ELSEVIER

International Immunopharmacology 6 (2006) 473–484

International Immunopharmacology

www.elsevier.com/locate/intimp

Activation of murine peritoneal macrophages by sulfated exopolysaccharide from marine microalgal *Gyrodinium impudicum* (strain *KG03*): Involvement of the NF-κB and JNK pathway

Sung-Yun Bae [a], Joung Han Yim [b], Hong Kum Lee [b], Suhkneung Pyo [a,*]

[a] *Division of Immunopharmacology, College of Pharmacy, Sungkyunkwan University, Suwon, Kyunggi-do, 440-746 South Korea*
[b] *Korea Polar Research Institute, KORDI, Ansan P.O. Box 29, Kyunggi-do, 425-600 Korea*

Received 29 June 2005; received in revised form 4 August 2005; accepted 21 September 2005

Abstract

This study examined the ability of microalgal sulfated exopolysaccharide (MSE) from marine microalgal *Gyrodinium impudicum* (strain *KG03*) to induce secretory and cellular responses in murine peritoneal macrophages. The cytotoxicity induced by preincubating tumor cells with MSE was demonstrated to be concentration-dependent. The MSE-induced tumoricidal activity was partially abrogated by a NO inhibitor, whereas the anti-TNF-α and anti-IFN-α/β antibodies as well as the scavengers of reactive oxygen intermediates had no effect. In addition, supernatants from murine peritoneal macrophages treated with MSE contained nitrite and their iNOS enzymatic activity was significantly increased. Therefore, the tumoricidal activity induced by MSE appeared to be mediated by the production of NO. Treating the macrophages with a JNK inhibitor (SP600125) partially blocked the tumoricidal activation and NO production induced by MSE, whereas inhibitors of the other kinases did not have an inhibitory effect. These results suggest that MSE induces NO production via the JNK dependent pathway. Furthermore, electrophoretic mobility shift assay analyses revealed that the MSE treatment induced the activation of the NF-κB transcription factor. Overall, these results indicate that the tumoricidal activity induced by MSE is mainly due to NO production, and the activation of macrophage by MSE is mediated probably via the NF-κB and JNK pathway.

Keywords: *Gyrodinium impudicum*; Microalgal sulfated exopolysaccharide; Macrophages; Tumoricidal activity; NF-κB; JNK

1. Introduction

There has been increasing interest in microorganisms as producers of high-molecular-weight polysaccharides in recent years, for the reason that these biopolymers often show advantages over the polysaccharides that are currently in use [1]. Algal fucans, a family of sulfated polysaccharides, have several biological activities [2]. Their use as drugs would offer the advantage of no potential risk of contamination with viruses or particles such as prions. Marine algae are a source of natural products with biological and pharmacological properties. Cell walls from marine algae characteristically contain sulfated polysaccharides that have a broad range of important biological properties comprising antioxidant effects, anticoagulant and antithrombotic activities [3,4]. Sulfated polysaccharides are also known to interfere with the

* Corresponding author. Tel.: +82 31 290 7713; fax: +82 31 292 8800.
E-mail address: snpyo@skku.ac.kr (S. Pyo).

1567-5769/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2005.09.009

adsorption and penetration of some enveloped viruses [5] and inhibit the development of Kaposi's sarcoma [6]. Sulfated residues are found in eukaryotic algal exopolysaccharides [7] as well as in some cyanobacterial polysaccharides [8]. *Gyrodinium impudicum* strain *KG03* produces mucous exopolysaccharide [9] and p-KG03, an exopolysaccharide, is a selective in vitro inhibitor of viral replication, particularly that of encephalomyocarditis virus (EMCV) [10]. In addition, previous reports have recently demonstrated the immunostimulatory effect of sulfated polysaccharide [11,12]. However, the mechanisms by which the components of marine algae exert their immunomodulation activity are still unknown.

Macrophages have been shown to be important components of the host defenses against bacterial infections and murine tumor cells (lymphoma and mastocytoma) [13,14]. Peritoneal macrophages can be stimulated by a variety of agents such as IFN-γ, lipopolysaccharides, or other microbial products [15–17] and some of these have also been shown to trigger the release of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and nitrite and to induce tumoricidal activity by the macrophages [18–22].

A large number of studies have shown that different signaling pathways participate in the activation of macrophages by various stimuli [23–26]. Previous studies have demonstrated that the NF-κB and mitogen-activated protein kinase (MAPK) pathway mediate the activation of human monocyte [27–29]. However, the cellular signaling and molecular mechanisms responsible for this activation by microalgal sulfated exopolysaccharide (MSE) are not known.

The aim of this study was to investigate the biological effects of microalgal sulfated exopolysaccharide from marine microalgal *G. impudicum* on macrophages.

2. Materials and methods

2.1. Algal strain and production of p-KG03

The microalgall strain, *KG03*, was originally isolated from a red tide bloom in Korea, and identified as *G. impudicum* (Fig. 1A and B) [10]. Briefly, bacteria-free cultures of the *G. impudicum* strain *KG*03 were grown in a M-KG03 medium in a 2-l photoreactor. The production conditions are as follows: 1% CO_2, 50 ml min^{-1} airflow, 22.5 °C, and light intensity of about 150 μE/m^2/s, and a 16-h light/8-h dark illumination cycle. The cells were removed from the culture medium by centrifugation at 12,000 ×*g* for 30 min at 4 °C. The sulfated polysaccharide (p-KG03) was isolated from the supernatant by adding two volumes of ethanol at 4 °C for 24 h and collecting the precipitate by filtration (Whatman GFF filter). The filtrate was then dissolved in deionized water, and reprecipitated by adding a 3% solution of cetyltrimethylammonium bromide (Cetavolon; Merk, Darmstadt, Germany). The precipitated Cetavolon–polysaccharide complex was collected by centrifugation at 10,000 ×*g* for 20 min at 4 °C, and redissolved in 10% NaCl. The precipitated polysaccharide component was recovered by adding 3 vol of ethanol. The extracted polysaccharide was dissolved in deionized water, and dialyzed against deionized water for two days. Further fractionation and purification were performed by gel chromatography on a Sepharose 4B column (Sigma Chemical Co., St. Louis, MO, USA), which was followed by elution with a 0.4 M NaCl buffer. The eluate was analyzed using the phenol-sulfuric acid method [30]. The carbohydrate-containing fractions were collected, dialyzed using Viva-flow (Sartorius, Germany), and then freeze-dried (Fig. 1C).

2.2. Mice, chemicals and reagents

The C57BL/6 male mice (6–8 weeks old, 17–21 g) were obtained from Charles River Breeding Laboratories (Atsugi, Japan). Unless otherwise indicated, all the chemicals were purchased from Sigma Chemical Co. (St Louis, MO). The

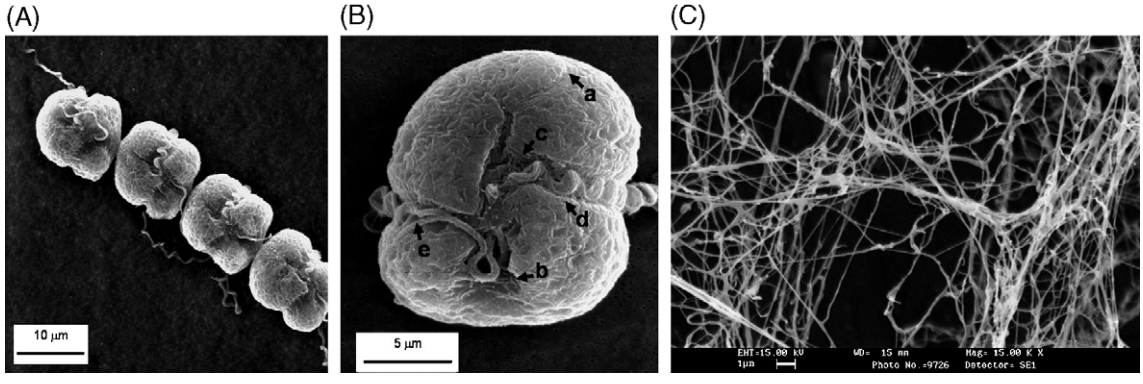


Fig. 1. Scanning electron micrographs of the *Gyrodinium impudicum* strain *KG03*. (A) Four-chained cells. (B) A single cell (a, apicon; b, hypocon; c, groove; d, transverse flagellum; e. longitudinal flagellum). (C) p-KG03 at 0.1% (w/v).

RPMI 1640 medium and fetal bovine serum were purchased from GIBCO (Grand Island, NY). NG-monomethyl-L-arginine (NMMA) was obtained from Calbiochem Co. (LaJolla, CA). The monoclonal antibodies to IFN-α/β and TNF-α antibody were purchased from BD Biosciences (San Jose, CA). The antibodies against JNKs, phospho-JNK (p-JNKs) and actin were obtained from Santa Cruz Biotechnology, USA. All tissue culture reagents as well as the thioglycollate broth were assayed for any endotoxin contamination using the Limulus lysate test (E-Toxate kit, Sigma) and the level of endotoxin was found to be <10 pg/ml.

2.3. Isolation of inflammatory peritoneal macrophages

The thioglycollate-elicited peritoneal-exudate cells were obtained from C57BL/6 male mice (6–8 weeks old, 17–21 g) after they were given an intraperitoneal injection of 1 ml Brewer. Thioglycollate broth (4.05 g/100 ml) (Difco Laboratories, Detroit, ML) followed by a lavage of the peritoneal cavity with 5 ml of medium 3–4 days later. The cells were washed twice and resuspended in RPMI-1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (RPMI-FBS). The macrophages were isolated from the peritoneal exudate cells using the method described by Um et al. [25]. The peritoneal exudate cells were seeded on teflon-coated petri dishes (100 × 15 mm) at densities of $5–6 \times 10^5$ cells/cm^2 and the macrophages were allowed to adhere for 2–3 h at 37 °C in a 5% CO_2 humidified atmosphere. Teflon-coated petri dishes were prepared by spraying them with aerosolized teflon (Fisher Scientific, Pittsburgh, PA), which was followed by sterilization using ultraviolet light for 3 h. The non-adherent cells were removed by washing the dishes twice with a 10 ml prewarmed medium and incubating the dishes for 10 min at 4 °C. The supernatants were then carefully removed and discarded, and the plates were washed once with a prewarmed Dulbecco's Phosphate Buffered Saline solution (PBS) (GIBCO). Cold PBS (15 ml) containing 1.5% FBS (PBS-FBS) was then added, which was followed by the addition of 0.3 ml of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages were removed by rinsing the plates 10 times using a 10 ml syringe. The viability of the detached cells was assessed by trypan blue exclusion and the proportion of macrophages was determined after cytoplasmic staining with acridine orange using fluorescence microscopy. More than 95% of the cell preparations were viable and contained >95% macrophages.

2.4. Macrophage-mediated cytotoxicity

The assay for macrophage cytotoxicity was based on an assay described elsewhere [31]. Briefly, the macrophages (1×10^5 cells/well) were plated into 96-well microtiter plates and incubated in various MSE concentrations for 18 h at 37 °C in a 5% CO_2 incubator. In some experiments, antibodies to cytokines or inhibitors of the metabolic pathway were included along with MSE. The macrophages were also pretreated with the JNK inhibitor (SP600125) prior to stimulation with MSE. The macrophages were washed with RPMI-FBS to remove the MSE and co-incubated with the B16 melanoma cells (ATCC, Rockville, MD) (1.0×10^4/wells; an initial effector : target cell ratio of 10 : 1) at 37 °C in a 5% CO_2 incubator. The cell density was then assessed by incubating the cells with 25 μg/ml MTT [3-(4,5-dimethylthiaozle-2-yl)-2,5-diphenyl-tetrazolium bromide] for a further 4 h. The formazan produced was dissolved in dimethyl sulfoxide and the optical density of each well was determined at a wavelength of 540 nm using a Molecular device microplate reader (Menlo Park, CA). The cytotolytic activity is expressed as the percentage tumor cytotoxicity as follows:

$$\%\ Cytotoxicity = \{1 - O.D.\ of\ [(target + macrophages) - macrophages] / O.D.\ of\ target\ (nontreated)\} \times 100$$

2.5. Nitrite determination

The amount of NO_2^- accumulated in the culture supernatants was measured using the assay system reported by Ding et al. [32]. Briefly, 100 μl of the supernatant was removed from each well and placed into an empty 96-well plate. After adding 100 μl Griess reagent to each well, the absorbance was measured at 550 nm using a Molecular Device microplate reader. The NO_2^- concentration was calculated from a $NaNO_2$ standard curve. The NO_2^- levels are indicative of the amount of NO production. The Griess reagent was prepared by mixing 1 part of 0.1% naphthylethylene diamine dihydrochloride in distilled water with 1 part of 1% sulfanilamide in 5% concentrated H_3PO_4.

2.6. Assay of NOS activity

The macrophages were seeded at 2×10^6 cells/ml in RPMI-1640 with 10% heat-inactivated FBS in 60 mm diameter tissue culture dishes. The cells were treated with MSE for 18 h at 37 °C in a 5% CO_2 humidified atmosphere. An aliquot of the conditioned medium was removed to determine the NO_2^- content. The macrophage monolayers were washed with PBS, scraped, and centrifuged at 180 ×g for 15 min at 4 °C. The cell pellets were resuspended in 500 μl of a sonication buffer (40 mM Tris–HCl, pH 7.9, 25 mM NaCl, containing 100 μM phenyl-methylsulfonylfluoride, 40 μg/ml trypsin inhibitor, 20 μg/ml leupeptin). The cells were lysed by sonication (10 s × 2, 60% output), and the cell debris was removed by centrifugation (180 ×g) for 15 min. The NOS activity was measured as described by Vodovotz et al. [33] in 20 mM Tris–HCl, pH 7.9 containing 4 μM FAD, 4 μM L-arginine and NADPH. The reaction was carried out for 120 min at 37 °C in a total volume of 100 μl in 96-well plates. A Griess assay was to determine the NO concentration.

2.7. Cytokine determination by ELISA

Peritoneal macrophages from treated or untreated male B57BL/6 mice were cultured for 20 h. The culture supernatants were collected and the TNF-α, IFN-α, IL-1 and IL-6 concentration in the culture supernatants was determined using Duo-Set Elisa kit (R and D System, Minneapolis, MN). Manufacturer's instructions were followed. Samples were assessed in triplicate relative to standards supplied by the manufacturer.

2.8. Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA)

The macrophages (2×10^6 cells/ml) were suspended in RPMI 1640 medium supplemented with 10% FBS and placed in 6-well plates (3 ml/well) and incubated at 37 °C. The cells were then incubated for 6 h after first exposing them to MSE and collected on ice before isolating the nuclear extracts. The cells were washed with ice-cold phosphate-buffered saline and suspended in 200 μl of a lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol). The cells were then allowed to swell on ice for 15 min, after which 12.5 μl of 10% nonidet P-40 was added. The tube was mixed thoroughly for 10 s using a Vortex mixer prior to centrifugation (10,000 $\times g$) at 4 °C for 3 min. The nuclear pellets obtained were resuspended in 25 μl of an ice-cold nuclear extraction buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol), and kept on ice for 15 min with intermittent agitation. The samples were subjected to centrifugation for 5 min at 4 °C, and the supernatant was stored at −70 °C. An aliquot was taken and the protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The EMSAs were carried out using a digoxigenin (DIG) gel shift kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's protocol. Briefly, the oligonucleotide 5′-AGTTGA*GGGGACTTTCC*CAGG-3′ containing the NF-B binding site was DIG-labeled using a 3′-end labeling kit, and the DNA probe was incubated with 10 μg of the nuclear extract at room temperature for 10 min. Subsequently, the protein–DNA complexes were separated on a 6% polyacrylamide gel, and electrically transferred to a nylon membrane (Boehringer Mannheim Biochemica) for chemiluminescence band-detection. The specificity of the binding was examined using competition experiments, where a 100-fold excess of the unlabeled oligonucleotide with the same sequence or unrelated oligonucleotide (5′-CTAGTGAGCC-TAAGGCCGGATC-3′) was added to the reaction mixture before adding the DIG-labeled oligonucleotide.

2.9. Western blot analysis

Western blot analysis was performed by a modification of a technique described elsewhere [34]. After the treatment, the cells were washed twice in PBS and suspended in a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP40, 100 μg/ml phenylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin). The cells were placed on ice for 30 min. The supernatant was collected after centrifugation at 15,000 g for 20 min at 4 °C. The protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Lab, Hercules, CA) with BSA (Sigma) as the standard. The whole lysates (20 μg) were resolved on a 7.5% SDS-polyacrylamide gel, transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington heights, IL) and probed with the appropriate antibodies. The blots were then developed using an enhanced chemoluminescence (ECL) kit (Amersham). In all immunoblotting experiments, the blots were reprobed with the anti-β-actin antibody as a control for the protein loading.

2.10. Statistical analysis

Each result is reported as means ± S.E.M. Statistical difference between the groups was determined by one-way analysis of variance (ANOVA) followed by Fisher's PLSD and the significant values are represented by an asterisk. ($*p < 0.05$).

3. Results

3.1. In vitro activation of macrophages with MSE

In order to determine if macrophages could be activated to express the tumoricidal activity in vitro, thioglycollate-elicited macrophages were treated with various MSE doses for 18 h and co-cultured with the B16 tumor cells, which were used as the targets because they are either TNF-α or NO sensitive. MSE (0.1–10 μg/ml) increased the level of cytotoxicity in the macrophages in a concentration-dependent manner (Fig. 2). MSE did not affect the cell viability but concentrations >10 μg/ml were found to be cytotoxic (data not shown). In addition, the effects of MSE were not due to the result of endotoxin contamination, which was found to be <10 pg/ml, as assessed by the limulus test.

3.2. Mediators of MSE-induced macrophage cytotoxicity

A number of compounds have been implicated in macrophage cytotoxicity. These include H_2O_2, TNF-α, NO, and interferon [35–38]. In order to determine if these compounds are involved in MSE-mediated cytotoxicity, an attempt was made to abrogate the macrophage cytotoxicity by inhibiting their production or by neutralizing their activity. The general design of these experiments was to preculture the macrophage with inhibitors (NMMA, catalase and superoxide dismutase) or antibodies (anti-TNF-α and anti-IFN-α/β), to challenge them with tumor cells, also in the presence of the inhibitors or antibodies, and to assess the cytotoxic activity. None of the inhibitors or antibodies at the concentrations used in this study affected the growth of tumor cells. In addition, the isotype-matched control antibodies had no effect on the cytotoxic

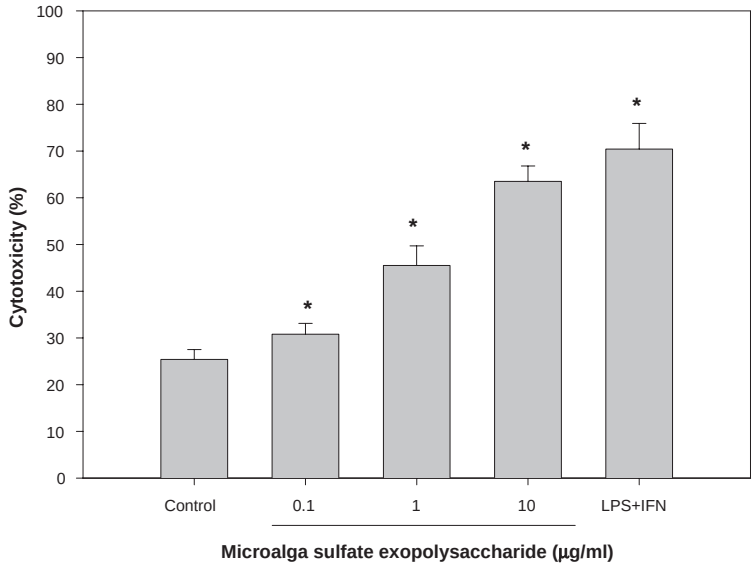


Fig. 2. Cytotoxicity of B16 tumor cells by MSE-activated macrophages. The peritoneal macrophages were stimulated with various doses of microalga sulfated exopolysaccharide (MSE) for 18 h. The macrophage tumoricidal activity was determined as described in Materials and methods. The data shown are the results of an initial effector/target ratio of 10 : 1. The results are reported as a mean ± S.E.M. of three independent experiments. As positive control, IFN-γ (100 U/ml) combined with LPS (1 μg/ml) was used. *: Significantly different from the control (no treatment); $p < 0.05$.

activity (data not shown). The role of H_2O_2 and the superoxide anion was evaluated by inactivating them with catalase and superoxide dismutase, respectively. The role of TNF and IFN was examined by neutralizing them with antibodies. The role of NO was determined by inhibiting its production with NMMA. The addition of catalase, superoxide dismutase, anti-TNF-α and anti-IFN-α/β antibodies had little effect on the cytotoxicity of the activated macrophages against the B16 tumor cells (Table 1). Moreover, MSE did not increase the rate of superoxide anion, hydrogen peroxide and cytokines production (IL-1, IL-6, TNF-α, and IFN) compared with that in the untreated cells (data not shown). Thus, reactive oxygen species and those cytokines did not appear to be involved in MSE-induced tumoricidal activity. In contrast, the NO inhibitor partially abrogated the MSE-induced cytotoxicity against the target (Table 1). Overall, these results suggest that NO is mainly involved in the tumoricidal activity of the MSE-stimulated macrophages.

Table 1
Inhibition of the tumoricidal activity of MSE-activated macrophages by the antibodies or inhibitors

Treatment[a]	% Cytotoxicity of target cells
None	22.52 ± 2.56
MSE[b] (10 μg/ml)	66.34 ± 4.05*
MSE + anti-TNF-α (500 U)	63.14 ± 1.37*
MSE + NMMA[c] (0.5 mM)	30.73 ± 3.54**
MSE + anti-IFN-α/β (500 U)	70.24 ± 2.18*
MSE + catalase (500 U)	65.38 ± 4.79*
MSE + superoxide dismutase (400 U)	72.96 ± 5.63*

[a] The macrophages were cultured for 18 h in the medium or in the medium supplement with microalga sulfated exopolysaccharide (MSE) (10 μg/ml) in the presence or absence of various antibodies or inhibitors. The macrophages were co-cultured for 18 h with the target at an initial effector/target cell ratio of 10 : 1. The results are reported as a mean + S.E.M. of three independent experiments.

[b] MSE, microalga sulfated exopolysaccharide.

[c] NMMA, N^G -monomethyl-L-arginine.

* Significantly different from the control (no treatment); $p < 0.05$.

** Significantly different from the MSE-treated; $p < 0.05$.

3.3. Induction of iNOS enzymatic activity in response to MSE

NO is produced by the enzymatic conversion of arginine in citrulline via the enzyme, iNOS. The functionality of the iNOS protein found in cells was examined by measuring the iNOS enzymatic activity in the cell extracts. As shown in Table 2, a significant increase in enzymatic activity compared with the untreated cells was observed in the MSE-treated

Table 2
Effect of MSE on the iNOS activity in macrophages

Treatment	iNOS specific activity (pmol NO/mg protein/min)
None	24 ± 6.3
MSE (1 μg/ml)	161 ± 18.7*
MSE (5 μg/ml)	383 ± 31.6*
MSE (10 μg/ml)	432 ± 22.4*
LPS (1 μg/ml) + IFN-γ (100 U/ml)	475 ± 33.8*

Peritoneal macrophages were plated in 100-mm diameter tissue culture dishes and treated for 18 h with the medium alone or microalga sulfated exopolysaccharide (MSE). The cell lysates were prepared and assayed for their iNOS enzymatic activity. The values represent the mean ± S.E.M. of three independent experiments.

* Significantly different from control (no treatment); $p < 0.05$.

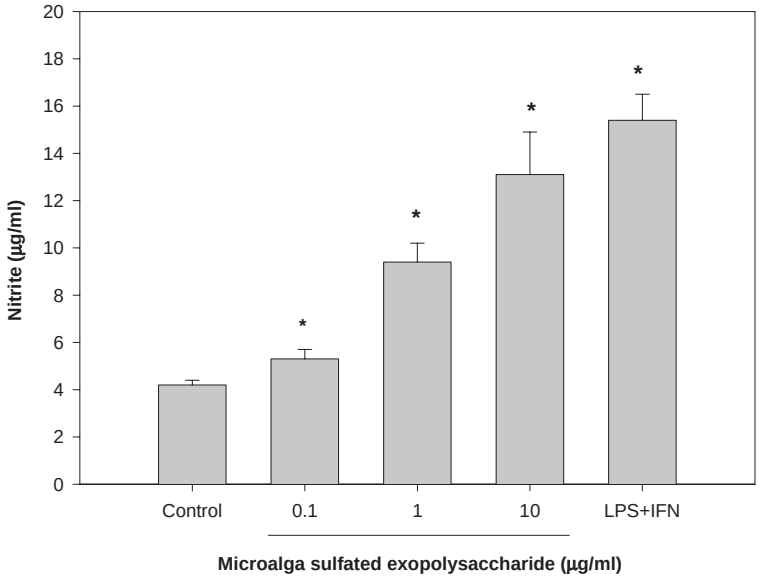


Fig. 3. Nitrite production from the peritoneal macrophages stimulated with MSE. The macrophages were treated with MSE for 18 h. The culture supernatants were collected and the nitrite level was measured, as described in Materials and methods. The results are mean ± S.E.M. of quintuplicates from one representative experiment. As a positive control, IFN-γ (100 U/ml) combined with LPS (1 μg/ml) was used. *: Significantly different from the control (no treatment); $p < 0.05$.

macrophages. In addition, NO production was observed in the supernatant obtained from the MSE-treated macrophages in a concentration-dependent manner (Fig. 3). These findings provide further evidence for the concept that NO is involved in the tumoricidal activity of MSE-stimulated macrophages.

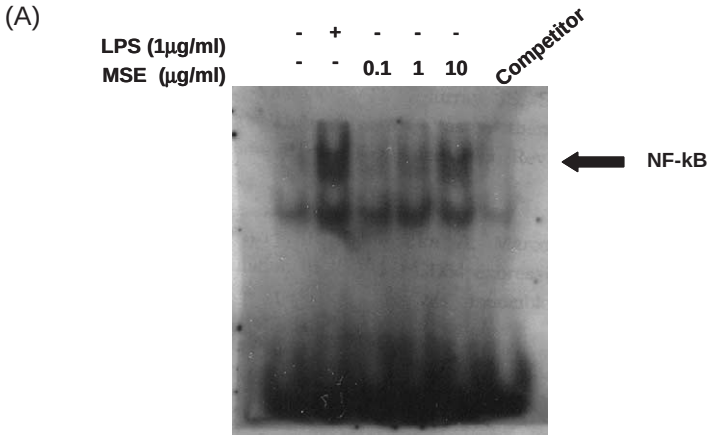
3.4. Involvement of NF-κB in the MSE-induced production of NO in macrophages

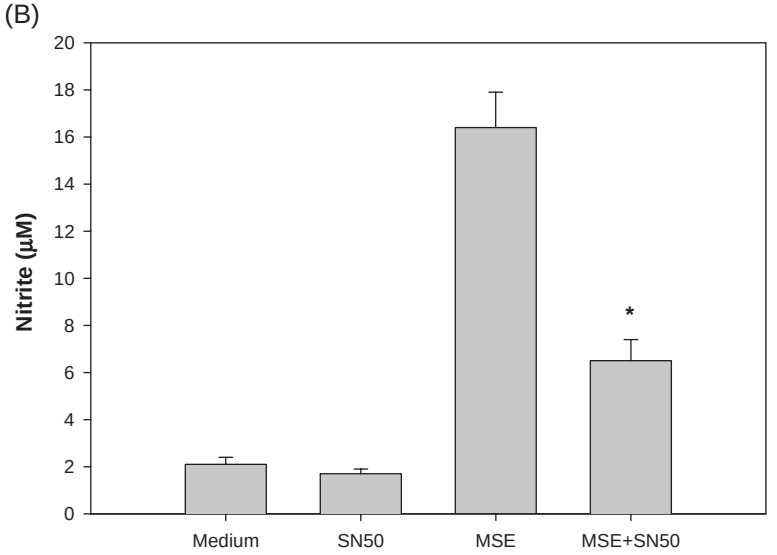
The ability of MSE to activate NF-κB was tested in an attempt to identify the nuclear factors that contribute to the activation of macrophages. As shown in Fig. 4A, the MSE treatment produced a marked increase in NF-κB DNA binding to its cognate in a concentration-dependent manner. This study next examined whether or not NF-κB is involved in the NO production induced by MSE. This is accomplished by analyzing the effect of NF-κB inhibitor, SN50, on nitrite production in the MSE-stimulate macrophages. The SN50 pretreatment partially suppressed the MSE-induced NO production (Fig. 4B). These results suggest that NF-κB is involved in the MSE-induced tumoricidal activity and NO production. To convincingly demonstrate the specificity of NF-κB activation by MSE in peritoneal macrophages, similar study was performed in the B16 tumor cells. As shown in Fig. 4C, SN50 did not inhibit the nitrite generation in tumor cells.

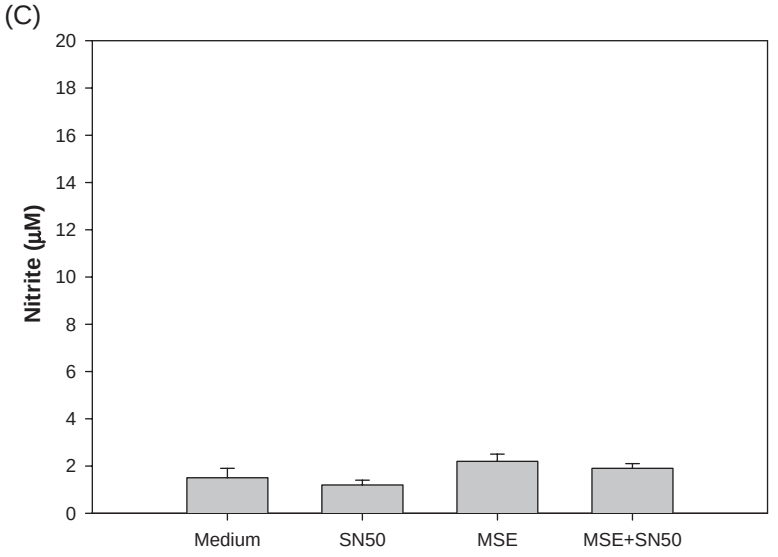
3.5. Effects of the kinase inhibitors on NO production and tumoricidal activity induced by MSE

The p38 MAPK inhibitor (SB203580), MEK1/2 inhibitor (U0126), JNK inhibitor (SP600125) and PKC inhibitor (staurosporin) were examined to determine if they interfere with the activation of macrophages by MSE. This is because the MAP kinase pathways are involved in controlling the production of inflammatory mediators such as NO, and given that the MSE treatments influence NO production and iNOS activity. To accomplish this, the macrophages were incubated for 18 h in medium with or without MSE along with a pretreatment with various inhibitors for 1 h. The macrophage monolayers were washed, and subsequently co-cultured with the B16 tumor cells. The level of cell lysis was determined 18 h later. As

Fig. 4. Involvement of NF-κB in NO production in the MSE-stimulated macrophages. (A) DNA-binding and the effects of MSE treatment on NF-κB DNA-binding activity in nuclear extracts of macrophages. The cells were treated with various MSE doses for 6 h. The cells were lysed and analyzed for their NF-κB DNA-binding activity with the use of an electrophoresis mobility shift assay, as described in Materials and methods. Lane 1 is the control; lane 2, LPS/IFN-γ; lane 3, MSE (0.1 μg/ml); lane 4, MSE (1 μg/ml); lane 5, MSE (10 μg/ml); lane 6, competition with the unlabeled NF-κB oligonucleotide with the nuclear extract of the MSE-treated cells. The experiment was performed in triplicate with identical results. (B) The macrophages were treated with NF-κB inhibitor, SN50 (10 μM), in the presence or absence of MSE prior to stimulation with MSE (10 μg/ml) for 18 h. The level of nitrite production was determined from the culture supernatant. The results are mean ± S.E.M. of quintuplicates from one representative experiment. *: Significantly different from MSE-treated; $p < 0.05$. (C) B16 tumor cells were treated with NF-κB inhibitor, SN50 (10 μM), in the presence or absence of MSE prior to stimulation with MSE (10 μg/ml) for 18 h. The level of nitrite production was determined from the culture supernatant.


(A)
LPS (1μg/ml)
MSE (μg/ml)
- + - - -
- - 0.1 1 10
Competitor
NF-kB


(B)
Nitrite (μM)
0
2
4
6
8
10
12
14
16
18
20
*
Medium
SN50
MSE
MSE+SN50


(C)
Nitrite (μM)
0
2
4
6
8
10
12
14
16
18
20
Medium
SN50
MSE
MSE+SN50

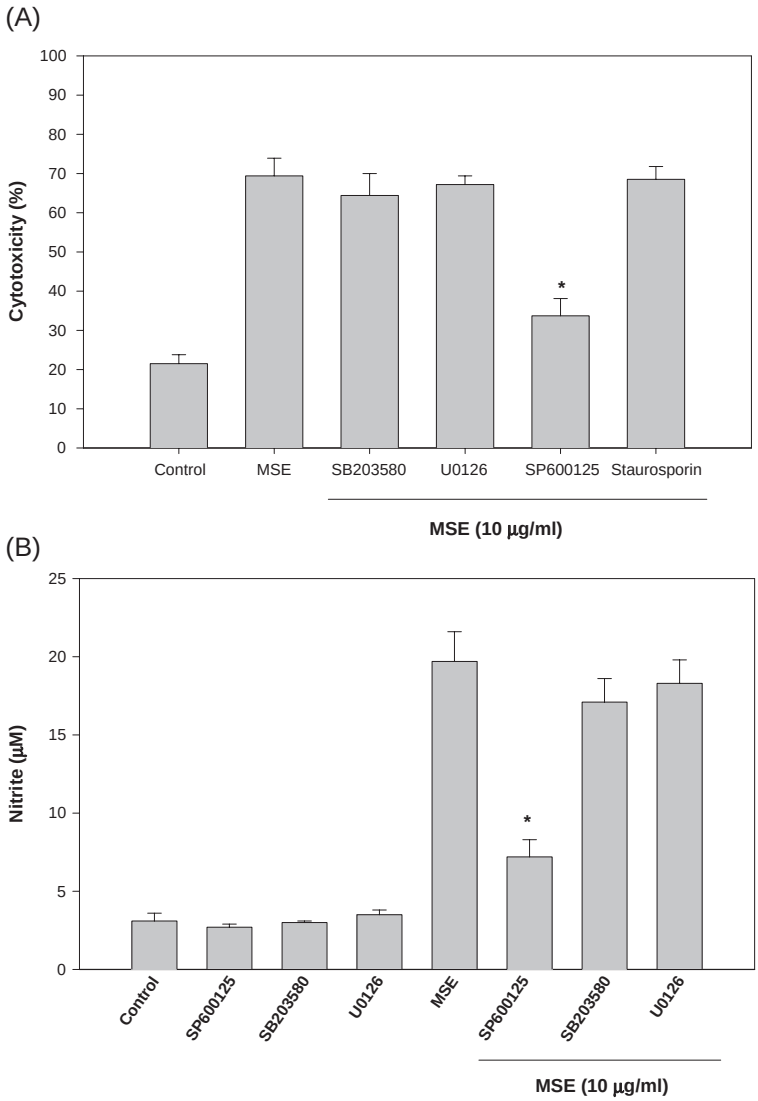


Fig. 5. Inhibition of the MSE-induced tumoricidal activation of macrophage by JNK inhibitor. The peritoneal macrophages were cultured for 18 h in the medium or in the medium supplemented with MSE (10 μg/ml) in the presence or absence of the various inhibitors. The macrophage tumoricidal activity was determined, as described in Materials and methods. The data shown here are the results at an initial effector/target ratio of 10 : 1. (B) The cells were treated with the JNK inhibitor, SP600125 (1 μM), in the presence or absence of MSE prior to stimulation with MSE (10 μg/ml) for 18 h. The level of nitrite production was determined from the culture supernatant. The results are mean ± S.E.M. of quintuplicates from one representative experiment. *: Significantly different from MSE-treated; $p < 0.05$.

shown in Fig. 5A, the presence of the JNK inhibitor during the activating stage inhibited the tumoricidal activation of macrophages by MSE, while pretreatments with the other inhibitors had little or no effect on the MSE-induced tumoricidal activity. At the concentrations used, none of the inhibitors that affected the growth of the tumor cells nor did they affect the cytotoxic activity. These results suggest that JNK is involved in the macrophage-mediated cytotoxicity induced by MSE. This study also examined whether or not the addition of a JNK inhibitor blocked the cytototoxic activity of the macrophage at the effector stage. The data shown in Table 3 demonstrate that the treatment of macrophages with SP600125 at the effector stage did not reduce the level of macrophage-mediated tumor cell lysis.

These results suggest that NO is involved in the tumoricidal activity of MSE-stimulated macrophages. Therefore, the next series of experiments were aimed at determining if treating the macrophages with the various inhibitors reduced their capacity to produce NO. As shown in Fig. 5B, SP600125 inhibited the MSE-stimulated NO production by 63%, whereas SB203580 produced a slight reduction in NO production. Overall, of the results suggest the involvement of JNK in the MSE induced NO production pathway. Further studies using western blot analyses demonstrated that MSE induced the

activation of JNK (Fig. 6). This suggests that JNK activation is essential for the tumoricidal activity of MSE as well as the MSE induced production of NO in macrophages.

4. Discussion

Reactive oxygen intermediates have been suggested to mediate the antitumor activity [36]. In these experiments, the reactive oxygen scavengers did not reverse the MSE-induced tumoricidal activity. Moreover, it was found that MSE did not increase the generation of superoxide anion or hydrogen peroxide, compared with that in the untreated cells (data not shown). Therefore, these mediators did not appear to play a role in the MSE-induced tumoricidal activity.

Sharon et al. reported that IFN produced a dose-dependent increase in the level of macrophage cytotoxicity to tumor cells [39]. In this study, the lack of IFN-α/β involvement in the MSE-induced tumoricidal activity was substantiated by the observation that the anti-IFN-α/β antibodies could not abolish the cytotoxic action of macrophages.

Activated macrophages produce NO, which inhibits mitochondrial respiration and results in cytostasis of the target cells [40]. Decker et al. [35] reported that TNF-α acts as an effector molecule in macrophage cell-mediated cytolysis against tumor cells that are highly sensitive to TNF-α. This study suggests that the reactive nitric oxide induced by MSE is involved in macrophage-mediated tumor cytolysis. In addition, treating the macrophages with MSE induced the NO synthase activity in the cytosol of the stimulated cells, suggesting that MSE induces the production of NO in these cells. However, the cytokines including TNF-α do not play a key role in macrophage-mediated death of the target cells, because the IL-1, IL-6, IFN-α/β and TNF-α levels were not affected by the MSE treatment.

Table 3
Lack of inhibitory effects of MSE on the iNOS activity in macrophages

Treatment[a]	% Cytotoxicity of target cells
None	18.4 ± 1.8
MSE (10 μg/ml)	65.4 ± 3.1
MSE+SP600125 (0.1 μM)	63.1 ± 6.4
MSE+SP600125 (0.5 μM)	68.2 ± 4.2
MSE+SP600125 (1 μM)	61.8 ± 6.7

[a] The macrophages were activated microalga sulfated exopolysaccharide (MSE) (10 μg/ml) for 18 h and washed, and then tumor cells were added with or without various concentrations of SP600125. The results are reported as a mean ± S.E.M. of three independent experiments.

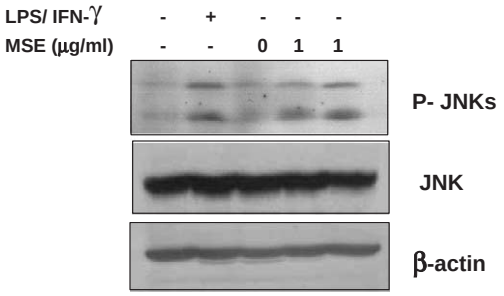


Fig. 6. Activation of JNK in MSE-treated cells. The cells were treated with MSE (10 μg/ml) and incubated for 10 h. The whole cell lysates were prepared and used for the p-JNKs, JNK, or actin Western with the respective antibodies. Each experiment was performed in triplicate with identical results.

Protein kinases play an important role in the signal transduction pathways that regulate the response of macrophages to external stimuli [24–29]. Moreover, previous studies have shown that the inhibitors of PKC and PTK can block the tumoricidal activity as well as production of various cytolytic molecules in macrophages [40,41]. These results show that the mechanisms by which MSE activates macrophages might involve JNK, which was confirmed by the observations that the effect of MSE on the activation of macrophages to tumoricidal activity was abrogated by an JNK inhibitor. This kinase inhibitor also inhibited the production of nitric oxide, which suggests the involvement of JNK in the process. These results also demonstrated that the treatment with SN50, which inhibits NF-κB nuclear translocation, inhibited the production of NO. This indicates that NF-κB is positively regulated by MSE in NO production. However, the data does not totally rule out the possibility that for macrophage activation, MSE can both directly and indirectly deliver divergent activation signals and trigger the synergistic signal transduction pathways. In support of this hypothesis, previous results suggested that other biologically active polysaccharides such as angelan, corn silk and safflower polysaccharide, activate several signaling pathways in macrophages [42–45]. It has been known that augmentation of NO production by LPS is dependent on the expression of iNOS, whose expression is, in turn, mediated by a series of signaling pathway, such as NF-κB [46,47] and mitogen-activated protein (MAP) kinases [48,49]. In addition, a recent study suggested that there is the potential for cross talk between the MAP kinase and NF-κB pathways in modulating the responsiveness of the macrophages exposed to external stimuli [50]. Therefore, the cell activation pathway of MSE might be similar to that of other stimulants such as LPS.

Moreover, MSE can trigger a complex interaction between multiple signal transduction pathways including the JNK signal molecule.

Although the membrane receptor of MSE has not been determined, it is possible that some membrane proteins such as CD14, TLR and CR3 act as receptors for MSE in macrophages. It is known that LPS binds to the LPS binding protein (LBP) and the LBP/LPS complex subsequently binds to CD14, which triggers cell activation [51,52]. The interaction between the ternary membrane CD14–LPS–LBP complex and the Toll like receptor 2 (TLR2) on the cell surface initiates the intracellular signal transduction for LPS in macrophages. Complement receptor CR3 has been implicated in the responsiveness to β-glucans [53]. Therefore, these membrane proteins are expressed on the surface of the macrophages and are involved in various cell–substrate interactions. However, the exact role of these membrane proteins in the response to MSE remains to be elucidated.

This study demonstrated that MSE augments the tumoricidal activity of macrophages that could be correlated with inducing the release of NO. Therefore, MSE might have potential therapeutic utility in cancer patients. However, it is possible that, in addition to the macrophage tumoricidal activity by MSE, macrophages activated in vivo by MSE may destroy the host cells. NO plays an important role in the pathogenesis of acute inflammatory diseases [54]. Moreover, NO plays important roles in the pathogenesis of gram-positive septic shock [55]. Therefore, NO overproduction by the MSE-activated macrophages might make a significant contribution to various pathological complications.

Macrophages have been shown to be an important component of the host defenses against a virus infection by inhibiting the intracellular replication of viruses (intrinsic resistance) and by killing virus infected cells (extrinsic cytotoxicity) [56]. It was found that MSE did not induce the antiviral activity of macrophages (data not shown), suggesting that mediators, which play a role in the MSE-induced tumoricidal activity, were not involved in the antiviral activity.

In summary, MSE can stimulate the production of NO, which plays a role in macrophage-mediated cytotoxicity. In addition, the JNK inhibitor blocked the ability of MSE to induce tumoricidal activity and NO production in macrophages. Furthermore, NF-κB plays a role in microalgal sulfated exopolysaccharide-induced macrophage activation. This suggests that the macrophage cellular and secretory activities induced by MSE occur via the NF-κB and JNK signal transduction pathways.

Acknowledgments

This study was supported by a grant to HKL from the Development of Marine Novel Compounds Program of the Korean Ministry of Maritime Affairs and Fisheries.

References

[1] Sutherland IW. Extracellular polysaccharides. In: Rechm J, Reed A, Puhler A, Stadler P, editors. Biotechnology-VI. Weinhem: VCH, 1996. p. 615–57.
[2] Boisson-Vidal C, Haroun F, Ellouali M, Blondin C, Fischer AM, Agostini A, et al. Biological activities of polysaccharides from marine algae. Drug Future 1995;20:1237–49.
[3] Zhang Q, Li N, Zhou G, Lu X, Xu Z, Li Z. In vivo antioxidant activity of polysaccharide fraction from *Porphyra haitanesis* (Rhodephyta) in aging mice. Pharmacol Res 2003;48(2): 151–5.
[4] Farias WRL, Valente AP, Pereira MS, Mourao PAS. Structure and anticoagulant activity of sulfated galactans. Isolation of a unique sulfated galactan from the red algae Botryocladia occidentalis and comparison of its anticoagulant action with that of sulfated galactans from invertebrates. J Biol Chem 2000;275(38):29299–307.
[5] Batinic D, Robey FA. The V3 region of the enveloped glycoprotein of human immunodeficiency virus type 1 binds sulfated polysaccharides and CD4-derived synthetic peptides. J Biol Chem 1992;267:6664–71.
[6] Wellstein S, Zugmaier G, Califano 3rd JA, Kern F, Paik S, Lipmann ME. Tumor growth dependent on Kaposi's sarcoma-derived fibroblast growth factor inhibited by pentosan polysulfate. J Natl Cancer Inst 1991;83(10):716–20.
[7] Bourgougnon N, Lahaye M, Chermann C, Kornprobst M. Composition and antiviral activities of a sulfated polysaccharide from *Schizymenia dubyi* (Rodophyta, Gigatiales) bioorganic. Med Chem Lett 1993;3:1141–6.
[8] Sudo H, Burgress G, Takemasa H, Nakamura N, Matsunaga T. Sulfated exopolysaccharide production by the halophilic cyanobacterium *Aphanocapsa halophyta*. Curr Microbiol 1995;30: 219–22.
[9] Park JG, Park YS. Comparison of morphological characteristics and the 24S rRNA sequences of *Cochlodinium polykrikoides* and *Gyrodinium impudicum*. J Kor Soc Ocean 1999;4(4):363–70.
[10] Yim JH, Kim SJ, Ahn SH, Lee JK, Rhie KT, Lee HK. Antiviral effects of sulfated exopolysacchride from the marine microalga *Gyrodinium impudicum* strain *kg03*. Mar Biotechnol 2004;6:17–25.
[11] Zhou G, Sun Y, Xin H, Zhang Y, Xu Z, Li Z. In vivo antitumor and immunomodulation activities of different molecular weight lambda-carrageenans from *Chondrus ocellatus*. Pharmacol Res 2004;50(1):47–53.
[12] Perez-Fernandez ME, Quesada E, Galvez J, Ruiz C. Effect of exopolysaccharide V2-7, isolated from *Halomonas eurihalina*, on the proliferation in vitro of human peripheral blood lymphocytes. Immunopharmacol Immunotoxicol 2000;22(1): 131–41.
[13] Hahn H, Kaufmann SH. The role of cell-mediated immunity in bacterial infections. Rev Infect Dis 1981;3:1221–50.

[14] Verstovsek S, Maccubbin D, Mihich E. Tumoricidal activation of murine resident peritoneal macrophage by interleukin 2 and tumor necrosis factor. Cancer Res 1992;52:3880–5.
[15] Dullens HFJ, De Weger RA, Van Der Maas M, Den Besten PJ, Vandebriel RJ, Den Otter W. Production of specific macrophage-arming factor precedes cytotoxic T lymphocyte activity in vivo during tumor rejection. Cancer Immunol Immunother 1989;30:28–33.
[16] Gautam S, Deodhar S. Generation of tumoricidal effector cells by human C-reactive protein and muramyl tripeptide: a comparative study. J Biol Response Modif 1989;8:560–9.
[17] Paulnock DM, Lambert LE. Identification and characterization of monoclonal antibodies specific for macrophages at intermediate stages in the tumoricidal activation pathway. J Immunol 1988;144:765–73.
[18] Arden L, Lansdorp P, De Groot E. A growth factor for B cell hybridomas produced by human monocytes. Lymphokines 1985;10:175–85.
[19] Arenzana-Seisdedos F, Virelizier J. Interferons as macrophage activating factors: II. Enhanced secretion of interleukin 1 by lipopolysaccharide-stimulated human monocytes. Eur J Immunol 1983;13:437–40.
[20] Choriki M, Freudenberg M, Calanos C, Poindron P, Bartholeyns J. Antitumoral effects of lipopolysaccharide, tumor necrosis factor, interferon and activated macrophages: synergism and tissue distribution. Anticancer Res 1989;9:1185–90.
[21] Stuehr DJ, Marletta MA. Synthesis of nitrite and nitrate in macrophage cell lines. Cancer Res 1987;47:5590–4.
[22] Keller R, Keist R, Frei K. Lymphokines and bacteria that induce tumoricidal activity, trigger a different secretory response in macrophages. Eur J Immunol 1990;20:695–8.
[23] Wright SD, Ramons RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990;249:1431–3.
[24] Dong Z, O'Brian CA, Fidler IJ. Activation of tumoricidal properties in macrophages by lipopolysaccharide requires protein-tyrosin kinase activity. J Leukoc Biol 1993;53:53–60.
[25] Um SH, Rhee DK, Pyo S. Involvement of protein kinase C and tyrosin kinase in tumoricidal activation of macrophage induced by *Streptoccus pneumoniae* type II capsular polysaccharide. Int Immunopharmacol 2002;2:129–37.
[26] Jaramillo M, Naccache PH, Olivier M. Monosodium urate crystals synergize with IFN-gamma to generate macrophage nitric oxide: involvement of extracellular signal-regulated kinase 1/2 and NF-kappa B. J Immunol 2004;172(9):5734–42.
[27] Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001;13:85–94.
[28] Caamano J, Hunter CA. NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions. Clin Microbiol Rev 2002;15:414–29.
[29] Schorey JS, Cooper AM. Macrophage signalling upon mycobacterial infection: the MAP kinases lead the way. Cell Microbiol 2003;5:133–42.
[30] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;38:350–6.
[31] Flick DA, Gifford GE. Comparison of in vitro cell cytotoxicity assays for tumor necrosis factor. J Immunol 1984;68:167–75.
[32] Ding AH, Nathan CF, Stuer DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol 1988;141:2407–12.
[33] Vodovotz Y, Bogdan C, Paik J, Xie QW, Nathan C. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor-B. J Exp Med 1993;178:605–13.
[34] Cho SJ, Kang NS, Park SY, Kim BO, Rhee DK, Pyo S. Induction of apoptosis and expression of apoptosis related genes in human epithelial carcinoma cells by *Helicobacter pylori* VacA toxin. Toxicon 2003;42(6):601–11.
[35] Decker T, Lohmann-Mathes ML, Gifford G. Cell associated tumor necrosis factor as a killing mechanism of activated cytotoxic macrophages. J Immunol 1987;130:957–62.
[36] Mavier P, Edgington TS. Human monocyte-mediated tumor cytotoxicity. I. Demonstration of an oxygen dependent myeloperoxidase-independent mechanism. J Immunol 1984;132:1980–4.
[37] Hibbs JB, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science 1987;235:473–6.
[38] Remels L, Fransen L, Huygen K, DeBaetselier P. Poly I:C activated macrophages are tumoricidal for TNF-α-resistant 3LL tumor cells. J Immunol 1990;144:4477–86.
[39] Sharon N, Shoham J, Passwell JH. Enhancement of human monocyte cytotoxicity by both interferon-γ and -β and comparison to other stimuli. Int J Immunopharmacol 1989;11:743–9.
[40] Goenka S, Das T, Sa G, Ray PK. Protein A induces NO production: Involvement of Tyrosin Kinase, Phospholipase C, and Protein Kinase C. Biochem Biophys Res Commun 1998;250:425–9.
[41] Shishodia S, Shrivastava A, Sodhi A. Protein kinase C: a potential pathway of macrophage activation with cisplatin. Immunol lett 1998;61:179–86.
[42] Jeon YJ, Han SB, Lee SH, Kim HC, Ahn KS, Kim HM. Activation of mitogen-activated protein kinase pathways by angelan in murine macrophages. Int Immunopharmacol 2001;1:237–45.
[43] Ando I, Tsukumo Y, Wakabayashi T, Akashi S, Miyake K, Kataoka T, et al. Safflower polysaccharides activate the transcription factor NF-kappa B via Toll-like receptor 4 and induce cytokine production by macrophages. Int Immunopharmacol 2002;2:1155–62.
[44] Kim KA, Choi SK, Choi HS. Corn silk induces nitric oxide synthase in murine macrophages. Exp Mol Med 2004;36:545–50.
[45] Yoon YD, Kang JS, Han SB, Park SK, Lee HS, Kang JS, et al. 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 2004;4:1477–87.
[46] Kim YM, Lee BS, Yi KY, Paik SG. Upstream NF-kappaB site is required for the maximal expression of mouse inducible nitric oxide synthase gene in interferon-gamma plus lipopolysaccharide-induced RAW 264.7 macrophages. Biochem Biophys Res Commun 1997;236:655–60.
[47] Liu SF, Ye X, Malik AB. In vivo inhibition of NF-kB activation prevents iNOS synthase expression and systemic hypotension in a rat model of septic shock. J Immunol 1997;159:3976–83.
[48] Heitmeier MR, Scarim AL, Corbett JA. Double stranded RNA induced nitric oxide synthase expression and IL-1 release by murine macrophages requires NF-kB activation. J Biol Chem 1999;273:15301–7.

[49] Chan ED, Riches DW. Potential role of the JNK/SAPK signal transduction pathway in the induction of iNOS by TNF-α. Biochem Biophys Res Commun 1998;253:790–6.
[50] Aga M, Watters JJ, Pfeiffer ZA, Wiepz GJ, Sommer JA, Bertics PJ. Evidence for nucleotide receptor modulation of cross talk between MAP kinase and NF-kappa B signaling pathways in murine RAW 264.7 macrophages. Am J Physiol Cell Physiol 2004;286:C923–30.
[51] Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 1995;13:437–57.
[52] Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. Structure and function of lipopolysaccharide binding protein. Science 1990;249:1431–3.
[53] Thornton BP, Vetvicka V, Pitman M, Goldman RC, Ross GD. Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). J Immunol 1996;156:1235–46.
[54] Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992;6:3051–64.
[55] Orman KL, Shenep JL, English BK. Pneumococci stimulate the production of the inducible nitric oxide synthase and nitric oxide by murine macrophages. J Infect Dis 1998;178:1649–57.
[56] Morahan PS, Connor JR, Leary KR. Viruses and the versatile macrophage. Br Med Bull 1985;410:150–210.