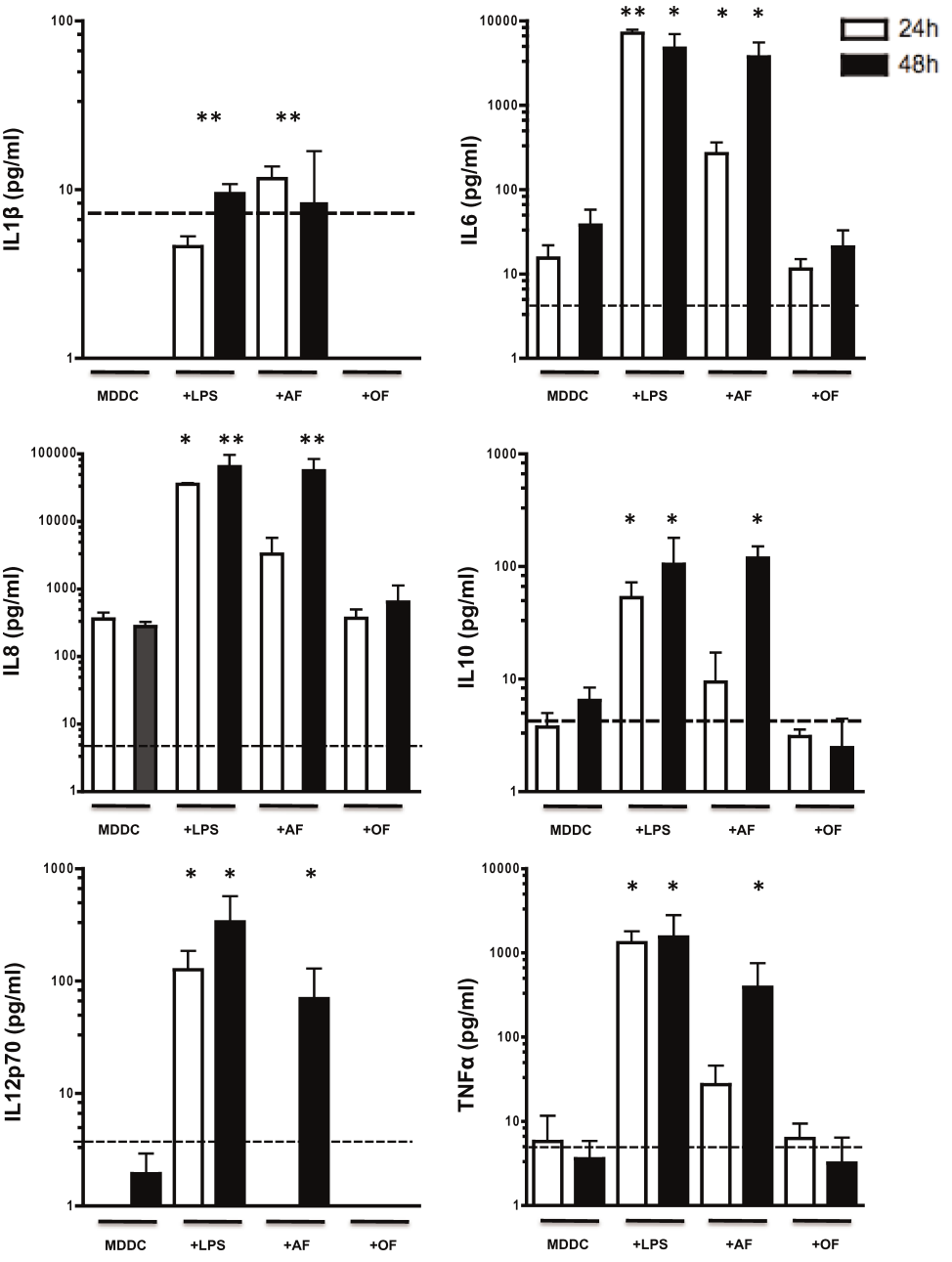

Figure 3. *P. alliacea* AF induces pro-inflammatory cytokine secretion. AF induces secretion of IL1β, IL6, IL8, IL10, IL12p70, and TNF-α after treatment for 24 and 48 h. AF displays similar behavior as LPS stimulation on MDDC, while OF did not show any effect. Each graph represents the mean of three independent experiments. Statistical significance is carried out comparing treated MDDCs and their controls using unpaired *t*-test ($^*p < 0.05$).

Table 2. Gene Expression Regulation of Dendritic Cells by *P. alliacea* Fractions

Gene Description	Fold Change				
Cytokines	LPS		AF		5F
	24 h	48 h	24 h	48 h	24 h
IL-1β	4.6	3.1	3.2	-2.5	3.6
IL-6	14.2*	3.4	1.7	−3.4	−2.3
IL-10	−4.6	−8.2*	−1.3	−6.2*	−1.8
IL-12p35	32.9*	193*	5.8*	−1.6	−1.8
TNFα	5.5	1.7	1.4	−2.4	1.5
TGFβ	−14.5*	−3.9	−7.8*	−1.7	−1.4
Transcription Factors					
NFκB	9*	2.7	2.9	1.1	−2

Note: Relative gene expression is calculated by $2^{-\Delta\Delta Ct}$ method. Data represents the mean of two independent experiments. Statistical significance is carried out comparing treated MDDCs with their controls using unpaired *t*-test (*$p < 0.05$).

some reports indicate that a hexanol-extract of the plant increases hematopoietic stem cell mobilization from the bone marrow (Quadros *et al.*, 1999). Although, the AF toxicity has been previously studied (Cavalcanti, 2008), the immunomodulatory activity has not been evaluated.

In the present work, we observed that human MDDCs cultured in the presence of *P. alliacea* aqueous and organic fractions induces distinct responses. The partial increase of CD86 expression by AF suggests an incomplete DC activation and therefore tolerance induction to antigens presented under these conditions (Lutz, 2002; Heath *et al.*, 2004; Blancoa *et al.*, 2008). However, AF is able to induce an increase of IL-1β, IL-6, TNF-α, IL-10, and IL-12p70 cytokines at mRNA and protein levels, suggesting that AF could have an unknown mechanism to activate dendritic cells and that this mechanism induces increased NF-κB.

For several years, it has been proposed that *P. alliacea* antitumor and immune-stimulant activities are due to the presence of dibenzyl trisulfide (DTS) (Rosner *et al.*, 2001). We have previously shown that the organic fraction, partially characterized by our group (Santander *et al.*, 2009), containing DTS, was unable to induce any detectable activity in dendritic cells, suggesting that other metabolites contained in AF could be involved in this activity.

Since cytokine secretion in human DCs is affected by AF, it could be suggested that the *P. alliacea* fraction could influence the cellular microenvironment and modulate the inflammatory immune response. Interestingly, secretion of IL-1β by DCs has been associated with inflammasome activation, an essential complex for priming CD8+ T cells against dying tumor cells, inducing IFN-γ secretion (Ghiringhelli *et al.*, 2009). This activity could partially explain the *P. alliacea* traditional use as an antitumor agent (Williams *et al.*, 2007).

In general, *P. alliacea* crude extracts are commonly used as anti-inflammatory agents (Germano *et al.*, 1993), an activity attributed to Th1-type cytokine production, like TNF-α, IL-6, and IL-1β and to the increase of Th2 cytokines as IL4 (Rossi *et al.*, 1993; Williams

et al., 2002; Urueña *et al.*, 2008; Cifuentes *et al.*, 2009). Del Valle *et al.* (1993) has reported that T lymphocytes treated with a *P. alliacea* ethanolic extract did not induce proliferation, however, a synergic activity appears when combined with PHA stimulation. A preclinical study showed that the lyophilized preparation of *P. alliacea* leaves (1200 μg/ml) has a protector effect on immunosuppressed (5-fluorouracil induced) Balb/c mice inducing lymphocytosis, neutrophilia and protector effects on B cells, together with an increase of bone marrow cellularity (Batista *et al.*, 2011).

The present work is the first study evaluating a differential immunomodulatory activity of *P. alliacea* fractions on immune system cells and providing relevant information concerning the different biological activities of aqueous and organic fractions. Currently, we are characterizing the AF metabolites and concentrating our study on primary metabolites as polysaccharides.

Acknowledgments

The authors acknowledge Nuala Mooney for her interesting contributions, the Pontificia Universidad Javeriana Science Faculty and the Fundación Universitaria Juan N. Corpas for their support to the present work and the Instituto Colombiano para el Desarrollo de la Ciencia y Tecnología "Francisco Jose de Caldas" (COLCIENCIAS) Bogotá, Colombia, for the financial support, grant number 324-2007. M.A. post doctoral fellow funded by France Foundation. S.P.S. and J.F.H. are PhD students sponsored by COLCIENCIAS.

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