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Immunomodulatory Effect of Arctigenin, a Lignan Compound, on Tumour Necrosis Factor-α and Nitric Oxide Production, and Lymphocyte Proliferation

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Abstract

We have investigated the immunomodulatory effects of arctigenin, a dibenzyl butyrolactone lignan compound, on tumour necrosis factor (TNF)-α and nitric oxide (NO) production, and lymphocyte proliferation.

Arctigenin inhibited strongly TNF-α production by lipopolysaccharide-stimulated murine macrophage RAW264·7 and differentiated human macrophage U937 with IC50 values of 5·0 and 3·9 μM, respectively, without displaying cytotoxicity. The TNF-α inhibitory effect of arctigenin in lipopolysaccharide-triggered RAW264·7 cells was increased by co-treatment with several known TNF-α inhibitors. It also potently attenuated T and B cell proliferation stimulated by concanavalin A and lipopolysaccharide in a dose-dependent manner with IC50 values of 2·9 and 14·6 μM, respectively. In contrast, the compound showed a different pattern in lipopolysaccharide- and interferon (IFN)-γ-induced NO production from RAW264·7 cells. Arctigenin inhibited NO release by IFN-γ signal, whereas it significantly enhanced lipopolysaccharide-triggered NO production in RAW264·7 cells.

The results suggested that arctigenin may regulate immune responses in activated macrophages and lymphocytes including TNF-α and NO production and lymphocyte proliferation.

Lignan compounds constitute a large and diverse group of phenylpropanoid metabolites found throughout the plant kingdom. These components are known for having various biological activities including antioxidative, bactericidal, fungicidal, antiviral, phytotoxic, anti-inflammatory and anti-cancer effects (Ayres & Loike 1990). Lignans can be divided into several different structural types. Among them, the most common compounds are the dibenzyl butyrolactones (arctigenin and savinin), furofurans (pinoresinol and eudesmin) and dibenzylbutanes (secoisolariciresinol).

Arctigenin is known to have several biological activities. It inhibited the replication cycle of human immunodeficiency virus (HIV) at the integration stage (Eich et al 1996; Vlietinck et al 1998), suppressed the receptor binding of platelet activating factor (Han et al 1996), induced the differentiation of mouse myeloid leukemia cells (Umehara et al 1996), showed strong cytotoxicity against HepG2 cell but little toxicity against normal liver cells (Chang liver cells) (Moritani et al 1996), and is known to be a cytostatic agent against human leukemic HL-60 cells without cytotoxicity (Hirano et al 1994).

Previously, we demonstrated that arctigenin potently inhibited TNF-α production from lipopolysaccharide-triggered murine macrophage in the screening study on TNF-α inhibitors (Cho et al, unpublished data). Since TNF-α production from lipopolysaccharide-activated macrophage is a general host immune response, it led us to analyse whether arctigenin was capable of modulating

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immune responses. Three kinds of assays were chosen, on the basis that they represented a range of in-vitro immunological phenomena likely to be performed by activated macrophage and lymphocytes. The data generated with these assays demonstrated that arctigenin, depending on assay, either inhibited or stimulated TNF-α and NO production, and T or B cell proliferation.

Materials and Methods

Animals

Eight-week-old BALB/c male mice were purchased from B & K Universal (Fremont, CA). The BALB/c mice were maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were freely available.

Materials

(−)-Arctigenin (Figure 1, purity: 97·5% by HPLC analysis) was a gift from Professor B. H. Han (Seoul National University, Seoul, Korea). A77,1726 (an active metabolite of leflunomide) was synthesized in the Chemistry Department of Daewoong R & D Center (Sungnam, Korea). Dibutyryl cyclicAMP (dbcAMP), nitro-L-arginine methyl ester (L-NAME), recombinant human interferon-γ (IFN-γ), concanavalin A, lipopolysaccharide (*E. coli* 0111:B4) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St Louis, MO). Foetal bovine serum and RPMI1640 were obtained from Gibco (Grand Island, NY). RAW264·7 and U937 cells were purchased from ATCC (Rockville, MD). All other chemicals were of Sigma grade.

Cell culture

RAW264·7 and U937 cells were maintained in RPMI1640 supplemented with 100 units mL^{-1} penicillin and 100 $\mu g\,mL^{-1}$ streptomycin, and 10% foetal bovine serum. Cells were grown at 37°C and 5% CO_2 in humidified air.

TNF-α production in-vitro

Stimulation of RAW264·7 cells. The inhibitory effect of arctigenin on TNF-α production was determined as described by Cho et al (1998a). Arctigenin solubilized with vehicle (89·9% propylene glycol, 10% ethanol and 0·1% dimethylsulphoxide) was diluted with RPMI1640. The final concentration of vehicle never exceeded 0·05% in the culture medium. In these conditions, none of the solubilization solvents altered TNF-α production in RAW264·7 cells. After pre-incubation of RAW264·7 cells (2×10^6 cells mL^{-1}) for 18 h in 24-well plates, lipopolysaccharide (1 $\mu g\,mL^{-1}$) and the various concentrations of testing samples were added to the wells for 4 h. Supernatants were assayed for TNF-α content using mouse TNF-α ELISA kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Differentiation and stimulation of U937 cells. Differentiation and stimulation of U937 cells were performed using the method of Sajjadi et al (1996). The differentiated U937 cells (2×10^6 cells mL^{-1}) were treated with 1 $\mu g\,mL^{-1}$ lipopolysaccharide for 6 h with arctigenin or positive control drug. Supernatants were harvested and assayed by ELISA.

Determination of nitric oxide (NO) production

After pre-incubation of RAW264·7 cells (1×10^6 cells mL^{-1}) for 18 h, the various concentrations of arctigenin or positive control drug with lipopolysaccharide (1 $\mu g\,mL^{-1}$) or IFN-γ (50 units mL^{-1}) were incubated for 24 h under the same conditions as reported by Ding et al (1988). Nitrite in culture supernatants was measured by adding 100 μL Griess reagent (1% sulphanilamide and 0·1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100-μL samples of medium for 10 min at room temperature. The optical density at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). The detection limit of the assay was 0·5 μM.

Splenocyte proliferation assay

Splenocytes were prepared from the spleens of mice killed by cervical dislocation under sterile conditions described previously (Cho et al 1998b). Briefly, splenocytes were released by teasing into RPMI1640 medium supplemented with 20 mM HEPES buffer. After removing red blood cells using 0·83% NH_4Cl–Tris buffer (pH 7·4), splenocytes were washed three times in Ca^{2+}–Mg^{2+} free Hank's balanced salt solution and resuspended to 5×10^6 cells mL^{-1} in RPMI1640 with 100 units mL^{-1} penicillin and 100 $\mu g\,mL^{-1}$ streptomycin, and 10% foetal bovine serum. Splenocytes (5×10^6 cells mL^{-1}) were cultured in 96-well plates in the presence or absence of T or B lymphocyte mitogens (concanavalin A and lipopolysaccharide) with arctigenin or positive control drugs in a total volume of 200 μL/well at the same conditions for 48 h. The proliferation assay was performed by MTT assay.

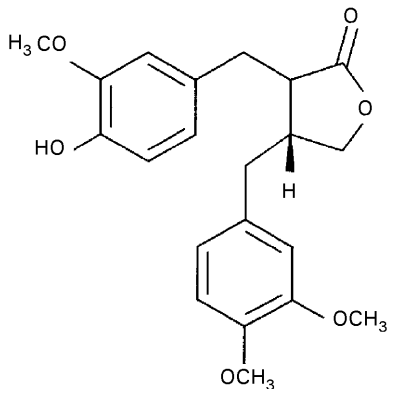

Figure 1. The chemical structure of (−)-arctigenin.

MTT assay (colorimetric assay) for measurement of cell proliferation

Cell proliferation was measured by conventional MTT assay. Four hours before culture termination, 10 μL MTT solution (10 mg mL^{-1} in phosphate buffered-saline, pH 7·4) was continuously cultured until termination. Culture was stopped by addition of 15% sodium dodecyl sulphate into each well for solubilization of formazan and the optical density at 570 nm ($OD_{570-630}$) was measured by a microplate Spectramax 250 microplate reader.

Cytotoxicity assay

Cytotoxicity of arctigenin in TNF-α and NO production assay was evaluated by MTT assay with minor modification as reported previously (Cho et al 1998a). The cell suspension of 1×10^6 cells mL^{-1} was plated in a 96-well plate. After 18-h culture, varying concentrations of testing compounds and stimuli were added to each well and cultured for another 4 (TNF-α assay) or 24 h (NO assay). The mean value of optical density content of four wells was used for calculating the viability (% control).

Statistical analysis

All values, expressed as mean ± s.e.m., were obtained from four independent observations performed in triplicate. The Student's *t*-test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference. $P \leq 0{\cdot}05$ was considered as statistically significant.

Results

Effect on TNF-α production

For comparison of the inhibitory effect of arctigenin in both murine and human macrophages, we used RAW264·7 cells and differentiated U937 cells stimulated by lipopolysaccharide. The optimal lipopolysaccharide concentration (1 μg mL^{-1}) and incubation time (4 or 6 h) for optimal TNF-α production were determined in preliminary experiments (data not shown) and used throughout the experiments.

Arctigenin inhibited strongly lipopolysaccharide-induced TNF-α production in murine and human macrophage cells in a dose-dependent manner (IC50 values 5·0 and 3·9 μM, respectively) without displaying cytotoxicity (Figure 2, Table 1). This suggested that arctigenin inhibited TNF-α production in human macrophage cells as well as in murine. Positive control drug dbcAMP significantly suppressed TNF-α production in a dose-dependent manner with IC50 values of 28·9 and 47·4 μM, respectively.

To assess additive or synergic effect, we treated arctigenin with several well-known TNF-α inhibi-

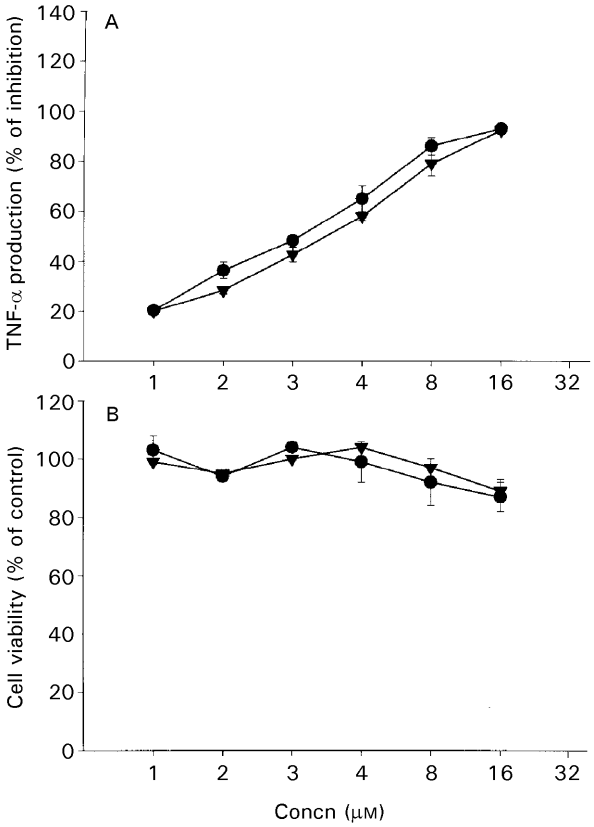

Figure 2. The inhibitory effect of arctigenin on (A) TNF-α production in RAW264·7 (▼) and differentiated U937 (●) cells and (B) cell viability. RAW264·7 and differentiated U937 cells (1×10^6 cells mL^{-1}) were stimulated by 1 μg mL^{-1} lipopolysaccharide with various concentrations of arctigenin. Supernatants were collected after 4 or 6 h and assayed by ELISA. Data represent mean ± s.e.m. of four independent observations performed in triplicate. Basal and stimulated level of TNF-α were 0·5–1·0 and 15–35 ng mL^{-1}, respectively. Cell viability under the same conditions was assayed by the MTT method as described in Materials and Methods.

Table 1. Inhibitory effect of arctigenin on TNF-α production in macrophages and lymphocyte proliferation.

Parameter	IC50 (μM)
TNF-α production	
RAW264·7	5·03 ± 0·37
U937	3·88 ± 0·48
Lymphocyte proliferation	
Concanavalin A	2·93 ± 0·05
Lipopolysaccharide	14·6 ± 2·11

Data represent mean ± s.e.m. of four independent observations performed in triplicate.

tors to lipopolysaccharide-activated RAW264·7 cells. As shown in Table 2, all tested inhibitors showed additive inhibitory effects.

Effect on lymphocyte proliferation

We examined the inhibitory effects of arctigenin on lymphocyte proliferation from splenocyte in the presence of common T or B cell mitogens by MTT assay. In this assay, concanavalin A was added at a concentration of 1 $\mu g\,mL^{-1}$ when splenocytes were cultured and lipopolysaccharide was added at a concentration of 10 $\mu g\,mL^{-1}$, as an optimal concentration. The proliferation of T lymphocytes treated by concanavalin A was increased significantly three to four times compared with untreated cells. As shown in Figure 3 and Table 1, arctigenin showed significant inhibitory effects in concanavalin A and lipopolysaccharide with IC50 values of 2·9 and 14·6 μM, respectively. Standard drugs, A77,1726 and dbcAMP in concanavalin A-stimulated splenocyte showed IC50 values of 6·7 and 603·5 μM, respectively.

Table 2. Effect of known TNF-α inhibitor on TNF-α inhibitory activity of arctigenin in lipopolysaccharide-stimulated RAW264·7 cells.

Treatment	% Inhibition	
	Alone	With arctigenin
None		44·1 ± 3·7
Herbimycin A	46·3 ± 5·3	93·0 ±
	38·2 ± 6·2	83·9 6·8
$_2$dbcAMP	46·6 ± 8·3	79·1 ± 3·2
2-Chloroadenosine	44·1 ± 3·7	80·8 ± 5·3

Protein kinase C inhibitor (staurosporin, 25 nM), protein tyrosine kinase inhibitor (herbimycin A, 500 nM), cAMP elevating agent (dbcAMP, 25 μM and PGE_2, 25 nM) and A_3 receptor agonist (2-chloroadenosine) were tested alone or in combination with arctigenin (4 μM) on TNF-α production in lipopolysaccharide-stimulated RAW264·7 cells. Supernatants were collected after 4 h and assayed by ELISA. Data represent mean ± s.e.m. of four independent observations performed in triplicate.

Effect on NO production

To evaluate the effect of arctigenin on NO production, the stable NO oxidation product, nitrite, was measured from the cell supernatant containing 0·5–1 μM NO as a basal level. When RAW264·7 cells were stimulated with 1 $\mu g\,mL^{-1}$ lipopolysaccharide and 50 units mL^{-1} IFN-γ for 24 h, NO produced approximately 30–55 μM in culture medium, more than 30–100-fold the amount at basal level. These conditions were applied to analyse suppressive effect on NO production by arctigenin and the positive control drug, L-NAME. As shown in Figure 4, arctigenin slightly inhibited NO production by IFN-γ signals in a dose-dependent manner at higher concentration, whereas NO production by lipopolysaccharide signal was significantly increased by arctigenin treatment. In the absence of stimuli, arctigenin did not stimulate or inhibit NO production at the tested concentration (data not shown). L-NAME also inhibited NO production in lipopolysaccharide- and INF-γ-stimulated RAW264·7 cells with IC50 values of 180 and 118 μM, respectively.

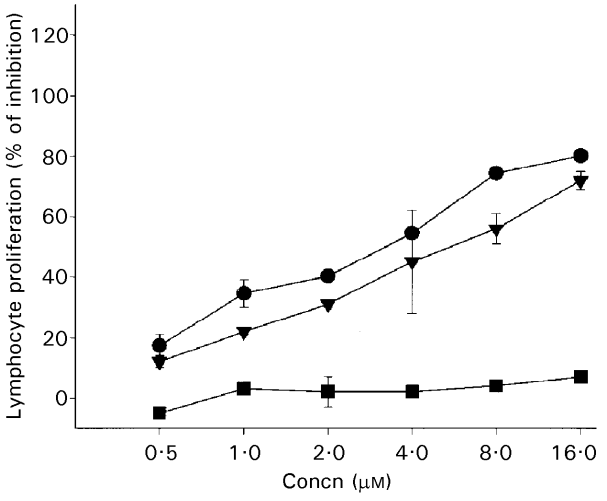

Figure 3. Effect of arctigenin on lymphocyte proliferation from mouse splenocytes in response to concanavalin A (●) and lipopolysaccharide (▼). (■, Normal cells.) Splenocytes (5×10^6 cells mL^{-1}) were incubated with various concentrations of arctigenin in the presence or absence of 1 $\mu g\,mL^{-1}$ concanavalin A or lipopolysaccharide for 48 h. Cell proliferation was assayed by the conventional MTT method. Data represent mean ± s.e.m. of four independent observations performed in triplicate. Basal and stimulated OD values of lymphocyte were 0·20–0·21 (basal), 1·40–1·45 (concanavalin A), and 0·45–0·50 (lipopolysaccharide).

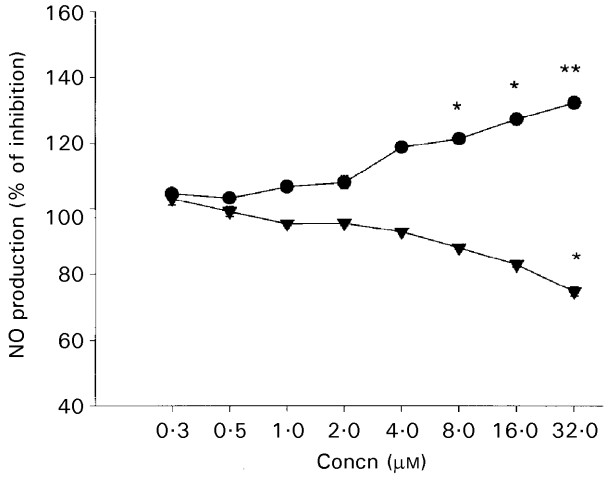

Figure 4. Effect of arctigenin on NO production stimulated by lipopolysaccharide (●) and INF-γ (▼). RAW264·7 cells (1×10^6 cells mL^{-1}) were incubated with various concentrations of arctigenin in the presence or absence of 1 μg mL^{-1} lipopolysaccharide or 50 units mL^{-1} IFN-γ for 24 h. Supernatants were collected and assayed for nitrite. Data represent mean ± s.e.m. of four independent observations. Basal and stimulated level of nitrite were $0{\cdot}5 \sim 1{\cdot}0$ μM (basal), $30 \sim 40$ μM (lipopolysaccharide) and $45 \sim 55$ μM (IFN-γ), respectively. *$P < 0{\cdot}05$ and **$P < 0{\cdot}01$ compared with controls (lipopolysaccharide or INF-γ alone).

Discussion

In the course of screening natural products to find novel anti-inflammatory drugs such as TNF-α inhibitors (Chae et al 1998; Cho et al 1998a,b,c), we have discovered that lignan compounds including eudesmin, pinoresinol, savinin and arctigenin have potent suppressive effects on cytokine production (Chae et al 1998; Cho et al 1998c). In particular, arctigenin strongly inhibited TNF-α production from murine macrophage stimulated by lipopolysaccharide. On the basis of these previous reports, we have examined the immunoregulatory effects of arctigenin on the immune response performed by activated macrophage and lymphocytes.

The data presented in this paper indicate that arctigenin can exert a significant immunomodulatory effect on mammalian immune response in-vitro. In particular, arctigenin significantly regulated the ability of macrophage and lymphocyte in response to mitogens such as lipopolysaccharide and concanavalin A. Thus, in differentiated U937 cells stimulated by lipopolysaccharide, RAW264·7 cells activated by lipopolysaccharide, and splenocytes treated by concanavalin A, arctigenin showed distinct modulatory activities in the production of TNF-α and NO, and the proliferation of T lymphocytes. These inhibitory activities (IC50) are summarized in Table 1. The effects were not due to non-specific cytotoxicity, since cell viability was not altered by arctigenin at the tested concentration showing the regulatory activities (Figures 2, 3 and 4), even though it significantly arrested RAW264·7 cell proliferation at concentrations higher than 40 μM for 24-h incubation (ED50 > 70 μM) (data not shown).

Since the pathophysiological consequences of several diseases are caused by the excessive release of TNF-α and proliferation of T lymphocyte (Moncada et al 1991; Panayi et al 1992; Sekut & Connolly 1996), it is conceivable that arctigenin may have beneficial clinical effects. It showed potent inhibitory activity on some inflammatory mediators as shown in our data. The inhibitory effects of the compound on TNF-α production and lymphocyte proliferation were higher than that of natural compounds isolated previously (Hwang et al 1996; Kang et al 1996; Chae et al 1998; Cho et al 1998b,c), common immunosuppressants such as leflunomide and L-cycloserine (Xu et al 1995; Tamma et al 1996), thalidomide (Shannon et al 1997), tyrphostin-related tyrosine kinase inhibitors (Ruetten & Thiemermann 1997) and pentoxifylline (D'Hellencourt et al 1996). Directly or indirectly TNF-α plays a central role in several steps of chronic inflammatory diseases and cancer, including angiogenesis, cell adhesion and release of other inflammatory mediators, and in DNA replication of HIV (Beutler 1995; Sekut & Connolly 1996; Jackson et al 1998). Arctigenin seems to have a wide range of therapeutic activities in these TNF-α-mediated diseases such as arthritis, tumour metastasis, even AIDS. Reports that arctigenin strongly inhibited HIV replication have led to its derivatives being developed for HIV therapy (Eich et al 1996; Vlietinck et al 1998). Our results suggest that arctigenin could inhibit DNA replication of HIV in either a direct or an indirect manner during chemotherapy for HIV infection.

The curative potential led us to evaluate possible in-vitro therapeutic potency through co-treatment with known TNF-α inhibitors (Table 2). TNF-α production from lipopolysaccharide-activated macrophage is regulated by a wide variety of related enzymes such as protein kinase C, protein tyrosine kinase and protein kinase A at the transcriptional or translational level (Lee et al 1994; Hwang et al 1996; Sekut & Connolly 1996; Ruetten & Thiemermann 1997). To understand the biosynthetic pathway, researchers have developed different kinds of enzyme inhibitors to block TNF-α production (Ruetten & Thiemermann 1997; Shannon et al 1997). The well-known TNF-α inhibitors enhanced the inhibitory activity of arctigenin (Table 2). This suggests that these kinds of drugs

may increase the therapeutic efficacy of arctigenin, although the data were obtained from in-vitro experiments.

An important observation of this study is that the increase in NO production by arctigenin did not relate to the decreased production of TNF-α (Figure 4). The formation of NO under inflammatory conditions from the guanidino nitrogen group of L-arginine is catalysed by inducible nitric oxide synthase (iNOS), expressed in response to pro-inflammatory stimuli (Moncada et al 1991; Wolkow 1998). The mechanism of iNOS induction by lipopolysaccharide is regulated by pro- and anti-inflammatory cytokines. For instance, endogenous release of TNF-α is involved in the process of iNOS induction (Spink et al 1995), indicating that inhibition of TNF-α results in decreasing NO release. In this study, however, we showed different results (Figure 4) at the same lipopolysaccharide signal, although arctigenin potently suppressed TNF-α production and slightly attenuated NO production by IFN-γ signal. Therefore, it seems possible that arctigenin may exert its enhancing effect on NO production by an independent manner, which requires further analysis, compared with TNF-α inhibition and IFN-γ signal. Since some of the literature has reported that arctigenin suppressed cAMP phosphodiesterase (Nishibe et al 1986) and the receptor binding of platelet activating factor (PAF) (Han et al 1996), the approach for analysis is likely to be focussed in terms of intracellular cAMP or PAF level. Indeed, there is some evidence that cAMP and PAF regulate TNF-α and NO production in a complicated manner (Im et al 1997; Wang et al 1997). Another possible approach to understand the opposing effects of arctigenin on lipopolysaccharide- and IFN-γ-induced NO production would be to consider the different regulatory roles in biochemical phenomena of the two signals, such as the different levels of EP2 and EP4 prostaglandin E receptors expressed by lipopolysaccharide and IFN-γ signals (Katsuyama et al 1998).

We have shown that arctigenin significantly modulated TNF-α and NO production and lymphocyte proliferation, suggesting that arctigenin may participate in an immunoregulatory effect in the host immune response. There have been a few reports on the immunomodulatory effects of lignans on cytokine production, inflammatory mediators, and lymphocyte activation and proliferation, and it is thought that these biological activities of arctigenin will help in the understanding of the immunoregulatory effects of lignans. Other immune responses by macrophage and lymphocytes should be tested to precisely define the functional modulation of arctigenin in-vivo.

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