Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 339 (2006) 137–144
BBRC
www.elsevier.com/locate/ybbrc

Dioscorin isolated from *Dioscorea alata* activates TLR4-signaling pathways and induces cytokine expression in macrophages

Shu-Ling Fu [a,*], Ya-Hui Hsu [b], Pei-Yeh Lee [a], Wen-Chi Hou [c], Ling-Chien Hung [a], Chao-Hsiung Lin [d], Chiu-Ming Chen [d], Yu-Jing Huang [a]

[a] *Institute of Traditional Medicine, National Yang-Ming University, 155, Sec.2, Li-Nong St., Taipei 11221, Taiwan*
[b] *Bureau of Food and Drug Analysis, Department of Health, Executive Yuan, 161-2, Kunyang Street, Nangang, Taipei 11221, Taiwan*
[c] *Graduate Institute of Pharmacognosy Science, Taipei Medical University, Taipei 11221, Taiwan*
[d] *Department of Life Science, National Yang-Ming University, 155, Sec.2, Li-Nong St., Taipei 11221, Taiwan*

Received 18 October 2005
Available online 9 November 2005

Abstract

The Toll-like receptor 4 (TLR4)-signaling pathway is crucial for activating both innate and adaptive immunity. TLR4 is a promising molecular target for immune-modulating drugs, and TLR4 agonists are of therapeutic potential for treating immune diseases and cancers. Several medicinal herb-derived components have recently been reported to act via TLR4-dependent pathways, suggesting that medicinal plants are potential resources for identifying TLR4 activators. We have applied a screening procedure to systematically identify herbal constituents that activate TLR4. To exclude possible LPS contamination in these plant-derived components, a LPS inhibitor, polymyxin B, was added during screening. One of the plant components we identified from the screening was dioscorin, the glycoprotein isolated from *Dioscorea alata*. It induced TLR4-downstream cytokine expression in bone marrow cells isolated from TLR4-functional C3H/HeN mice but not from TLR4-defective C3H/HeJ mice. Dioscorin also stimulated multiple signaling molecules (NF-κB, ERK, JNK, and p38) and induced the expression of cytokines (TNF-α, IL-1β, and IL-6) in murine RAW 264.7 macrophages. Furthermore, the ERK, p38, JNK, and NF-κB-mediated pathways are all involved in dioscorin-mediated TNF-α production. In summary, our results demonstrate that dioscorin is a novel TLR4 activator and induces macrophage activation via typical TLR4-signaling pathways.

Keywords: Dioscorin; *Dioscorea alata*; TLR4; NF-κB; MAPK; Cytokine

The innate immunity is activated immediately after infection for fighting against the invading pathogens and therefore is the first line of host defense. The innate immune system requires a series of pattern-recognition receptors (PRRs) that recognize specific pathogen-associated molecular patterns (PAMPs) [1]. Recognition of PAMPs by PRRs results in the expression of effector molecules, such as cytokines, chemokines, and co-stimulatory molecules, which subsequently mediate inflammatory reactions and adaptive immune responses. The Toll-like receptor (TLR) family members have been found to be crucial for innate immunity [2]. To date, 11 TLR family members (TLR1–11) have been identified [3]. Ligands for different TLR receptors have also been characterized [3,4].

TLR4 is mainly expressed in monocytes, neutrophils, macrophages, and dendritic cells [5]. It has been demonstrated that the constitutive expression of TLR4 induces upregulation of inflammatory cytokines [6]. Additional evidence linking TLR4 to the innate immunity comes from the demonstration that TLR4 is the receptor for lipopolysaccharides (LPS); mice with either a spontaneous mutation of the TLR4 gene (C3H/HeJ) or a targeted disruption of the gene (C57BL/10ScCR and C57BL/10ScN) are

* Corresponding author. Fax: +886 2 28225044.
E-mail address: slfu@ym.edu.tw (S.-L. Fu).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.005

hyporesponsive to LPS [7]. In addition to LPS, TLR4 recognizes several endogenous ligands, such as fibrinogen, hyaluronic acid, heparan sulfate, Tamm–Horsfall glycoprotein, and β-defensin. These ligands are proposed to trigger TLR4-mediated inflammatory reactions or adaptive immune responses [8–10].

TLR4-mediated signaling pathways have been extensively studied using LPS as the prototype ligand. Stimulation of TLR4 triggers either the MyD88 (Myeloid differentiation factor 88)-dependent or the MyD88-independent signaling pathway. In the MyD88-mediated TLR4 signaling, MyD88 interacts with TRAK [interleukin-1 (IL-1) receptor-associated kinase] family and activates TRAF6 (tumor-necrosis factor-receptor-associated factor 6), which ultimately leads to the activation of nuclear factor-κB (NF-κB), and p42/p44ERK, p38, and JNK (Jun N-terminal kinase). In the MyD88-independent TLR4 signaling, NF-κB is also subsequently activated. Upon activation, NF-κB translocates from cytoplasm to nucleus and functions as a transcription factor to induce NF-κB-mediated cytokine expression including IL-1, IL-6, TNF-α, and IFN-γ [11].

Since TLR4 plays essential roles in the activation of host defense mechanisms, much effort has been made to identify agents that activate TLR4 signaling, as a way to induce immune responses in order to treat diseases. Many medicinal plants are widely applied as immune modulators and have been safely used as ingredients of traditional medicines or food supplements for a long period in many oriental countries. Particularly, several medicinal plant-derived natural compounds, purified polysaccharides, purified proteins, or crude extracts have recently been reported to act on TLR4-dependent pathways to stimulate immune responses [12–17]. These observations suggest that medicinal plants can be used as resources for the discovery of effective but non-toxic TLR4 agonists. In the present study, we established a screening procedure to search for novel TLR4-activating medicinal herbal components. We found a novel TLR4 activator, dioscorin, which is originally purified from *Dioscorea alata* [18,19]. We also further investigated the dioscorin-induced signaling pathways and cytokine expression profiles in RAW 264.7 macrophages.

Materials and methods

Chemicals and antibodies. Lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4) and polymyxin B sulfate were purchased from Sigma (St. Louis, MO). Pyrogen-free water was purchased from Taiwan Biotech (Taoyuan, Taiwan). PD98059, SB203580, LY294002, PDTC, and SP600125 were purchased from Calbiochem (La Jolla, CA). Anti-ERK1/2 antibody was purchased from BD Biosciences (San Diego, CA), and anti-actin antibody was from Sigma–Aldrich (St. Louis, MO). Antibodies against phospho-ERK1/2 (Thr202/Tyr204) (clone E10), p38, phospho-p38 (Thr180/Tyr182), JNK, and phospho-JNK (Thr183/Tyr185) were purchased from Cell Signaling (Beverly, MA).

Preparation of purified herbal components or herbal water extracts. Pure natural compounds were purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in DMSO. All herbs (raw materials) were purchased from local drug stores. To make herbal water extracts, 1 g of powdered herbs was resuspended in 10 ml sterile water, stirred overnight at 4 °C, and sterilized through 0.22 μm filter (Millipore, Bedford, MA). Purified proteins and polysaccharides are gifts from Dr. Wen-Chi Hou in the Graduate Institute of Pharmacognosy Science, Taipei Medical University, Taipei, Taiwan. LPS and Polymyxin B sulfate were both dissolved in pyrogen-free ddH_2O.

Cells and culture medium. The murine macrophage cell line RAW 264.7 was purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan). DMEM was purchased from Biochrom KG (Berlin, Germany), and bovine calf serum (BCS) was from HyClone (Logan, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel), and trypsin was from Invitrogen (Carlsbad, CA). Antibiotics (penicillin and streptomycin), L-glutamine, and sodium pyruvate were purchased from Invitrogen (Carlsbad, USA). Both RAW 264.7 and its derived cell line RAW 264.7/Luc-P1 (see below) were regularly cultured at 37 °C in 5% CO_2 incubator in DMEM supplemented with 10% heat-inactivated BCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μM L-glutamine, and 1 mM sodium pyruvate. Hematopoietic cells isolated from bone marrow were cultured at 37 °C in 5% CO_2 incubator in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μM L-glutamine, and 1 mM sodium pyruvate.

Experimental animals. C3H/HeN and C3H/HeJ were originally provided by the Animal Center at National Cheng-Kung University (Tainan, Taiwan) and maintained at the Animal Center of National Yang-Ming University (Taipei, Taiwan). Full-length *tlr4* cDNA from both types of mice was cloned and sequenced to validate that C3H/HeN and C3H/HeJ mice carry wild type *tlr4* and defective *tlr4* gene, respectively.

Generation of a LPS-responsive cell clone with stably integrated reporter gene. Transfection of RAW 264.7 cells with pELAM1-Luc and pCI-puro plasmids has been described previously [20]. The pELAM1-Luc consists of NF-κB-responsive region from endothelial leukocyte adhesion molecule I (ELAM1), followed by the reporter gene firefly *luciferase*. The plasmid pCI-puro directs expression of the puromycin-resistant gene. Forty-eight hours after transfection, the cells were grown in puromycin-supplemented medium (6 μg/ml) for 1 week. Limiting dilutions of puromycin-resistant cells were then performed to obtain individual clones. Eighteen clones were picked, expanded in puromycin-supplemented medium (3 μg/ml), and assayed for LPS responsiveness by luciferase assays as described below. The clone showing the strongest luciferase activity upon LPS treatment (designated as RAW 264.7/Luc-P1) was used for all subsequent experiments.

Luciferase assays in RAW 264.7/Luc-P1 macrophage cells. RAW 264.7/Luc-P1 cells were seeded in 24-well plates at the density of 4×10^5 cells per well and grown overnight in 5% CO_2 incubator at 37 °C. After treated with positive control LPS or drugs for 5 h, cells were harvested and lysed in 100 μl lysis reagent. Cell lysates (20 μl) were then mixed with 100 μl luciferin right before luminescence detection. The luminescence resulted from luciferase activity was measured using an AutoLumat LB953 (Berthold Technologies, Bad Wildbad, Germany). All reagents for luciferase assays were purchased from Promega (Madison, WI).

Isolation and culture of hematopoietic cells from mouse bone marrow. C3H/HeN and C3H/HeJ mice were sacrificed and sterilized with 70% alcohol. Under an aseptic condition, femurs were collected and cut with scissors. The bone marrow was exposed and flushed with medium using a 10 ml syringe attached to a 25-gauge needle. Medium containing the bone marrow cells was transferred into a 50 ml conical centrifuge tube and centrifuged for 10 min at 800 rpm. The supernatant was discarded and pellets were resuspended in 10 ml medium using an 18-gauge needle to separate aggregates and produce single-cell suspensions. Bone marrow cells were transferred to 100-mm tissue culture plates. Non-adherent cells were removed and adherent cells were maintained for subsequent experiments.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Cells were resuspended at the desired concentration (10^5 or 10^6 cells/ml) into 60-mm tissue culture plates, grown overnight, and treated with vehicle or drugs for 5 h. Total RNA was isolated using Trizol reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Extracted RNA

was treated with a DNA-free kit (Ambion, Austin, TX) to remove DNA. One microgram of DNA-free total RNA was reverse-transcribed using ThermoScript RT-PCR system (Invitrogen, Carlbad, CA). Synthesized cDNA was amplified using *Taq* DNA polymerase (BIOTOOLS B&M Labs, Madrid, Spain) with the indicated primers. The sequences of primers used in this report were as follows: (1) mouse TNF-α—sense, AAC TTCGGGGTGATCGGTCC; antisense, CAAATCGGCTGACGGTGT GGG; (2) mouse TLR4—sense, CAGCTTCAATGGTGCCATCA; antisense, CTGCAATCAAGAGTGCTGAG; (3) mouse IL-1β—sense, TAC AAGGAGAACCAAGCAACGACA; antisense, TGTCGTTGCTTGGT TCTCCTTGTA; (4) mouse IL-6—sense, GCCAGAGTCCTTCAGAGA GAGATACAG; antisense, CCCAACATTCATATTGTCAG; (5) mouse iNOS—sense, TGGGAATGGAGACTGTCCCAG; antisense, GGGATC TGAATGTGATGTTTG; (6) mouse GADPH—sense, TGTGATGG GTGTGAACCACGAG; antisense, TGCTGTTGAAGTCGCAGGA GAC. Expected PCR products for TNF-α, TLR4, IL-1β, IL-6, iNOS, and GADPH are 331, 438, 287, 343, 306, and 471 bp, respectively. Thirty amplification cycles were performed for TNF-α, IL-1β, IL-6, and iNOS (PCR: 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min); 30 amplification cycles for TLR4 (PCR: 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min); 20 cycles for GADPH (same PCR condition as TLR4). The PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

Western blot. 10^6 cells were seeded in six-well plates for 16–18 h, treated with vehicle or drugs, washed with ice-cold phosphate-buffered saline (PBS), scraped, and lysed with 200 μl RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% (w/v) sodium deoxycholate, 5 mM EDTA (pH 8.0), and 1 mM EGTA (pH 8.0)] plus protease inhibitor cocktail (Sigma, St. Louis, MO) for 15 min on ice. The lysates were clarified by high-speed centrifugation (13,000*g*, 4 °C). Aliquots of the protein extracts were quantified by the Bradford protein assay (Bio-Rad, Hercules, CA). Fifty microgram protein lysate was loaded and analyzed using SDS–PAGE gels, transferred to PVDF membranes (Millipore, Bedford, MA), and incubated with blocking solution (5% non-fat milk in TBS/0.1% Tween 20) for 1 h. Primary antibody and secondary horseradish peroxidase-conjugated antibody diluted in 1% BSA/TBS/0.1% Tween 20 were sequentially added to and incubated with the membranes for 5–24 and 1 h, respectively. Protein bands were visualized by chemiluminescence using the ECL detection system (Amersham Biosciences, Uppsala, Sweden).

Enzyme-linked immunosorbent assay (ELISA). RAW 264.7 cells (5×10^5) were seeded in 24-well plates and grown overnight in 5% CO_2 incubator at 37 °C. Cells were then treated with various inhibitors for 1 h and incubated with LPS or dioscorin for another 5 h. The medium was then harvested for measurement of TNF-α using the DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN).

Statistical analysis. Data are expressed as mean values ± standard error. Data were compared by two-sample *t* test. Differences were considered statistically significant for $p < 0.05$.

Results

Systematic screening for herbal components that activate NF-κB

The strategy for screening TLR4-dependent agents is described as in Fig. 1. In part I, candidate natural compounds/herbal components that activate NF-κB are first selected by a NF-κB activity-based reporter assay. In part II, NF-κB-activating natural products/herbal components from part I are then incubated with bone marrow cells isolated from wild type or TLR4-defective mice. Those natural products induced expression of TNF-α and IL-1β in wild type cells but not in TLR4-defective cells are considered to act as TLR4-activating drugs. We choose NF-κB as the target molecule since NF-κB is a crucial signal transducer mediating TLR4-downstream signaling in both MyD88-dependent and MyD88-independent pathways. Moreover, the NF-κB-responsive region in the reporter construct is derived from the promoter of ELAM1 (E-selectin) that has been previously used to detect TLR4-downstream NF-κB activation [21,22]. The *luciferase* is used as the reporter gene, because the protein product of this gene is easily measurable and displays low endogenous activity in mammalian cells. The well-known potent TLR4 agonist, LPS, is included in this study as the positive control. Since NF-κB-based reporter assay is fairly efficient, we do not limit our screening only to known immune-modulated agents.

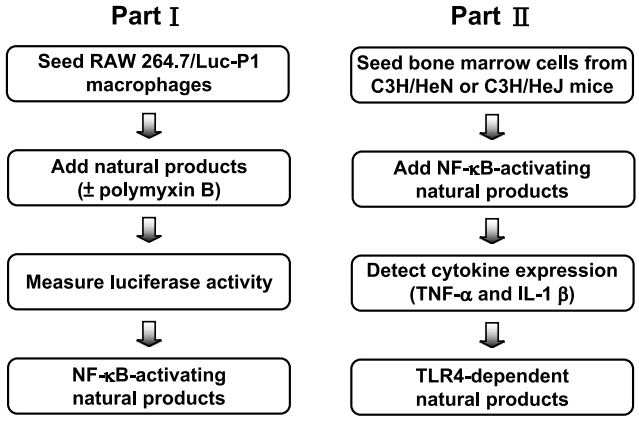

Fig. 1. Flowchart depicting the screening of TLR4-activating natural products. (Part I) Identification of NF-κB activators by the reporter assays. RAW 264.7/Luc-P1 cells were treated with vehicle or plant components in the absence or presence of polymyxin B (10 μg/ml), and then their luminescence was measured to determine NF-κB activity. Polymyxin B-treated plant components showing activity threefold higher than that of vehicle-only assays are defined as NF-κB activators. (Part II) Identification of TLR4-dependent activators. Candidate natural components identified from part I were further incubated with bone marrow cells isolated from TLR4-functional or TLR4-defective mice, and their ability to stimulate cytokine production was measured. Natural products that selectively stimulated cytokine production in C3H/HeN cells are considered as TLR4 activators.

We had tested a total of 50 medicinal plant-derived nature compounds, proteins or crude herbal water extracts. The treatment concentrations used were as follows: 10 μM for pure compounds, 100 μg/ml for purified polysaccharides and proteins, or 1 mg/ml for herbal extracts. The treatment time is 5 h. Polymyxin B, a known pharmacological antagonist of LPS, was premixed with each tested agent to exclude potential LPS activity contaminated within these samples. Agents showing activity threefold higher than the vehicle-only, in the presence of polymyxin B, were considered to be NF-κB activators. Some of the screening data is shown in Table 1. As expected, LPS exhibited NF-κB-driven luciferase activity, and the LPS response was completely blocked by polymyxin B. Furthermore, previously identified TLR4 activators, such as taxol and *Acanthopanax senticosus*, also showed significant NF-κB activity (Table 1). The above data indicate the feasibility

Table 1
The effects of herbal compounds on NF-κB activation in RAW 264.7/Luc-P1 cells with/without polymyxin B

Drug	Concentration	Polymyxin B (10 μg/ml)	Relative luciferase activity (fold)
ddH_2O		−	1.49 ± 0.04
		+	1.29 ± 0.01
DMSO	0.1%	−	0.81 ± 0.06
		+	0.85 ± 0.07
LPS	100 ng/ml	−	42.78 ± 2.38[d]
		+	1.22 ± 0.09
Taxol[a]	10 μM	−	7.43 ± 0.54[d]
		+	7.55 ± 1.29[d]
Chrysin[a]	10 μM	−	1.01 ± 0.04
		+	1.02 ± 0.15
Dioscorin[b]	100 μg/ml	−	66.42 ± 2.37[d]
		+	61.07 ± 4.42[d]
A. senticosus[c]	1 mg/ml	−	30.57 ± 0.63[d]
		+	9.62 ± 2.09[d]
Bupleurum chinense[c]	1 mg/ml	−	14.35 ± 0.21[d]
		+	1.00 ± 0.06
Rubus chingii[c]	1 mg/ml	−	1.67 ± 0.15
		+	0.90 ± 0.05

Data are presented as means ± standard error from three independent experiments.
[a] Pure compound.
[b] Purified protein.
[c] Herbal water extract.
[d] Luciferase activity >3-fold.

of using this reporter assay to pre-screen NF-κB-activating TLR4 agonists. In some cases, such as the crude extract from *Bupleurum chinense*, the NF-κB activity was completely abolished upon polymyxin B treatment (Table 1), indicating potential LPS contamination in this herbal extract.

One of the novel NF-κB activators we identified is dioscorin, which is the glycoprotein isolated from *D. alata* using a procedure described previously [18,19]. As shown in Table 1, dioscorin stimulated NF-κB activity in the absence or presence of polymyxin B. To rule out the possibility that the amount of polymyxin B we added during drug screen might be not enough to remove potential LPS contamination in purified dioscorin, we also treated dioscorin samples with a higher amount of polymxyin B. As shown in Fig. 2, dioscorin activated NF-κB in a concentration-dependent manner, and treatment with a higher amount of polymyxin B did not reduce the activity of dioscorin. We have applied SDS–PAGE and mass spectrometry to confirm the purity and identity of dioscorin fraction used in this study. The data are shown in Fig. 1 in the section of "Supplementary data." Two bands of similar sizes on SDS–PAGE gel were detected, and mass spectrometric analysis revealed these two bands corresponding to dioscorin A and dioscorin B, both being known isoforms of dioscorin protein. Protein bands retrieved from SDS–

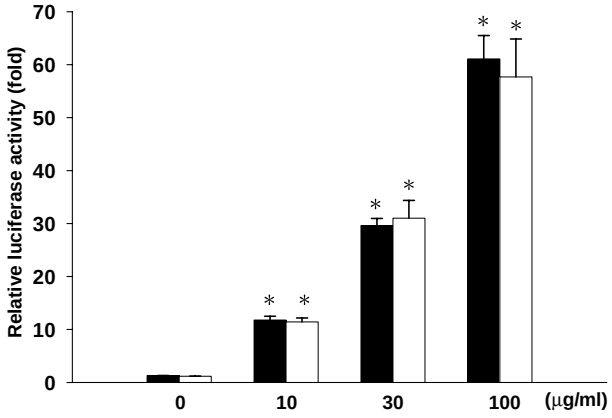

Fig. 2. Dioscorin exhibits NF-κB activity in a concentration-dependent manner. Various amounts of dioscorin were mixed with polymyxin B (■ 10 μg/ml or □ 20 μg/ml) and applied to RAW 264.7/Luc-P1 cells for 5 h. Luciferase assays were performed as described under Materials and methods. Data are presented as means ± standard error from three independent experiments. (*) Values are significantly different from those of vehicle-treated cells ($p < 0.05$).

PAGE gel, containing both isoforms, still exhibit NF-κB-activating ability (Fig. 1, Supplementary data). Furthermore, we used HPLC to collect the major components in dioscorin fraction used in this study. As shown in Fig. 2 in the section of "Supplementary data," HPLC fraction collected from the major peak contains dioscorin protein and still exhibits NF-κB-activating ability in RAW 264.7 cells. Taken together, our data demonstrated that dioscorin activates NF-κB in RAW 264.7 macrophages.

Dioscorin induces expression of TNF-α and IL-1β in a TLR4-dependent manner

We next examined whether dioscorin acts via the TLR receptor using C3H/HeJ and C3H/HeN mice. C3H/HeJ mice carrying a missense point mutation within *Tlr4* are endotoxin-resistant, while the other strain C3H/HeN mice containing wild type *Tlr4* are endotoxin-sensitive. Bone marrow cells of C3H/HeJ and C3H/HeN strain were isolated, treated with pyrogen-free water, positive control LPS, or dioscorin (10 μg/ml) for 5 h, and their expression of TNF-α, IL-1β, and *Tlr4* was measured by RT-PCR. As shown in Fig. 3, dioscorin induced expression of TNF-α and IL-1β in bone marrow-derived cells of C3H/HeN, but not in those of C3H/HeJ mice. Polymyxin B treatment of dioscorin did not affect the expression of both cytokines. We therefore conclude that dioscorin induces the pro-inflammatory cytokines in a TLR4-dependent manner. *Tlr4* expression was detected in both types of cells. The coding region of the *tlr4* gene from these cells was subcloned and sequenced to validate that bone marrow cells from C3H/HeN mice contain wild type *tlr4* but the cells from C3H/HeJ mice carry the defective *tlr4* in which a missense mutation resulting in a substitution of proline at amino acid 712 with histidine (data not shown). This result is consistent with previous report [7].

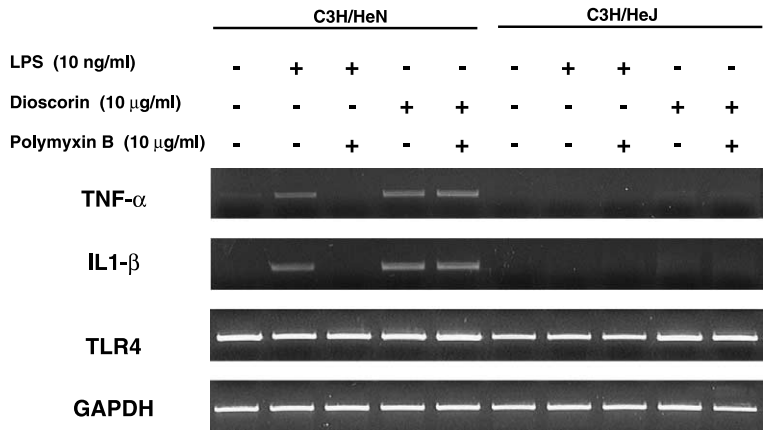

Fig. 3. Dioscorin stimulates cytokine expression in bone marrow cells in a TLR4-dependent manner, Bone marrow-derived cells from C3H/HeJ (TLR4-defective) and C3H/HeN (wild type) mice were treated with sterile water (negative control), LPS (the positive control), or dioscorin (10 μg/ml) for 5 h. Expression of each indicated gene was measured by RT-PCR as described in the section of Materials and methods. Data are one representative example out of three independent experiments. GAPDH expression serves as the internal control.

Dioscorin induces TLR4-downstream cytokine expression in RAW 264.7 macrophages

Upon activation by TLR4 ligands, macrophages can become effector cells that release pro-inflammatory cytokines and reactive oxygen species to eliminate foreign antigens. To test whether dioscorin can activate macrophages, we examined the expression of TLR4-downstream cytokines and inducible nitric oxide synthase (iNOS) in dioscorin-treated RAW 264.7 macrophages. As shown in Fig. 4, dioscorin stimulated expression of TLR4-downstream genes, such as TNF-α, IL-1β, IL-6, and iNOS, in RAW 264.7 macrophages.

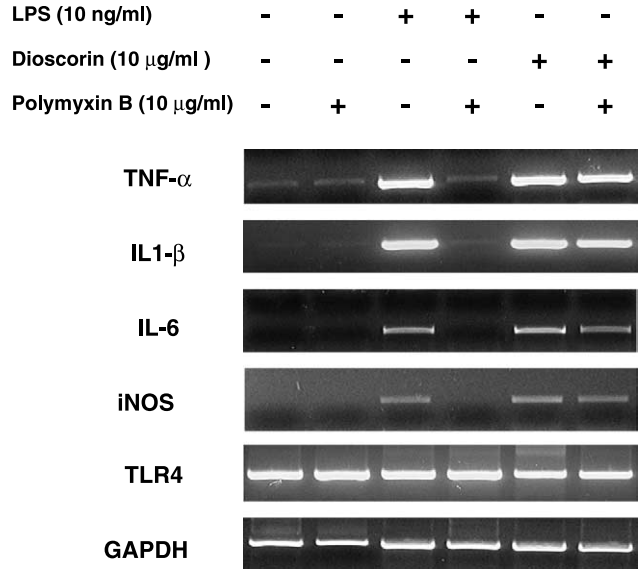

Fig. 4. Dioscorin induces expression of cytokines and iNOS in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with sterile water or dioscorin (10 μg/ml) for 5 h and was measured their expression of TNF-α, IL-1β, IL-6, and iNOS genes, by RT-PCR as described under Materials and methods. Data are one representative example of three independent experiments. GAPDH expression serves as the internal control.

Dioscorin activates MAPK-mediated signaling pathways

In addition to NF-κB, recent reports have shown that MAPK family members are also involved in TLR4-downstream signaling [11]. We therefore examined whether these signaling pathways were stimulated by dioscorin in RAW 264.7 macrophages. Western blots were performed to detect amount of both total and active forms of MAPK proteins (ERK, p38, and JNK) in vehicle- or dioscorin-treated cells. As shown in Fig. 5, activation of all MAPK members was detected in dioscorin-treated macrophages. This result and Fig. 2 demonstrate that dioscorin induced the activation of NF-κB, JNK, p38, and ERK1/2, all of which are known downstream effector molecules of the TLR4 signaling.

Both NF-κB and MAPKs are involved in dioscorin-induced TNF-α expression

One of the cytokines induced by TLR4-dependent pathway in macrophages is TNF-α. The NF-κB and MAPKs (ERK, JNK, and p38) signaling pathways were previously found to be involved in the TL4-dependent induction of TNF-α by LPS agonists [12,14,23–27]. Our data described above indicate that dioscorin can activate both NF-κB and MAPKs. To further examine which pathways are required for dioscorin-mediated TNF-α stimulation, we treated cells with dioscorin in the presence of various inhibitors (PD98059 for blocking MEK; SB203580 for p38; PDTC for NF-κB; and SP600125 for JNK), and measured the effect of each inhibitor on TNF-α production by ELISA. The effects of these inhibitors on LPS-induced TNF-α production were also determined for comparison. In the presence of these inhibitors, TNF-α production was clearly repressed, suggesting that ERK, JNK, p38, and NF-κB-mediated pathways are all required for dioscorin-mediated

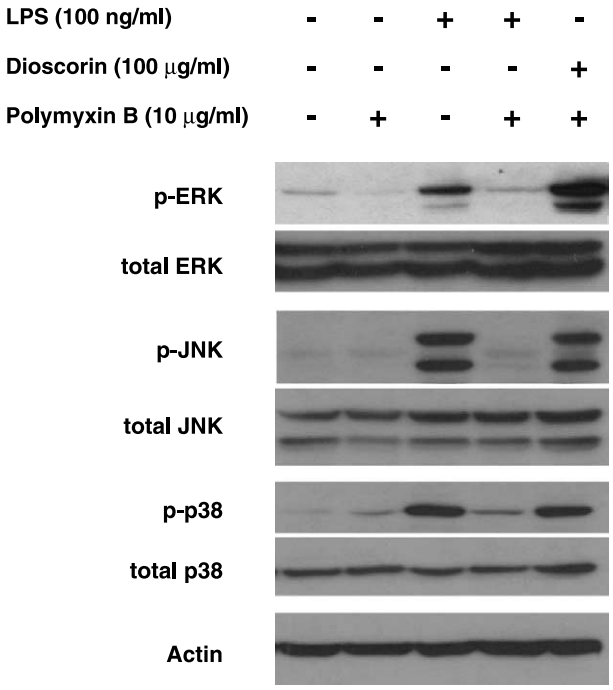

Fig. 5. Dioscorin activates MAP kinase-signaling pathways in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with sterile water or polymyxin B-treated dioscorin (100 μg/ml) for 20 min. Expression of total and activated forms of MAPK family proteins was determined by Western blot as described under Materials and methods. Actin expression serves as the internal control. Data are one representative example of three independent experiments.

TNF-α production (Fig. 6A). The result is similar to that of LPS-treated RAW 264.7, despite the fact that the inhibitory effects by various inhibitors are slightly different when LPS and dioscorin are compared (Fig. 6). In summary, NF-κB and MAPKs (ERK, p38, and JNK) are all involved in dioscorin-induced TNF-α expression.

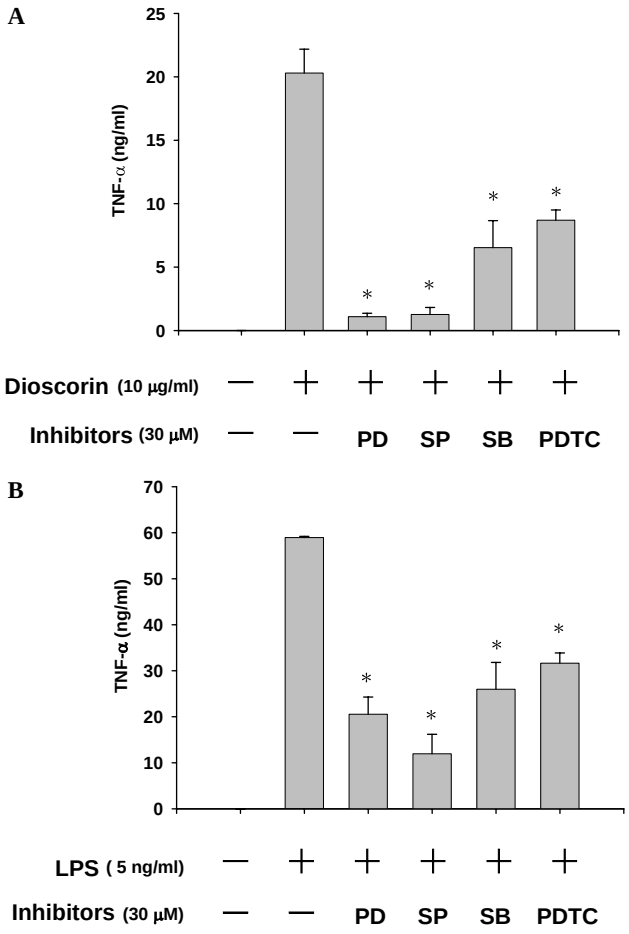

Fig. 6. The effects of various pharmacological inhibitors on dioscorin- or LPS-induced TNF-α induction. RAW 264.7 macrophages were treated with inhibitors PD98059, SB203580, SP600125 or PDTC (all at the final concentration of 30 μM) for 1 h and then treated with polymyxin B-treated dioscorin [10 μg/ml (A)] or LPS [5 ng/ml; (B)] for 5 h. Culture medium was then collected and assayed for TNF-α production using ELISA as described in the section of Materials and methods. Data are shown as means ± standard error from three independent experiments. (*) Values are significantly different from those obtained from dioscorin- or LPS-treated cells in the absence of inhibitors ($p < 0.05$).

Discussion

In the present study, we demonstrated a screening strategy allowing efficient identification of natural products that activate TLR4. The NF-κB-based reporter assays serve as rapid pre-screen of potential TLR4 activators. The fact that previously reported TLR4-activating taxol and herbal extract of *A. senticosus* also exhibit significant NF-κB activity in our assays supports the feasibility of our screening strategy. Furthermore, several NF-κB activators identified in our assays, including dioscorin, were found to be TLR4 activators (Fig. 3 and data not shown). This relatively high correlation between NF-κB activators and TLR4 agonists in our screening which may be due to the NF-κB-responsive region used in this study is from the promoter of the E-selectin, a TLR4-downstream target gene previously shown in microarray analysis [28]. Indeed, this reporter construct has been widely used to detect TLR4-downstream NF-κB activation [21,22,29]. We have tried another NF-κB-based reporter plasmid, which consists of five tandemly repeated NF-κB-binding sites. This reporter was not responsive to TLR4 agonists, such as LPS and taxol, in RAW 264.7 macrophages (data not shown). Therefore, flanking sequences around NF-κB-binding sites may play crucial roles in the mediation of the genuine activation of NF-κB. However, we cannot rule out the possibility that some TLR4-activating herbal extracts may function through NF-κB-independent signaling pathway(s) and these TLR4 agonists would then be overlooked in our screening.

Many researchers have pointed out that the potential contamination of experimental agents with microbial-derived immunostimulatory molecules, such as LPS, may lead to false identification of TLR4 agonists [30,31]. We have previously shown that potential LPS contamination in the herbs should be concerned (described in Ref. [20]). Actually, in our screening crude water extracts from a few herbs completely lost NF-κB activity upon polymyxin B treatment, suggesting that these herbs may be contami-

nated with LPS during cultivation, storage or extraction. However, since crude herbal extracts contain complex components, it is possible that certain constituents of these herbal extracts exhibit LPS-like properties. It is worth noting that the data in the present study confirmed that the results obtained with dioscorin are not due to LPS contamination. Since supplement with polymyxin B did not block activation of the signaling molecules (NF-κB, ERK, JNK, and p38) and cytokine expression triggered by dioscorin. Furthermore, the lack of TNF-α and IL-1β responses to dioscorin in *Tlr4*-defective mice suggest that there is no contamination with bacterial lipoproteins.

We also investigated the possible TLR4-downstream signaling pathways and cytokine production initiated by dioscorin. Both NF-κB and MAPK-signaling proteins were activated by dioscorin. Induction of the pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and iNOS was also observed. Moreover, inhibition of both NF-κB and MAPK signaling affected dioscorin-mediated TNF-α. Similar signaling pathways and cytokine profiles were previously found in TLR4 activation by LPS and polysaccharides of medicinal herbs [12,14,23–27]. Based on these observations, we conclude that dioscorin acts via the typical TLR4-signaling pathways.

Yam species have long been considered as health-promoting plants. Dried slices of yam tubers are frequently used as medicinal plants, and the fresh tubers are a popular food supplement in Asia. The immune-stimulating activity of yam-derived mucopolysaccharide has been recently reported [32]. Dioscorin as tested in this study is a glycoprotein originally isolated from the tuber of yam (*D. alata* cv. Tainong No. 1). This protein has been previously shown to exhibit carbonic anhydrase, trypsin inhibitor, and angiotensin converting enzyme inhibitory activities [18,19]. In this study, we show that dioscorin can also activate TLR4-dependent signaling pathway and induce cytokine expression in macrophages, which has not been described in any previous publication. Our data support the idea that yam is an immune-promoting medicinal plant.

Macrophages play an essential role in host protection against pathogen and tumors. They are important effector cells that eliminate pathogens as part of the innate immune responses and activate T_H cells for adaptive immunity. Activated macrophages exhibited greater phagocytic activity and increased secretion of various anti-pathogen mediators such as cytokines, and reactive oxygen and nitrogen intermediates. In the present study, we showed that dioscorin stimulated cytokine production (e.g., IL-1, IL-6, and TNF-α) and iNOS expression in a macrophage cell line, indicating that dioscorin treatment can lead to macrophage activation. Whether dioscorin can stimulate activation of other immune cells, such as dendritic cells, remains to be tested. In addition, several TLR4 ligands, such as taxol, are found to be species-specific. It will be of interest to determine whether dioscorin fraction also activates human macrophages. Activated macrophages, producing high levels of iNOS, IL-12, and TNF-α, are potent effector cells that exhibit tumor-killing properties [33]. Moreover, TLR4 agonists, such as synthetic compounds and OK432, have been shown to act as immune-promoting adjuvant and anticancer agent [22,34]. The potential therapeutic effects of dioscorin remained to be further determined.

Acknowledgments

We thank Dr. Tsun-Hsien Chuang at the Scripps Research Institute (La Jolla, USA) for providing plasmid pELAM1-Luc. We also thank Dr. Ming-Ji Fann at the Yang-Ming University (Taipei, Taiwan) for valuable suggestions on the manuscript. This work was supported by grants from the National Science Council, Taiwan (NSC 93-2320-B010-067 and NSC 93-2752-B010-004-PAE).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.11.005.

References

[1] C.A. Janeway Jr., R. Medzhitov, Innate immune recognition, Annu. Rev. Immunol. 20 (2002) 197–216.
[2] R. Medzhitov, Toll-like receptors and innate immunity, Nat. Rev. Immunol. 1 (2001) 135–145.
[3] E. Lien, R.R. Ingalls, Toll-like receptors, Crit. Care Med. 30 (2002) S1–S11.
[4] K. Takeda, T. Kaisho, S. Akira, Toll-like receptors, Annu. Rev. Immunol. 21 (2003) 335–376.
[5] M. Muzio, N. Polentarutti, D. Bosisio, P.P. Manoj Kumar, A. Mantovani, Toll-like receptor family and signalling pathway, Biochem. Soc. Trans. 28 (2000) 563–566.
[6] R. Medzhitov, P. Preston-Hurlburt, C.A. Janeway Jr., A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity, Nature 388 (1997) 394–397.
[7] A. Poltorak, X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, B. Beutler, Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, Science 282 (1998) 2085–2088.
[8] S.T. Smiley, J.A. King, W.W. Hancock, Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4, J. Immunol. 167 (2001) 2887–2894.
[9] M.D. Saemann, T. Weichhart, M. Zeyda, G. Staffler, M. Schunn, K.M. Stuhlmeier, Y. Sobanov, T.M. Stulnig, S. Akira, A. von Gabain, U. von Ahsen, W.H. Horl, G.J. Zlabinger, Tamm–Horsfall glycoprotein links innate immune cell activation with adaptive immunity via a Toll-like receptor-4-dependent mechanism, J. Clin. Invest. 115 (2005) 468–475.
[10] A. Biragyn, P.A. Ruffini, C.A. Leifer, E. Klyushnenkova, A. Shakhov, O. Chertov, A.K. Shirakawa, J.M. Farber, D.M. Segal, J.J. Oppenheim, L.W. Kwak, Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2, Science 298 (2002) 1025–1029.
[11] S. Akira, Toll-like receptor signaling, J. Biol. Chem. 278 (2003) 38105–38108.
[12] I. Ando, Y. Tsukumo, T. Wakabayashi, S. Akashi, K. Miyake, T. Kataoka, K. Nagai, Safflower polysaccharides activate the transcription factor NF-kappa B via Toll-like receptor 4 and induce cytokine

production by macrophages, Int. Immunopharmacol. 2 (2002) 1155–1162.
[13] Y.D. Yoon, S.B. Han, J.S. Kang, C.W. Lee, S.K. Park, H.S. Lee, H.M. Kim, Toll-like receptor 4-dependent activation of macrophages by polysaccharide isolated from the radix of *Platycodon grandiflorum*, Int. Immunopharmacol. 3 (2003) 1873–1882.
[14] S.B. Han, Y.D. Yoon, H.J. Ahn, H.S. Lee, C.W. Lee, W.K. Yoon, S.K. Park, H.M. Kim, Toll-like receptor-mediated activation of B cells and macrophages by polysaccharide isolated from cell culture of *Acanthopanax senticosus*, Int. Immunopharmacol. 3 (2003) 1301–1312.
[15] H.Y. Hsu, K.F. Hua, C.C. Lin, C.H. Lin, J. Hsu, C.H. Wong, Extract of Reishi polysaccharides induces cytokine expression via TLR4-modulated protein kinase signaling pathways, J. Immunol. 173 (2004) 5989–5999.
[16] M. Okamoto, E.G. Oh, T. Oshikawa, S. Furuichi, T. Tano, S.U. Ahmed, S. Akashi, K. Miyake, O. Takeuchi, S. Akira, K. Himeno, M. Sato, S. Ohkubo, Toll-like receptor 4 mediates the antitumor host response induced by a 55-kilodalton protein isolated from *Aeginetia indica* L., a parasitic plant, Clin. Diagn. Lab. Immunol. 11 (2004) 483–495.
[17] K. Kawasaki, S. Akashi, R. Shimazu, T. Yoshida, K. Miyake, M. Nishijima, Mouse toll-like receptor 4·MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol, J. Biol. Chem. 275 (2000) 2251–2254.
[18] W.C. Hou, H.J. Chen, Y.H. Lin, Dioscorins from different *Dioscorea* species all exhibit both carbonic anhydrase and trypsin inhibitor activities, Bot. Bull. Acad. Sin. 41 (2000) 191–196.
[19] F.L. Hsu, Y.H. Lin, M.H. Lee, C.L. Lin, W.C. Hou, Both dioscorin, the tuber storage protein of yam (*Dioscorea alata* cv. Tainong No. 1), and its peptic hydrolysates exhibited angiotensin converting enzyme inhibitory activities, J. Agric. Food Chem. 50 (2002) 6109–6113.
[20] Y.H. Hsu, S.L. Fu, Detection of endotoxin contamination in Chinese herbs by NF-κB activity-based reporter assays, J. Food Drug Anal. 12 (2004) 34–39.
[21] Y. Bulut, E. Faure, L. Thomas, H. Karahashi, K.S. Michelsen, O. Equils, S.G. Morrison, R.P. Morrison, M. Arditi, Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway, J. Immunol. 168 (2002) 1435–1440.
[22] L.D. Hawkins, S.T. Ishizaka, P. McGuinness, H. Zhang, W. Gavin, B. DeCosta, Z. Meng, H. Yang, M. Mullarkey, D.W. Young, D.P. Rossignol, A. Nault, J. Rose, M. Przetak, J.C. Chow, F. Gusovsky, A novel class of endotoxin receptor agonists with simplified structure, toll-like receptor 4-dependent immunostimulatory action, and adjuvant activity, J. Pharmacol. Exp. Ther. 300 (2002) 655–661.
[23] C.D. Dumitru, J.D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J.H. Lin, C. Patriotis, N.A. Jenkins, N.G. Copeland, G. Kollias, P.N. Tsichlis, TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway, Cell 103 (2000) 1071–1083.
[24] T.D. Geppert, C.E. Whitehurst, P. Thompson, B. Beutler, Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway, Mol. Med. 1 (1994) 93–103.
[25] J.L. Swantek, M.H. Cobb, T.D. Geppert, Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK, Mol. Cell. Biol. 17 (1997) 6274–6282.
[26] B. van den Blink, N.P. Juffermans, T. ten Hove, M.J. Schultz, S.J. van Deventer, T. van der Poll, M.P. Peppelenbosch, p38 mitogen-activated protein kinase inhibition increases cytokine release by macrophages in vitro and during infection in vivo, J. Immunol. 166 (2001) 582–587.
[27] Y.D. Yoon, J.S. Kang, S.B. Han, S.K. Park, H.S. Lee, J.S. Kang, H.M. Kim, Activation of mitogen-activated protein kinases and AP-1 by polysaccharide isolated from the radix of *Platycodon grandiflorum* in RAW 264.7 cells, Int. Immunopharmacol. 4 (2004) 1477–1487.
[28] I. Baltathakis, O. Alcantara, D.H. Boldt, Expression of different NF-kappaB pathway genes in dendritic cells (DCs) or macrophages assessed by gene expression profiling, J. Cell. Biochem. 83 (2001) 281–290.
[29] C.A. Byrd-Leifer, E.F. Block, K. Takeda, S. Akira, A. Ding, The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol, Eur. J. Immunol. 31 (2001) 2448–2457.
[30] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (2004) 499–511.
[31] B. Gao, M.F. Tsan, Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages, J. Biol. Chem. 278 (2003) 174–179.
[32] E.M. Choi, S.J. Koo, J.K. Hwang, Immune cell stimulating activity of mucopolysaccharide isolated from yam (*Dioscorea batatas*), J. Ethnopharmacol. 91 (2004) 1–6.
[33] A.H. Klimp, E.G. de Vries, G.L. Scherphof, T. Daemen, A potential role of macrophage activation in the treatment of cancer, Crit. Rev. Oncol. Hematol. 44 (2002) 143–161.
[34] M. Okamoto, T. Oshikawa, T. Tano, G. Ohe, S. Furuichi, H. Nishikawa, S.U. Ahmed, S. Akashi, K. Miyake, O. Takeuchi, S. Akira, Y. Moriya, S. Matsubara, Y. Ryoma, M. Saito, M. Sato, Involvement of Toll-like receptor 4 signaling in interferon-gamma production and antitumor effect by streptococcal agent OK-432, J. Natl. Cancer Inst. 95 (2003) 316–326.