

Immunomodulatory Effects of Polar Lichens on the Function of Macrophages In Vitro

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Abstract Lichen species were collected from King George Island (Antarctica) and were screened for their immunomodulatory effect. Among the lichens tested, the methanol extract (CR-ME) of *Caloplaca regalis* showed the highest nitric oxide (NO) production in murine peritoneal macrophages. Therefore, this study further examined the ability of *C. regalis* to induce secretory and cellular responses in macrophages. Macrophages were treated with various concentrations of CR-ME for 18 h. The CR-ME treatment induced tumoricidal activity and increased the production of tumor necrosis factor- α (TNF- α) and nitric oxide by macrophages. However, CR-ME had a little effect on the levels of reactive oxygen species, interleukin-1 and IFN- γ in CR-ME-treated macrophages. The CR-ME-induced tumoricidal activity was partially abrogated by a NO inhibitor and the anti-TNF- α antibody. Thus, the tumoricidal effect of CR-ME appeared to be mainly mediated by NO and TNF- α production from macrophages. Treating the macrophages with a p38 mitogen-activated protein kinase (MAPK) inhibitor partially blocked the tumoricidal activation induced by CR-ME, whereas inhibitors of the other kinases did not have an inhibitory effect. These results suggest that CR-ME induces the tumoricidal activity via the p38 MAPK-dependent pathway. Furthermore, electrophoretic mobility shift assay analyses revealed that the CR-ME

treatment induced the activation of the NF- κ B transcription factor. Overall, these results indicate that the tumoricidal activity induced by CR-ME is mainly due to TNF- α and NO production, and the activation of macrophage by CR-ME is mediated probably via the p38 MAPK and NF- κ B pathway. Our results may also provide some leads in the development of new immunomodulating drugs.

Keywords Antarctic · *Caloplaca regalis* · Immunomodulating activity · Macrophages · NF- κ B · p38 MAPK

Introduction

There has been increasing interest in the flora and fauna of the Antarctic in recent years. In the Antarctic region, lichens form the main part of the flora and many lichen species are found (Smith 1984). Lichens represent a source of natural products formed by symbiotic associations between heterotrophic fungi and cyanobacteria or algae. Lichens and their natural products have been used for cosmetics, food, and natural remedies (Choudhary et al. 2005; Müller 2001). In addition, they exert a broad range of important biological actions comprising antibiotic, antimycobacterial, antiviral, antiinflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic effects (Oksanen 2006). Moreover, previous reports have demonstrated the immunostimulatory effect of lichen-derived polysaccharides including lichenan and isolichenan from *Cetraria islandica* and thamnolan from *Thamnolia subuliformis* (Ingolfsdottir et al. 1994; Olafsdottir et al. 1999). However, there is still large a potential for further industrial screening and research on lichen products, and their therapeutic potential remains pharmaceutically unexploited.

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Macrophages have been shown to be important components of the host defenses against bacterial infections and murine tumor cells (lymphoma and mastocytoma; Hahn and Kaufmann 1981; Verstovsek et al. 1992). Peritoneal macrophages can be stimulated by a variety of agents such as IFN- γ , lipopolysaccharide (LPS), or microbial products (Martin and Edwards 1994; Dong et al. 1993; Um et al. 2000; Bae et al. 2006), 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 (Choriki et al. 1989; Stuehr and Marletta 1987; Keller et al. 1990).

A large number of studies have shown that different signaling pathways participate in the activation of macrophages by various stimuli (Dong et al. 1993; Um et al. 2000; Jaramillo et al. 2004). Previous studies have demonstrated that the NF- κ B and mitogen-activated protein kinase (MAPK) pathway mediate the activation of macrophages (Kim et al. 1997; Bae et al. 2006; Schorey and Cooper 2003). However, published scientific information regarding the basic mechanisms of lichen products at the molecular level involved in their immunomodulating activity is scarce.

The aim of this study was to investigate the immunomodulatory effects of methanol extract from various polar lichen species on murine peritoneal macrophages in vitro.

Materials and Methods

Preparation of extract L1 (*Usnea aurantiaco-atra*), L2 (*Usnea antatica*), L3 (*Stereocaulon alpinum* Laurer), L5 (*Ramalina terebrata* Hook and Taylor), L6 (*Caloplaca* sp.), L8 (*Lecanora* sp.), L9 (*Cladonia borealis*), and L17 (*Caloplaca regalis*) were collected from the Korean Antarctic Research Station site on King George Island (61°50'–62°15' S and 57°30'–59°01' W), Antarctica. Voucher specimens were deposited in the Polar Lichen Herbarium, Korea Polar Research Institute, KOPRI, Incheon, South Korea. The dried samples of eight lichens were extracted in methanol–water (90:10 v/v) at room temperature. The methanol extract was filtered and evaporated under vacuum to obtain the dried methanol extracts. These dried methanol extracts were dissolved in DMSO at 100 mg/ml and their effects tested on the function of macrophage.

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, USA). The RPMI 1640 medium and fetal bovine serum were purchased from GIBCO (Grand Island, NY, USA). N^G -monomethyl-L-

arginine (NMMA) and MAPKs inhibitors (SB203580, PD98059, and SP600125) were obtained from Calbiochem Co. (LaJolla, CA, USA). TNF- α antibody, IL-1, and TNF- α enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D System (Minneapolis, MN, USA). The IFN- γ ELISA kit was purchased from Alexis Biochemicals (Switzerland). The antibodies against p38, phospho-p38 (p-p38) 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.

Isolation of inflammatory peritoneal macrophages The thioglycollate-elicited peritoneal exudate cells were obtained from C57BL/6 male mice after they were given an intraperitoneal injection of 1 ml Brewer thioglycollate broth (4.05 g/100 ml; Difco Laboratories, Detroit, MI, USA) 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, USA) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μ g/ml; RPMI–FBS). The macrophages were isolated from the peritoneal exudate cells as described previously (Um et al. 2000). The peritoneal exudate cells were seeded on Teflon-coated petri dishes (100 \times 15 mm) at densities of 5–6 \times 10⁵ cells/cm², and the macrophages were allowed to adhere for 2–3 h at 37°C in a 5% CO₂-humidified atmosphere. Teflon-coated petri dishes were prepared by spraying them with aerosolized Teflon (Fisher Scientific, Pittsburgh, PA, USA), 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 was then added, which was followed by the addition of 0.3 ml of 0.1 M ethylenediamine tetraacetic acid (EDTA; pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages were removed by rinsing the plates ten 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.

Macrophage-mediated cytotoxicity The assay for macrophage cytotoxicity was based on an assay described

elsewhere (Bae et al. 2006). Briefly, the macrophages (1×10^5 cells/well) were plated into 96-well microtiter plates and incubated with various concentrations of CR-ME for 18 h at 37°C in a 5% CO₂ incubator. In some experiments, antibodies to cytokine or inhibitor of the metabolic pathway were included along with CR-ME. The macrophages were also pretreated with inhibitors of the signaling pathway prior to stimulation with CR-ME. The macrophages were washed with RPMI-FBS to remove the CR-ME and co-incubated with the B16 melanoma cells (ATCC, Rockville,

MD, USA; 1.0×10^4 /wells; an initial effector/target cell ratio of 10:1) at 37°C in a 5% CO₂ incubator. The cell density was then assessed by incubating the cells with 25 µg/ml MTT [3-(4,5-dimethylthiazole-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, USA). The cytotoxic activity is expressed as the percentage tumor cytotoxicity as follows:

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

Nitrite determination Peritoneal macrophages were seeded at a density of 1×10^6 cells in 60 mm culture dishes and cells treated with the crude methanol extracts of lichens for 18 h. The amount of NO₂⁻ accumulated in the culture supernatants was measured as described previously (Ding et al. 1988). 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 NO₂⁻ concentration was calculated from a NaNO₂ standard curve. The NO₂⁻ levels are indicative of the amount of NO production. The Griess reagent was prepared by mixing one part of 0.1% naphthyl-ethylene diamine dihydrochloride in distilled water with one part of 1% sulfanilamide in 5% concentrated H₃PO₄.

Determination of intracellular Reactive Oxygen Species (ROS) Peritoneal macrophages were seeded at a density of 1×10^6 cells in 60 mm culture dishes and cells treated with CR-ME for 18 h. Cells were stained at 37°C for 15 min with 5 µM CM-H₂-DCFDA (Molecular Probes, Eugene, OR, USA), an ROS sensitive dye, for ROS measurement. The intracellular ROS level was measured by flow cytometry (Win BRYTE HS, Bio-Rad, USA). Data are expressed as percentage of untreated control from triplicate samples and at least 10,000 cells were analyzed at each experiment.

Cytokine Determination by ELISA Peritoneal macrophages were treated with CR-ME for 18 h. The culture supernatants were collected and the TNF-α, IFN-γ, and IL-1 concentrations in the culture supernatants were determined using DuoSet Elisa kit (R&D System). The manufacturer's instructions were followed. Samples were assessed in triplicate relative to the standards supplied by the manufacturer.

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 six-well plates (3 ml/well) and incubated at 37°C. The cells were then incubated for 6 h after first exposing them to CR-ME 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 ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (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×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'-AGTTGAGGGGACTTCCAGG-3' containing the NF-κB 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'-CTAGTGAGCCTAAGG CCGGATC-3') was added to the reaction mixture before adding the DIG-labeled oligonucleotide.

Western blot analysis Western blot analysis was performed by modification of a technique described elsewhere (Bae et al. 2006). 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, USA) 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 difluoride membrane (Amersham, Arlington Heights, IL, USA) 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 re-probed with the anti-β-actin antibody as a control for the protein loading.

Statistical analysis Each result is reported as means ± SEM. Two-way analysis of variance was used for analysis of differences among groups, and the significant values are represented by an asterisk. (* $p < 0.05$).

Results

In vitro activation of macrophages with CR-ME We chose to examine the effects of the methanol extracts from all eight lichen species on the level of nitric oxide in peritoneal macrophages since nitric oxide has been proposed as a principal mediator of macrophage antimicrobial and tumoricidal activity, and exhibited a wide diversity of physiological activities in the immune system (Lowenstein and Snyder 1992; Lorsbach et al. 1993). The methanol extracts of various polar lichens were tested for their ability to induce NO production at a concentration between 1 and 100 µg/ml. As shown in Table 1, the extract of *C. regalis* (L17: CR-ME) showed the highest NO production in peritoneal macrophages at 100 µg/ml as compared to other lichens. Similar results were also found at 1 and 10 µg/ml (data not shown). These results lead us to suggest that CR-ME as a cell-eliciting agent has the potential to induce secretory and cellular responses in macrophages. Therefore, the present study was conducted to further investigate the effects of CR-ME on macrophage function.

In order to determine if macrophages could be activated to express the tumoricidal activity in vitro, thioglycollate-elicited macrophages were treated with various concentrations (1–100 µg/ml) of CR-ME for 18 h and co-cultured

Table 1 Nitrite production from the peritoneal macrophages stimulated with the methanol extracts from eight polar lichens

Test lichens (100 µg/ml)	Nitrite (µM)
None	1.43±0.04
L1	2.71±0.16
L2	1.97±0.32
L3	1.89±0.06
L5	0.53±0.06
L6	2.47±0.16
L8	3.15±0.27
L9	1.63±0.08
L17	14.63±0.47*

The macrophages were treated with eight extracts for 18 h. The culture supernatants were collected and the nitrite level was measured, as described in "Materials and Methods". The results are mean ± SEM of quintuplicates from one representative experiment

* $p < 0.05$, significantly different from the control (no treatment)

with the B16 tumor cells, which were used as the targets because they are either TNF-α or NO sensitive. CR-ME significantly increased the level of cytotoxicity in the macrophages at 10 and 100 µg/ml (Fig. 1). These data suggest that CR-ME could modulate the production of secretory molecules related to tumoricidal activities of macrophages. CR-ME did not affect the cell viability but concentrations >100 µg/ml were found to be cytotoxic (data not shown). In addition, the effects of CR-ME were not due to the result of endotoxin contamination, which was found to be <10 pg/ml, as assessed by the Limulus lysate test.

Effect of CR-ME on the production of cytokines and Cytotoxic Molecules by Macrophages Once activated, tumoricidal macrophages produce a large number of cytotoxic molecules. These include H₂O₂, TNF-α, NO, IL-1, and IFN-γ (Mavier and Edgington 1984; Decker et al. 1987; Jeon et al. 2001; Lovett et al. 1986; Fultz et al. 1993). We determined the secretory effect of CR-ME on the production of TNF-α, IL-1, and IFN-γ by macrophages. Peritoneal macrophages were treated with various concentrations of CR-ME for 18 h and culture supernatants were assayed for cytokines. As shown in Fig. 2, treatment of the macrophages with CR-ME resulted in an increase in TNF-α production at 100 µg/ml compared to untreated cultures, while the production of IL-1 and IFN-γ were not significantly altered by the treatment with CR-ME. In the next set of experiments, we determined whether nitric oxide and reactive oxygen species was produced by peritoneal macrophage in treatment with CR-ME (Fig. 3). The supernatants in well plate exposed to CR-ME for 18 h were measured using the Griess method. ROS was determined by staining CR-ME-treated cells with CM-H₂-DCFDA (5 µM). CR-ME treatment resulted in an increase

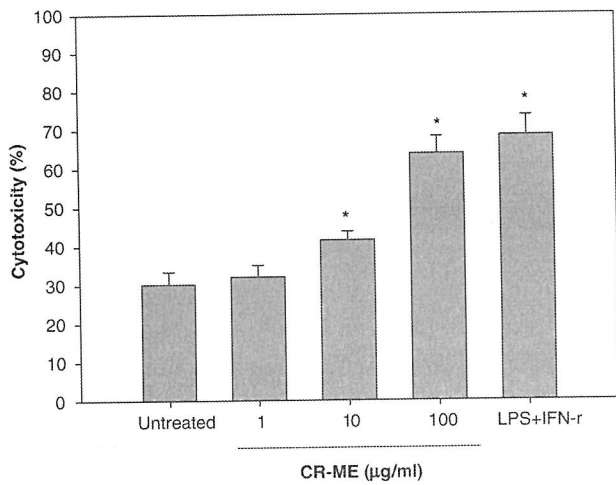


Fig. 1 Cytotoxicity of B16 tumor cells by CR-ME-activated macrophages. The peritoneal macrophages were stimulated with various doses of methanol extract of *Caloplaca regalis* microalga (CR-ME) for 18 h. The macrophage tumoricidal activity was determined as described in “Materials and Methods”. The data shown are the results of an initial effector/target ratio of 10:1. The results are reported as a mean ± SEM of quintuplicates from a representative experiment. As a positive control, IFN-γ (100 U/ml) combined with LPS (1 μg/ml) was used. **p*<0.05, significantly different from the control (no treatment)

in NO production at 100 μg/ml. In contrast, CR-ME did not affect the production of ROS.

The present data demonstrate that anti-TNF-α antibody and the NO inhibitor, NMMA, were able to abrogate, in part, the tumoricidal activities of CR-ME-exposed macrophages against target (Table 2). At the concentrations employed, none of the inhibitor or antibody affected the growth of the tumor cells, and the isotype-matched control antibodies had no effect on cytotoxic activity (data not shown). These findings provide further evidence for the concept that TNF-α and NO are involved in the tumoricidal activity of CR-ME-stimulated macrophages.

Activation of p38 MAPK and NF-κB in CR-ME-treated macrophage The MAPK and NF-κB signaling pathways have been implicated in macrophage activation (Kim et al. 1997; Bae et al. 2006; Schorey and Cooper 2003). In addition, the MAP kinase pathways are involved in controlling the production of inflammatory mediators such as NO, and the CR-ME treatments influence NO and TNF-α production. The p38 MAPK inhibitor (SB203580, 10 μM), MEK1/2 inhibitor (U0126, 10 μM), and JNK inhibitor (SP600125, 1 μM) were examined to determine if they interfere with the activation of macrophages by CR-ME. To accomplish this, the macrophages were incubated for 18 h in medium with or without CR-ME along with a pretreatment with various inhibitors for 1 h. The macrophage monolayers were washed and subsequently co-

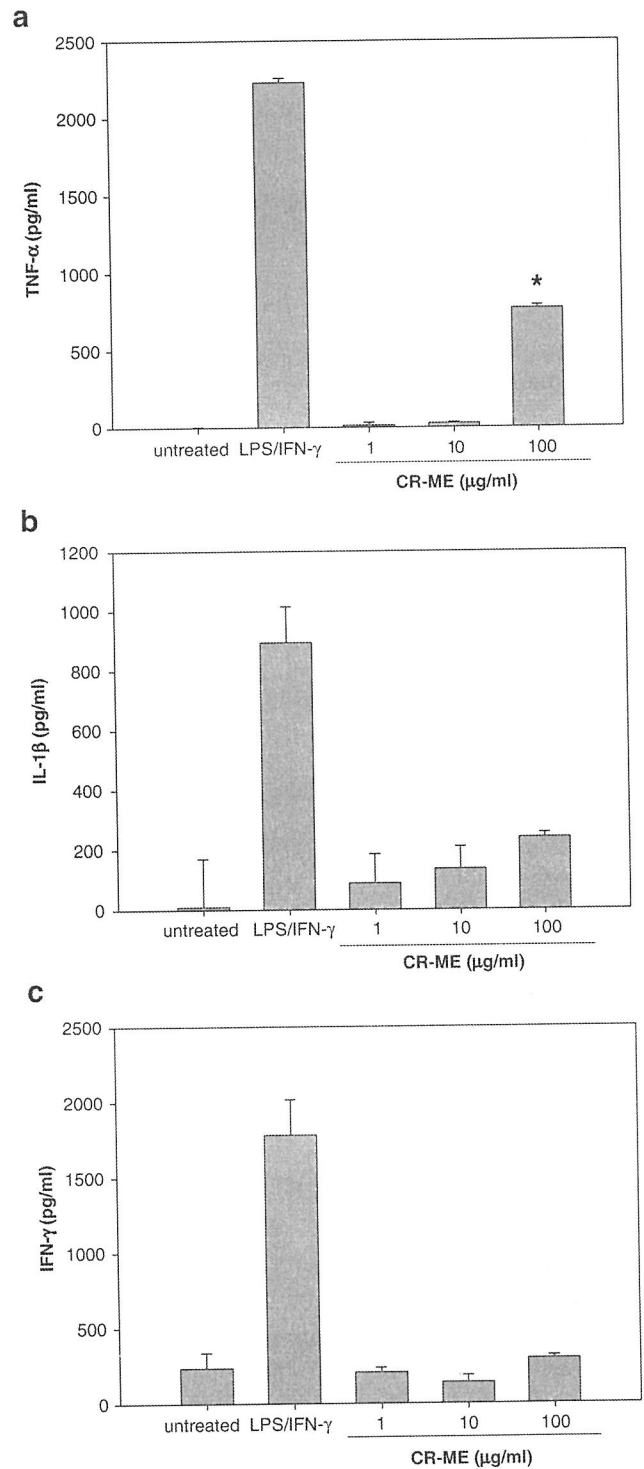


Fig. 2 a–c TNF-α, IL-1, and IFN-γ production by peritoneal macrophages stimulated by CR-ME. Macrophages were treated with CR-ME for 18 h. Culture supernatants were collected, and the levels of TNF-α, IL-1, and IFN-γ were measured by ELISA, respectively. The results shown are the mean ± SEM of quintuplicates from a representative experiment. **p*<0.05, significantly different from the control (no treatment)

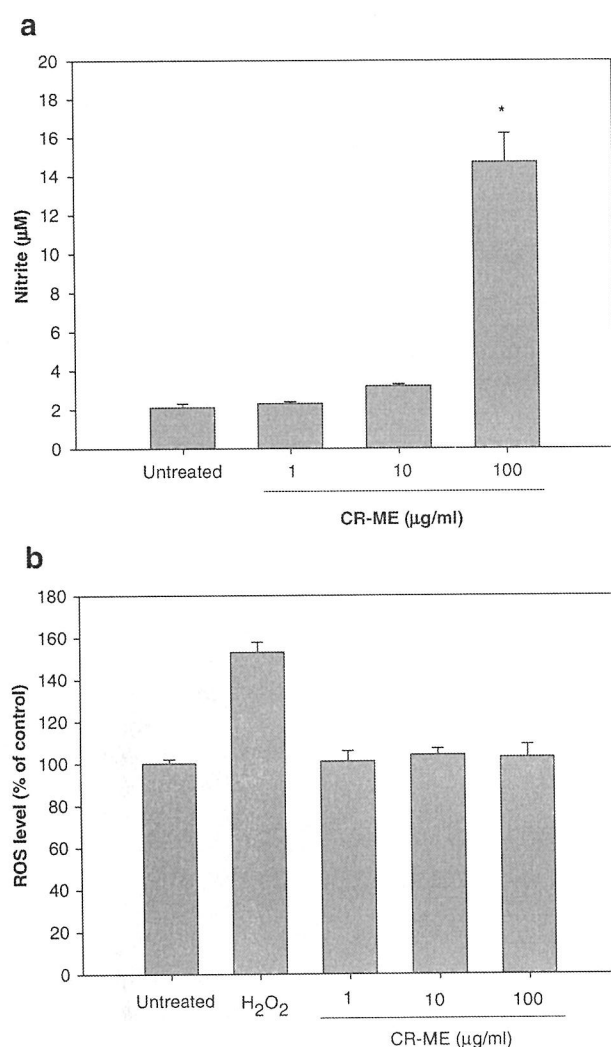


Fig. 3 Nitrite (a) and ROS (b) production from the peritoneal macrophages stimulated with CR-ME. The macrophages were treated with CR-ME for 18 h. **a** The culture supernatants were collected and the nitrite level was measured, as described in "Materials and Methods". **b** The macrophages were incubated either with CM-H₂-DCFDA (5 µM) for 15 min at 37°C for the ROS level. Stained cells were analyzed by flow cytometry. The results are mean ± SEM of quintuplicates from one representative experiment. As a positive control, H₂O₂ (0.1 mM) was used. * $p < 0.05$, significantly different from the control (no treatment)

cultured with the B16 tumor cells. The level of cell lysis was determined 20 h later. As shown in Fig. 4a, the presence of the p38 MAPK inhibitor during the activating stage inhibited the tumoricidal activation of macrophages by CR-ME, while pretreatments with the other inhibitors had little or no effect on the CR-ME-induced tumoricidal activity. At the concentrations used, none of the inhibitors affected the cytotoxic activity. These results suggest that p38 MAPK is involved in the macrophage-mediated cytotoxicity induced by CR-ME.

Since our results suggest that NO and TNF- α are involved in the tumoricidal activity of CR-ME-stimulated macrophages, the next series of experiments were aimed at determining if treating the macrophages with p38 MAPK inhibitor reduces their capacity to produce NO and TNF- α . As shown in Table 3, SB203580 inhibited the CR-ME-stimulated NO production by 54%, whereas TNF- α production was slightly reduced by the inhibitor. Overall, the results suggest that p38 MAPK is mainly involved in the CR-ME-induced NO production pathway. Further studies using Western blot analyses demonstrated that CR-ME induced the activation of p38 MAPK (Fig. 4b). This suggests that p38 MAPK activation is essential for the tumoricidal activity of CR-ME as well as CR-ME-induced production of NO in macrophages.

It is well known that NF- κ B plays an important role in macrophage activation via the transcription of inflammatory mediators downstream of multiple types of stimulatory events. As p38 MAP kinase had been demonstrated to be activated by CR-ME, the ability of CR-ME to activate NF- κ B was tested in an attempt to identify the nuclear factors that contribute to the activation of macrophages. As shown in Fig. 5, the CR-ME treatment produced a marked increase in NF- κ B DNA binding to its cognate in a concentration-dependent manner.

Discussion

In the present study, our data demonstrate that methanol extract obtained from *C. regalis*, a polar lichen, has an immunomodulatory activity which is not reported for this lichen so far.

Reactive oxygen species have been suggested to mediate the antitumor activity (Mavrier and Edgington 1984). In

Table 2 Inhibition of the tumoricidal activity of CR-ME-activated macrophages by the antibody or inhibitor

Treatment ^a	Percent cytotoxicity of target cells
None	27.2±3.3
CR-ME (100 µg/ml)	68.6±6.2*
CR-ME + anti-TNF- α (500 U)	38.4±2.2**
CR-ME + NMMA (0.5 mM)	33.7±3.8**

^a The macrophages were cultured for 18 h in the medium or in the medium supplemented with methanol extract of *Caloplaca regalis* (CR-ME; 100 µg/ml) in the presence or absence of TNF- α antibody or NMMA. The macrophages were co-cultured for 20 h with the target at an initial effector/target cell ratio of 10:1. The results are reported as a mean ± SEM of quintuplicates from a representative experiment

CR-ME Methanol extract of *C. regalis*, NMMA, N^G-monomethyl-L-arginine

* $p < 0.05$, significantly different from the control (no treatment)

** $p < 0.05$, significantly different from the CR-ME-treated cells

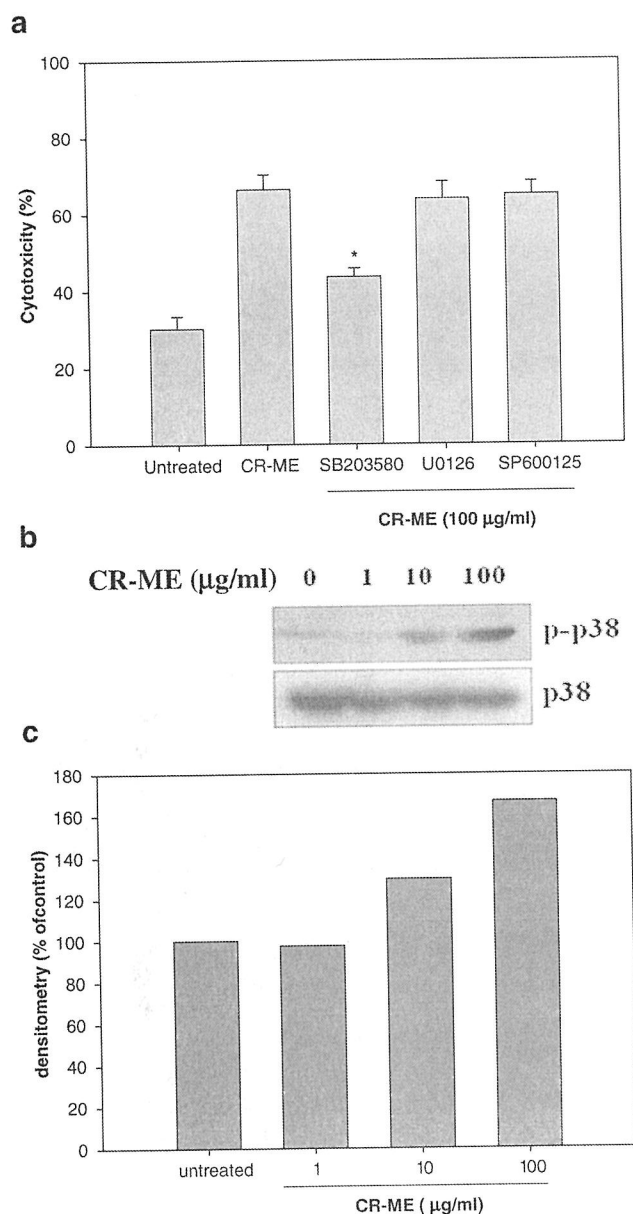


Fig. 4 a Inhibition of the CR-ME-induced tumoricidal activation of macrophage by p38 MAPK inhibitor. The peritoneal macrophages were cultured for 18 h in the medium or in the medium supplemented with CR-ME (100 μg/ml) in the presence or absence of the various inhibitors. The macrophage tumoricidal activity was determined, as described in "Materials and Methods". The data shown here are the results at an initial effector/target ratio of 10:1. The results are mean ± SEM of quintuplicates from one representative experiment. **b** Activation of p38 MAPK in CR-ME-treated cells. The cells were treated with CR-ME (100 μg/ml) and incubated for 10 h. The whole cell lysates were prepared and used for the p-p38 or p38 MAPK Western with the respective antibodies. As a positive control, IFN-γ (50 U/ml) combined with LPS (1 μg/ml) was used. **c** Each value was measured by densitometric analysis of the immunoblot based on the density of the band in the untreated control as 100%. Similar observations were obtained in three other experiments. **p*<0.05, significantly different from CR-ME-treated cells

Table 3 Inhibition of the MSE-induced NO and TNF-α production by p38 MAP inhibitor

Treatment ^a	Nitrite (μM)	TNF-α (pg/ml)
None	2.3±0.2	11.1±3.9
CR-ME (100 μg/ml)	7.8±0.1*	2,578.4±89.6*
CR-ME + SB203580 (10 μM)	3.6±0.1**	2,351.0±5.9**

^a The macrophages were cultured for 18 h in the medium or in the medium supplemented with methanol extract of *Caloplaca regalis* (CR-ME; 100 μg/ml) in the presence or absence of p38 MAPK inhibitor (SB203580). The culture supernatants were collected and the levels of nitrite and TNF-α were measured, as described in "Materials and Methods". The results are reported as a mean ± SEM of quintuplicates from a representative experiment
 CR-ME Methanol extract of *C. regalis*
 **p*<0.05, significantly different from the control (no treatment)
 ***p*<0.05, significantly different from the CR-ME-treated

these experiments, it was found that CR-ME did not increase the generation of ROS, compared with that in the untreated cells (data not shown). Therefore, these mediators did not appear to play a role in the CR-ME-induced tumoricidal activity.

Activated macrophages produce NO, which inhibits mitochondrial respiration and results in cytoxicity of the target cells (Goenka et al. 1998). Decker et al. (1987) reported that TNF-α acts as an effector molecule in macrophage cell-mediated cytotoxicity against tumor cells that are highly sensitive to TNF-α. Treating the macrophages with CR-ME induced the production of NO and TNF-α, suggesting that the reactive nitric oxide and TNF-α induced by CR-ME are involved in macrophage-mediated tumor cytotoxicity. However, IL-1 and IFN-γ do not play a key role in macrophage-mediated death of the target cells because the levels of these cytokines were not affected by the CR-ME treatment.

Protein kinases play an important role in the signal transduction pathways that regulate the response of macrophages to external stimuli (Dong et al. 1993; Jaramillo et al.

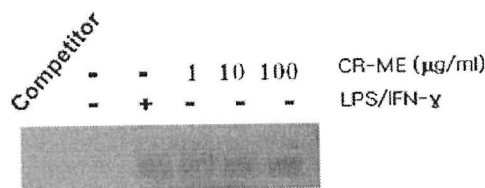


Fig. 5 Activation of NF-κB in the CR-ME-stimulated macrophages. DNA binding and the effects of CR-ME treatment on NF-κB DNA-binding activity in nuclear extracts of macrophages. The cells were treated with various CR-ME doses for 6 h. The cells were lysed and analyzed for their NF-κB DNA-binding activity with the use of an electrophoresis mobility shift assay, as described in "Materials and Methods". The experiment was performed in triplicate with identical results. As a positive control, IFN-γ (50 U/ml) combined with LPS (1 μg/ml) was used

2004; Schorey and Cooper 2003). Moreover, previous studies have shown that the inhibitors of PKC and PTK can block the tumoricidal activity as well as the production of various cytolytic molecules in macrophages (Goenka et al. 1998). These results show that the mechanisms by which CR-ME activates macrophages might involve p38 MAPK, which was confirmed by the observations that the effect of CR-ME on the activation of macrophages to tumoricidal activity was abrogated by a p38 MAPK inhibitor. This kinase inhibitor also inhibited the production of nitric oxide, whereas TNF- α production was slightly reduced by the inhibitor. This suggests that p38 MAPK is mainly involved in the macrophage-mediated NO production induced by CR-ME. However, the data do not totally rule out the possibility that for macrophage activation, CR-ME 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 compounds, such as angelan and safflower polysaccharide, activate several signaling pathways in macrophages (Jeon et al. 2001; Ando et al. 2002). 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- κ B (Kim et al. 1997) and mitogen-activated protein kinases (Chan and Riches 1998). In addition, a recent study suggested that there is the potential for cross talk between the MAP kinase and NF- κ B pathways in modulating the responsiveness of the macrophages exposed to external stimuli (Aga et al. 2004). Therefore, the cell activation pathway of CR-ME might be similar to that of other stimulants such as LPS. Moreover, CR-ME can trigger a complex interaction between multiple signal transduction pathways including the p38 MAPK signal molecule. Based on these findings, low levels of TNF- α production by CR-ME might synergistically act with other cytotoxic molecules including NO to mediate tumoricidal activity of macrophages using divergent activation signals.

This study demonstrated that CR-ME augments the tumoricidal activity of macrophages that could be correlated with inducing the release of NO and TNF- α . Therefore, CR-ME might have potential therapeutic utility in cancer patients. However, it is possible that, in addition to the macrophage tumoricidal activity by CR-ME, macrophages activated in vivo by CR-ME may destroy the host cells. NO and TNF- α play an important role in the pathogenesis of acute inflammatory diseases (Nathan 1992; Clark 2007). Thus, NO and TNF- α overproduction by the CR-ME-activated macrophages might make a significant contribution to various pathological complications.

In summary, CR-ME can stimulate the production of NO and TNF- α , which play a role in macrophage-mediated cytotoxicity. In addition, the p38 MAPK inhibitor blocked

the ability of CR-ME to induce tumoricidal activity in macrophages. Furthermore, NF- κ B plays a role in CR-ME-induced macrophage activation. This suggests that the macrophage cellular and secretory activities induced by CR-ME occur via the NF- κ B and p38 MAPK signal transduction pathways. In addition, our results may provide some leads in the development of new immunomodulating drugs.

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