JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Polysaccharide from *Ganoderma atrum* Evokes Antitumor Activity via Toll-like Receptor 4-Mediated NF-κB and Mitogen-Activated Protein Kinase Signaling Pathways

Shenshen Zhang, Shaoping Nie,* Danfei Huang, Jianqin Huang, Yawei Wang, and Mingyong Xie*

State Key Laboratory of Food Science and Technology, Nanchang University, 235 Nanjing East Road, Nanchang, Jiangxi 330047, China

ABSTRACT: *Ganoderma atrum* has been used as a traditional Chinese medicine for centuries. In this study, the antitumor activity of a novel *G. atrum* polysaccharide (PSG-1) was investigated in vitro and in vivo using S180 tumor-bearing mice. The results showed that PSG-1 significantly inhibited the proliferation of S180 via the activation of macrophages in a dose-dependent manner. PSG-1-primed macrophages exhibited a higher tumoricidal activity than untreated macrophages. Administration of PSG-1 significantly inhibited the growth of transplantable sarcoma S180-bearing mice and increased macrophage phagocytosis and the levels of cytokines and nitride oxide. Expression of Toll-like receptor (TLR) 4 in the membrane was markedly increased in PSG-1-treated groups, suggesting that it may be a possible receptor for PSG-1. PSG-1 also promoted the translocation of the p65 subunit of NF-κB from cytosol to nucleus and the degradation of IκBα. Moreover, the phosphorylation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1/2, and c-Jun N-terminal kinase in macrophages was improved by PSG-1 in a dose-dependent manner. Therefore, it is suggested that PSG-1 may elicit its antitumor effect by improving immune system functions through TLR4-mediated NF-κB and MAPK signaling pathways.

KEYWORDS: *Ganoderma atrum polysaccharide, antitumor, immunity, Toll-like receptor 4, mitogen-activated protein kinase*

INTRODUCTION

Cancer is one of the most serious diseases in developed countries and the second largest deadly disease in developing countries.[1] There is a close relationship between the occurrence, growth, and decline of tumor and immune states. Immunomodulation may make a great contribution to the prevention and cure of neoplastic diseases.[2] Anticancer treatment based on exploiting the host's own antitumor defense mechanism can be beneficial. The enhancement or potentiation of host immune response has been recognized as a possible way of inhibiting tumor growth without harming the host.[3] Therefore, it is very important to develop novel antitumor drugs with improved immunity potential.

Polysaccharides from plants, epiphyte, and animal extracts, such as mushroom, algae, and higher plants, are an interesting source of additives in the food and drug industries.[4] They have been widely studied due to their broad spectrum of therapeutic properties, relatively low toxicity, few side effects, and unique biological, chemical, and physical properties.[5] Especially, their immunomodulatory, anti-inflammatory, and antitumor effects have recently attracted more and more attention in the biochemical and medical fields.[6] However, some polysaccharides, such as the *Ganoderma lucidum* polysaccharide, do not show any direct inhibitory effect against the proliferation and apoptosis of S180 or HL-60 and have pronounced antitumor effects in vivo.[7] All of these activities may be due to their contribution to the enhancement of immune function in the body.

The identification of mammalian Toll-like receptors (TLRs) represented an important advance in our understanding of innate immunity to microbial pathogens. The TLRs may recognize endogenous ligands induced during innate immune-responses.[8] They are involved in the activation of immunocompetent cells such as DCs and macrophages and thus participate in the innate defense against bacterial infection.[9] Several TLRs have been identified in blood macrophages and cells of the myelomonocytic lineage.[10] TLR4 is predominately activated by LPS and lipoteichoic acid; it directs an immune response primarily through the activation of mitogen-activated protein kinases (MAPKs) and NF-κB, which results in the activation of macrophages to become cytokine-producing cells and in the proliferation of B cells.[11,12] Recently, a number of studies have demonstrated that polysaccharides isolated from *Platycodon grandiflorum*, *Phellinus linteus*, and *Acanthopanax senticosus* could activate the TLR4 signaling pathway.[9,13,14] However, to the best of our knowledge, it has not been reported yet whether the antitumor activity can be induced by polysaccharides isolated from plants via TLR4-mediated NF-κB and MAPK pathways.

Ganoderma, a popular medicinal mushroom, has been used as a home remedy in traditional Chinese medicine in many Asian countries.[15] Following the views of ancient Chinese medical scholars, people in Asian countries have been widely using *G. lucidum* for promoting health and longevity.[16] *G. lucidum* has also been used to treat various human diseases such as allergy, arthritis, bronchitis, gastric ulcer, hyperglycemia, hypertension, chronic hepatitis, hepatopathy, insomnia, nephritis, neurasthenia, scleroderma, inflammation, and cancer.[17] Recent studies emphasized its potential in the treatment of viral infections.[18]

Received: January 28, 2013
Revised: March 20, 2013
Accepted: March 20, 2013
Published: March 20, 2013

dx.doi.org/10.1021/jf4004225 | *J. Agric. Food Chem.* 2013, 61, 3676−3682

Numerous studies have demonstrated that extracts of *G. lucidum* inhibited tumor growth in vitro and in several mouse models of tumor, and the extracts have also been used as health promotion supplements.[19] However, the mechanism of this effect is not well understood. *Ganoderma atrum* has been widely used in Asian countries for centuries. Recently, a polysaccharide was isolated from *G. atrum* in our laboratory (named PSG-1, with a purity of >99.8%). Its primary structural features and molecular weight were characterized, and this polysaccharide has been shown to possess potent antioxidant activity.[20] We have further shown that PSG-1 possesses potent immunopotentiating activity against CTX-induced immunosuppression in vivo and may enhance the antitumor effect of CTX by triggering the mitochondrial pathway in S180-bearing mice.[21,22] It is thus necessary to further determine its functional properties for wide applications in pharmaceutical industries. The present study was aimed to investigate its antitumor and immunomodulatory activities in S180 tumor-bearing mice and provide insights into the underlying mechanisms.

MATERIALS AND METHODS

Materials. *G. atrum* was cultivated and collected in Ganzhou, Jiangxi Province, China. The species was identified by Dr. Zhihong Fu, a professor at Jiangxi University of Traditional Chinese Medicine. PSG-1 was extracted and purified following our previously published method.[20] 5-Fluorouracil (5-Fu) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture products were obtained from Life Technologies (Paisley, Scotland). IL-1β and TNF-α ELISA kits were purchased from SenXiong Biotech (Shanghai, China), and dimethyl sulfoxide (DMSO) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium and fetal bovine serum (FBS) were from Gibco (Grand Island, NY, USA). All other reagents were of analytical grade.

Cell Line and Animals. The S180 cell line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, China. BALB/c mice, weighing 20.0 ± 2.0 g, were provided by Beijing HFK Biotechnology Co. (Certificate SCXK (jing) 2009-0004, Beijing, China). The mice were acclimatized for 1 week before being used for the study. All animals used in this study were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996), and all procedures were approved by the Nanchang University Medical College Animal Care Review Committee.

MTT Assay. To determine the effect of PSG-1 on the inhibition of S180 cell proliferation, cell survival was measured using the MTT assay. A single cell suspension (4×10^4 cells/mL) was placed in 96-well plates. The cells were allowed to grow for 4 h. Then the old medium was replaced with fresh medium, and 10−320 μg/mL PSG-1 was added into the wells for determining its effect on cell growth. The plate was read at 570 nm using a microplate reader (Thermo, Shanghai, China).

Macrophage Cytotoxicity Assay. The assay for macrophage cytotoxicity was based on an assay described elsewhere.[23] Briefly, the mouse peritoneal macrophages (5×10^5 cells/mL) were plated into 96-well microtiter plates and incubated in various concentrations of PSG-1 (0−320 μg/mL) for 48 h at 37 °C. The macrophages were washed with RPMI 1640 to remove the stimulants and co-incubated with S180 cells for 48 h; the ratio of macrophages to target cells was approximately 25:1 at the beginning of each experiment. The percentage of growth inhibition of S180 cells was determined by using the MTT method. Cytolytic activity was expressed as the percentage of tumor cytotoxicity, as follows:

$$\%\ \text{cytotoxicity} = 1 - \left\{ \frac{\text{O.D. of [(target + macrophages) − macrophages]}}{\text{O.D. of target (untreated)}} \right\}$$

Measurement of in Vivo Antitumor Activity. S180 tumor cells (approximately 5×10^6 per 0.2 mL) were implanted subcutaneously into the right hind groin of the mice, and these mice were randomized into five groups containing eight mice each. Twenty-four hours after tumor implantation, the tumor-bearing mice were orally treated with PSG-1 at a dose of 50, 100, or 200 mg/kg body weight (BW) every day for 2 weeks. The positive control was given with 5-Fu at 20 mg/kg BW/day), and the normal group and control group received equal amounts of saline. On day 15, the mice were sacrificed. Tumors, thymuses, and spleens were extirpated, weighed, and fixed in Heidenhain's Susa fluid. The inhibition ratio was calculated with the following formula: inhibitory rate (%) = $[(C - T)/C] \times 100$, where C is the average tumor weight of the negative control group and T is that of the treatment group. The thymus and spleen indices were expressed as the thymus or spleen weight relative to BW.

Macrophage Phagocytosis. Tumor-bearing mice were sacrificed by cervical dislocation. After stimulation by intraperitoneal injection of 4 mL of PBS solution, peritoneal macrophages were aseptically prepared from peritoneal exudates of tumor-bearing mice as quickly as possible. Macrophage phagocytosis for chicken red blood cells (CRBCs) was measured after Giemsa staining, and CRBC were examined with light microscopy as described.[24] The number of macrophage-ingesting CRBCs in at least 100 cells was calculated by direct visual enumeration on a light microscope. The phagocytic rate (PR) was calculated as follows:

$$\text{PR}\,(\%) = \frac{\text{no. of macrophages − ingesting CRBCs}}{\text{total no. of macrophages}} \times 100$$

Determination of Cytokine Production in Peritoneal Macrophages (MΦ). After the mice were sacrificed, MΦ were collected under aseptic conditions and incubated (1×10^6cells/well) in 96-well culture plates. Nonadherent cells were removed after 2 h of incubation at 37 °C in a 5% CO_2 atmosphere. After a further incubation for 48 h, the supernatants were collected for the measurement of IL-1β and TNF-α levels using commercial ELISA kits according to the manufacturer's instructions. The absorbance was measured at 450 nm in an ELISA reader (Thermo, Shanghai, China).

Lymphocyte Proliferation assay. Spleens were aseptically removed from control and tumor-bearing mice treated with PSG-1 and gently homogenized in RPMI 1640 complete medium. The splenic cells (100 μL) were set up in 96-well plates (1×10^6cells/well). After the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in the presence of Con A (final concentration = 5 μg/mL) or LPS (final concentration = 10 μg/mL) for 48 h, 20 μL of MTT (5 mg/mL) was added to each well and incubated for a further 4 h. After the incubation, the cell suspensions were centrifuged at 1500 rpm for 6 min, the supernatants were removed, and then 150 μL of DMSO was added. The absorbance at 570 nm was measured in an automatic ELISA plate reader. The experiment was performed in triplicate.

NO Measurement. The mouse MΦ were collected from tumor-bearing mice; adherent macrophages (5×10^5cells/well) were placed in a 96-well plate and incubated in complete RPMI 1640 medium for 48 h. Nitrite content in the culture medium was determined by Griess reaction. At the end of the culture period, 100 μL/well of cell culture medium was incubated with an equal volume of Griess solution (1% sulfanilamide, 0.1% naphthylethyldiamine dihydrochloride, and 2% phosphoric acid) at room temperature for 10 min. The absorbance at 540 nm was read, and the concentrations of NO_2^- were determined from a least-squares linear regression analysis with a sodium nitrite standard curve.

Cytokine Assay. Peritoneal macrophages were harvested and purified from tumor-bearing mice. The concentrations of IL-1β and TNF-α in the culture supernatants were determined by ELISA kits

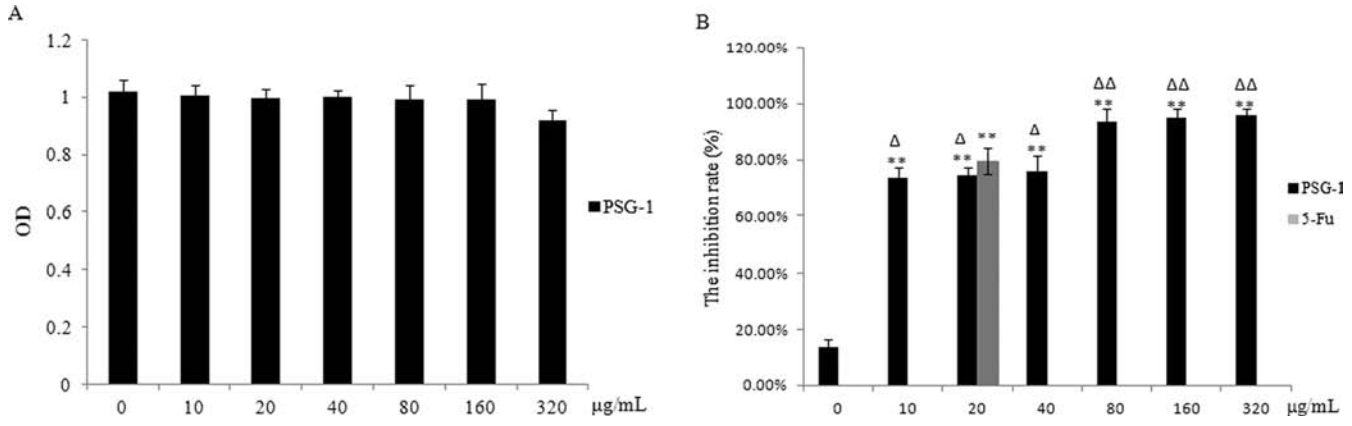

Figure 1. (A) Inhibition effect of PSG-1 on S180 in vitro at different concentrations. S180 cells were incubated at 37 °C for 48 h in the presence or absence of PSG-1. After incubation, cell survival was assayed by the MTT method. There was no significant difference ($P > 0.05$), compared with the control group. Percent of inhibition = (1 − treated/untreated control) × 100%. (B) Effect of PSG-1 on macrophage-mediated cytotoxicity against tumor cells. Peritoneal macrophages were isolated from mice and cultured on a 96-well U-bottom microtiter plate with PSG-1 for 24 h. Then, S180 cells were added and incubated for another 24 h. After culture, the inhibition of proliferation of S180 cells was analyzed. (∗) $P < 0.05$ and (∗∗) $P < 0.01$, compared with the untreated group; (Δ) $P < 0.05$ and (ΔΔ) $P < 0.01$, compared with the 5-Fu control group.

Table 1. Effects of PSG-1 on Inhibitory Rate, and Spleen and Thymus Indices in S180-Bearing Mice

group	concentration (mg/kg)	inhibitory rate (%)	thymus index[a] (mg/g)	spleen index[a] (mg/g)
normal control			1.483 ± 0.361*##	4.314 ± 0.372**
S180 control			1.251 ± 0.153##	7.592 ± 0.442##
5-Fu	20	47.49	0.221 ± 0.012**	4.382 ± 0.253**
PSG-1	50	28.32	1.410 ± 0.325*##	9.574 ± 0.423**##
	100	34.78	1.793 ± 0.143**##	9.661 ± 0.346**##
	200	46.52	2.555 ± 0.331**##	10.490 ± 0.352**##

[a]Values are the mean ± SEM of eight mice. *, means significantly different from S180 control group at $P < 0.05$; **, means significantly different from S180 control group at $P < 0.01$; #, means significantly different from 5-Fu control group at $P < 0.05$; ##, means significantly different from 5-Fu control group at $P < 0.01$.

Table 2. Effects of PSG-1 on Immunity Modulatory in S180-Bearing Mice

group	concentration (mg/kg)	A_{570} Con A[a] (OD)	A_{570} LPS A[a] (OD)	phagocytic rate (%)
normal control		0.431 ± 0.022*##	0.501 ± 0.032**##	32.361
S180 control		0.243 ± 0.016#	0.282 ± 0.024#	20.421
5-Fu	20	0.202 ± 0.020*	0.237 ± 0.021*	16.324
PSG-1	50	0.283 ± 0.034*#	0.329 ± 0.019*##	40.248
	100	0.389 ± 0.019**##	0.456 ± 0.020**##	46.478
	200	0.422 ± 0.027**##	0.513 ± 0.023**##	54.371

[a]Values are the mean ± SEM of eight mice. *, means significantly different from S180 control group at $P < 0.05$; **, means significantly different from S180 control group at $P < 0.01$; #, means significantly different from 5-Fu control group at $P < 0.05$; ##, means significantly different from 5-Fu control group at $P < 0.01$.

(SenXiong Biotech, Shanghai, China) according to the manufacturer's protocol.

SDS-PAGE and Western Blot Analysis. The MΦ were harvested from tumor-bearing mice, and then lysates (20 μg of total protein) were separated on 10 or 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). Blots were blocked for 1 h at room temperature in TBST containing 0.2% Tween 20 and 5% BSA. They were then incubated overnight at 4 °C with rabbit polyclonal antibodies: anti-TLR4, anti-NF-κB p65, and anti-IκBα (Cell Signaling Technology, Beverly, MA, USA); antiphospho-ERK, antiphospho-p38, antiphospho-JNK, anti-ERK, anti-p38, and anti-JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following three washings with TBST, membranes were incubated with secondary HRP-conjugated goat anti-rabbit IgG for 1 h. After a further four 10 min washings, the blots were developed using the Enhanced Chemiluminescence detection kit (Beyotime, Shanghai, China) by following the manufacturer's instructions.

Statistical Analysis. All experiments were repeated at least three times, and values represent the means from three independent experiments, each performed in triplicate. One-way analysis of variance (ANOVA) was used to determine the significance of differences between treatment groups. Values are expressed as the mean ± SEM. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Antitumor Activity of PSG-1 in Vitro. Antitumor activity of PSG-1 was examined in vitro. Figure 1A shows the growth of S180 cells in the presence or absence of various additives in 48 h incubation. There was no significant suppressive effect of PSG-1 on the proliferation of these cells.

Effect of PSG-1 on Activation of Macrophages. To determine the effect of PSG-1 on the ability of macrophages to kill S180 cells, macrophages were exposed to PSG-1 and then

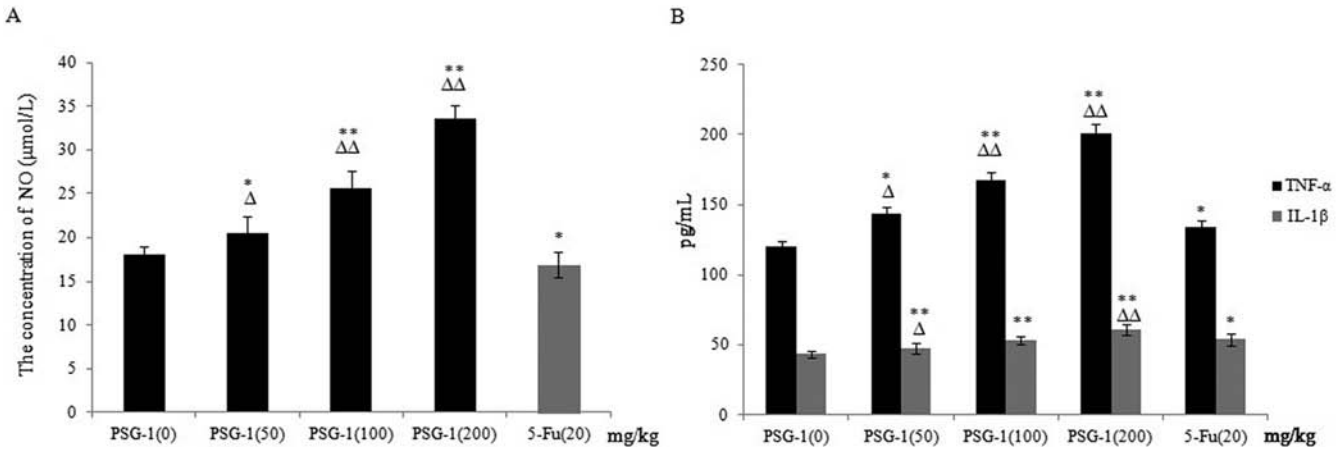

Figure 2. Effect of PSG-1 on peritoneal macrophage NO, IL-1β, and TNF-α production in S180-bearing mice. The contents of NO, IL-1β, and TNF-α in the culture supernatants were determined by ELISA as described in the text. The values are presented as the mean ± SEM (n = 3). (*) P < 0.05 and (**) P < 0.01 versys S180 control group; (Δ) P < 0.05 and ($\Delta\Delta$) P < 0.01 versus 5-Fu control group.

cocultured with S180 tumor cells. As shown in Figure 1B, PSG-1-primed macrophages enhanced tumoricidal activity in a dose-dependent manner, with a 7-fold increase at 320 μg/mL of PSG-1 as compared to untreated macrophages. The degree of antitumor activity of PSG-1 (40 μg/mL) was similar to that of 20 μg/mL of the positive control, 5-Fu.

Antitumor Effect of PSG-1 in S180-Bearing Mice. Table 1 shows the antitumor effect of PSG-1 at various doses in the S180-bearing mouse model in comparison with 5-Fu. PSG-1 at all three doses exhibited an excellent antitumor effect, with inhibitory ratios of 28.32, 34.78, and 46.52%, respectively. Moreover, the relative weights of spleen and thymus were important indices for nonspecific immunity. The thymus index and spleen index of the mice were calculated to explore the mechanism of antitumor activity. Interestingly, PSG-1 significantly increased thymus and spleen indices in mice (Table 1). Thus, immunostimulation evoked by PSG-1 may be the way to inhibit the growth of S180 tumor cells. Although 5-Fu effectively inhibited tumor growth, thymus and spleen indices were significantly lower than the controls.

Effect of PSG-1 on Proliferative Activity of Splenic Lymphocyte. Lymphocyte proliferative responses are often used to evaluate the functional capacity of T- and B-lymphocyte immunity. Con A stimulates T-cell and LPS stimulates B-cell proliferation.[25] As shown in Table 2, Con A- and LPS-induced splenocyte proliferation in the S180-bearing mice was significantly enhanced by PSG-1 at doses of 50, 100, and 200 mg/kg BW (P < 0.01). PSG-1 at 200 mg/kg could regulate cellular immunity in S180-bearing mice nearly to that of the controls. The splenocyte proliferation in the 5-Fu-treated group was significantly lower than controls (P < 0.05).

Effect of PSG-1 on Phagocytosis of Peritoneal Macrophages. Activated macrophages participate in both specific and nonspecific immune reactions and are also the "bridge cells" of these two kinds of immune reactions.[26] The phagocytosis of peritoneal macrophages isolated from the mouse model is shown in Table 2. A significant reduction in macrophage phagocytosis was induced in the S180 control group. The phagocytic rate in the 5-Fu-treated group was significantly lower than that of the negative control group, suggesting that the immune function damaged by 5-Fu was significant. Interestingly, PSG-1 markedly increased macrophage phagocytosis in a dose-dependent manner. The phagocytic rates in the PSG-1-treated groups were 1.2−1.7-fold that of the normal control group.

Effect of PSG-1 on NO, TNF-α, and IL-1β Production. It is well-known that activated macrophages release various cytokines, which play a role in modulating immune responses. Macrophages also act as a killer against tumor cells when these cells are potentially activated by some stimuli.[27] NO is known to be one of the major toxic and proinflammatory mediators in acute and chronic inflammatory diseases as well as in normal defense reactions; thus, we examined the effects of PSG-1 or 5-Fu administration on the levels of NO and cytokines in macrophages of tumor-bearing mice. As shown in Figure 2, the level of NO in the 5-Fu-treated group was lower than in the model control group, which indicated 5-Fu could not promote the secretion of NO. However, compared with the model control group, NO, IL-1β, and TNF-α productions were significantly increased in the PSG-1-treated groups (P < 0.05 or P < 0.01), especially at the dose of 200 mg/kg BW. In addition, the cytokines level was dramatically higher than that in the 5-Fu-treated group. Therefore, PSG-1 treatment activated MΦ and enhanced secretions of cytokines and NO.

PSG-1 Stimulates the TLR4-Mediated NF-κB Pathway. TLRs were identified as important membrane receptors involved in the activation of macrophages and found to play an essential role in the recognition of microbial components.[28] To determine the involvement of TLR4 in the activation of macrophages, we examined the expression of TLR4 using Western blot analysis. The results showed that the expression of TLR4 in the membrane was markedly increased in PSG-1-treated groups (Figure 3A).

As described previously, signals originated from TLRs are known to activate NF-κB. Translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα, so nuclear translocation of NF-κB is directly linked to IκBα degradation and phosphorylation.[29] Because p65 is the major component of NF-κB activated by LPS in macrophages, we next investigated whether PSG-1 also stimulates the translocation of the p65 subunit of NF-κB from the cytosol to the nucleus after its release from IκBs. We found that the amount of NF-κB p65 in the nucleus was markedly increased in PSG-1-treated groups in a dose-dependent manner (Figure 3C). The nuclear translocation of the NF-κB transcription factor was preceded by the degradation

dx.doi.org/10.1021/jf4004225 | J. Agric. Food Chem. 2013, 61, 3676−3682

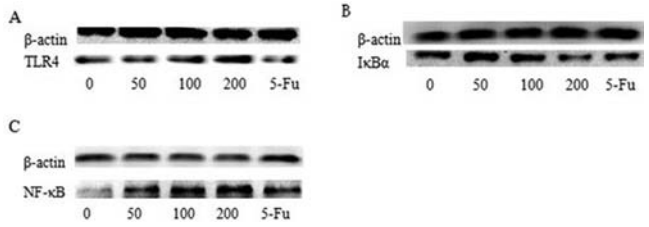

Figure 3. Western blot analysis of TLR4-mediated signaling pathways. Macrophages from each tumor-bearing mouse were collected. (A) Total cell extracts were prepared and Western blotted for TLR4 using specific TLR4 antibodies. (B) Cytosolic extracts of the cells were prepared and Western blotted for IκBα using specific anti-IκBα antibodies. (C) Nuclear extracts were prepared for the Western blotting of p65 of NF-κB using specific anti-p65 monoclonal antibody. This experiment was repeated three times, producing similar results.

and phosphorylation of IκBα.[30] To determine whether PSG-1 could affect the degradation and phosphorylation of IκBα, we measured the IκBα content in macrophages of tumor-bearing mice. Western blot analysis of cell extracts with antibodies specific to IκBα showed that PSG-1 enhanced IκBα degradation dose dependently (Figure 3B).

Involvement of MAPK Pathway in PSG-1-Induced Antitumor Responses. The MAPKs play critical roles in the regulation of cell growth and differentiation and in cellular responses to cytokines and stresses. MAPKs are also known to be important for the transcriptional activation of NF-κB.[31] To investigate whether the activation of macrophages by PSG-1 is mediated through the MAPK pathway, we examined the effect of PSG-1 on the phosphorylation of ERK1/2, JNK1/2, and p38 MAPK in macrophages by Western blotting with phosphospecific antibodies. As shown in Figure 4, PSG-1 induced the

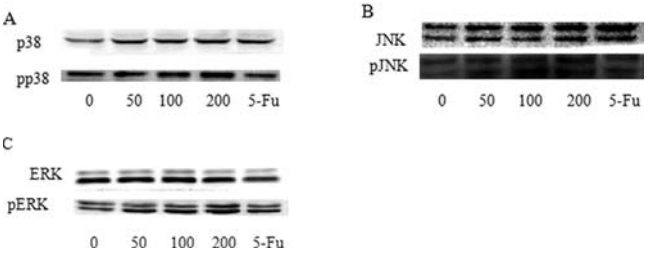

Figure 4. Involvement of the p38 MAPK pathway in PSG-1-induced antitumor responses. Collected peritoneal macrophages from tumor-bearing mice, whole cell lysates, were analyzed by Western blot analyses using antibodies against the activated MAPK. The data shown are representative of three independent experiments.

activation of ERK1/2, JNK1/2, and p38 MAPK in a dose-dependent manner. Interestingly, the levels of nonphosphorylated ERK, JNK, and p38 MAPK were unaffected by PSG-1.

■ DISCUSSION

Cancer is a formidable problem for people. The great majority of chemical compounds that have been identified as cytotoxic to cancer cells are also toxic to normal cells.[32] Therefore, it is very important to identify novel antitumor substances with little toxicity to the host. The discovery and identification of new antitumor drugs that can potentiate the immune function have become an important goal of research in immunopharmacology and oncotherapy. This study has demonstrated the antitumor effect of PSG-1, a polysaccharide from *G. atrum*, and its immunomodulative activity.

Previous results from our laboratory strongly suggested that PSG-1 significantly suppressed tumor growth in CT26 tumor-bearing mice, which could activate macrophages via TLR4-dependent signaling pathways, improve immunity, and inhibit tumor growth.[33] To further investigate the antitumor effects of PSG-1, in the present research we studied the effect of PSG-1 on the growth of S180 cells in vitro. We also investigated the antitumor effect of PSG-1 on mouse transplantable S180 sarcomas and the effect of PSG-1 on the immunological parameters in S180 tumor-bearing mice. Our findings suggested that PSG-1 could significantly inhibit the growth of S180 tumors transplanted in mice and induce changes in the immunological parameters. However, treatment with PSG-1 could not inhibit tumor cells in vitro, suggesting that the antitumor activity of PSG-1 may be largely due to its immunostimulatory properties.

The immune system plays an important role in antitumor defense. Many papers have suggested that antitumor activity of the polysaccharides was also mediated through enhancement of the immune response.[5] In this study, a significant tumor suppression effect of PSG-1 was observed in the tumor-bearing animal model. The spleen and thymus are the important immune organs; spleen and thymus indices reflects the immune function of the organism. PSG-1 could increase the relative weights of the spleen and thymus compared with 5-Fu, the positive control, which had a high tumor inhibitory rate but with an immunosuppressive effect on immune organs. We concluded that PSG-1 might suppress the tumor growth in vivo and that its protective immunity against tumor was composed of both specific and nonspecific immunity.

The phagocytic function of macrophages is one of the most important nonspecific immune responses of the body. Macrophages protect the host by phagocytosis, present antigens to lymphocytes, and release numerous cell factors that regulate the activity of other cells.[34] In this study, we found that PSG-1 significantly increased macrophage phagocytosis of CRBCs in S180 tumor-bearing mice, which suggested a role of PSG-1 in the activation of macrophages. The specific immune response includes humoral and cellular immunity, and it is mediated specifically by T cells, including NK cells. The cellular immune response is weakened in cancer patients or tumor-bearing animals. The lymphocyte proliferation response is demonstrated by the capacity to elicit an effective T- and B-cell immunity.[35] Con A and LPS are known to stimulate the proliferation of T cells and B cells, respectively. The present study showed that PSG-1 significantly promoted the Con A- and LPS-stimulated splenocyte proliferation in tumor-bearing mice. The results indicated that PSG-1 could increase the activities of macrophages and lymphocyte cells and enhance the specific and nonspecific immunity in tumor-bearing mice.

NO is a short-lived free radical synthesized from L-arginine by NO synthase; it contributes to the killing of microorganisms and tumor cells by activated macrophages and mediates a variety of biological functions as an intracellular messenger molecule.[36] Activated macrophages also produce a variety of cytokines. Cytokines regulate both cellular and humoral immune responses by affecting immune cell proliferation, differentiation, and functions. TNF-α and IL-1β induce the proliferation of other cells and the expression of various genes via the autocrine and paracrine systems. TNF-α could cause apoptosis of tumor-associated endothelial cells, resulting in

tumor necrosis.[37] It also plays a pivotal role in host defense and can induce the expression of a number of other immunoregulatory and inflammatory mediators.[38] The present results showed that PSG-1 significantly increased the releases of NO, TNF-α, and IL-1β in macrophages of S180-bearing mice. Therefore, PSG-1 played an important role in antitumor activity through the release of effective molecules NO, TNF-α, and IL-1β. The modulation of NO, TNF-α, and IL-1β production possibly contributed to the therapeutic effects of PSG-1.

The function of immune cells largely relies on the structures and functions of their membranes.[39] Recently, TLRs were identified as important membrane receptors involved in the activation of macrophages by a variety of pathogens.[28] One of the most famous TLR4 ligands, other than those from bacterial sources, is paclitaxel.[40] Recently, there have been several reports demonstrating TLR-dependent activation of macrophages by polysaccharides, so we investigated the expression of TLR4 in macrophages of tumor-bearing mice in this study. PSG-1 was found to cause an increase in TLR4 expression. In contrast, 5-Fu, a positive control, had no effect on TLR4 expression. We concluded that TLR4 probably was one of the membrane receptors of PSG-1.

The stimulation of TLRs leads to the activation of several MAPK pathways, suggesting that TLRs have or share a common signaling pathway.[41] The activation of a MAPK pathway subsequently induces gene expression by activating several transcription factors, including NF-κB and AP-1.[42] NF-κB is a dimeric transcription factor formed by the hetero- or homodimerization of proteins in the Rel family, including p65 and p50. These NF-κB molecules act through distinct signaling pathways that converge on the activation of an IκBα kinase (IKK); IKK activation initiates IκBα phosphorylation at specific amino-terminal serine residues.[43] Ando et al. reported that safflower polysaccharides activate the transcription factor NF-κB via TLR4 and induce cytokine production by macrophages.[13] In addition, IκBα phosphorylation and degradation can induce NF-κB activation.[44] We found that PSG-1 promoted the translocation of the p65 subunit of NF-κB from the cytosol to the nucleus after its release from IκBs and dose dependently promoted the degradation of IκBα in macrophages.

The activation of NF-κB is regulated by cellular kinases such as MAPKs.[45] MAPKs, a highly conserved family of protein serine/threonine kinases, include the p38, ERK1/2, and JNK subgroups.[46,47] The activation of p38, ERK1/2, and JNK is considerred to be a hallmark of LPS-induced signal transduction. Therefore, to further confirm the mechanism of NF-κB activation by PSG-1, we investigated the roles of p38, ERK, and JNK signaling in the pathway induced by PSG-1 in tumor-bearing macrophages. The phosphorylations of p38, ERK1/2, and JNK were enhanced in PSG-1-treated mice in a concentration-dependent manner. We concluded that PSG-1 probably acted on the TLR4 receptors on macrophages, signaled through the p38 MAPK pathway, and then activated NF-κB.

In summary, we found that PSG-1 significantly inhibited the growth of transplantable tumors in mice, whereas it could not kill S180 directly in vitro. Furthermore, it significantly increased the levels of NO, TNF-α, and IL-1β, secreted by peritoneal macrophages, and splenocyte proliferation in S180-bearing mice, which indicated that PSG-1 could improve the immune response. The signaling mechanism may be that PSG-1 induced macrophage activation through TLR4-mediated NF-κB and MAPK (p38, ERK1/2, and JNK) signaling pathways, initiating the release of cytokines, such as TNF-α and IL-1β, and effector molecules such as NO in macrophages. The results suggested that PSG-1 had antitumor activity by improving immune system functions and could act as an antitumor agent with immunomodulatory activity. We concluded that PSG-1 could be explored as a novel therapeutic agent in the field of pharmaceutical and functional foods.

AUTHOR INFORMATION

Corresponding Author

*Phone/fax: +86-791-83969009 (M.X.) or +86-791-88304452 (S.N.). E-mail: myxie@ncu.edu.cn (M.X.) or spnie@ncu.edu.cn (S.N.).

Funding

Financial support for this study by the Key Program of National Natural Science Foundation of China (No. 31130041), the National Natural Science Foundation of China (31071532), the National Key Technology R&D Program of China (2012BAD33B06), the Objective-Oriented Project of State Key Laboratory of Food Science and Technology (SKLF-MB-201001), the Training Project of Young Scientists of Jiangxi Province (Stars of Jing gang), and the Exploring Project of State Key Laboratory of Food Science and Technology (SKLF-TS-201107) is gratefully acknowledged.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BSA, bovine serum albumin; BW, body weight; ConA, concanavalin A; CRBC, chicken red blood cells; IκB, inhibitor protein-kappa B; LPS, lipopolysaccharide; MΦ, peritoneal macrophages; TLR, Toll-like receptor; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; NO, nitride oxide; PBS, phosphate-buffered saline; PSG-1, *Ganoderma atrum* polysaccharide; TBST, Tris-buffered saline with Tween

REFERENCES

(1) Jema, A.; Bray, F.; Center, M. M.; Ferlay, J.; Ward, E.; Forman, D. Global cancer statistics. *CA Cancer J. Clin.* **2011**, *61*, 69−90.

(2) Mitchell, M. S. Immunotherapy as part of combinations for the treatment of cancer. *Int. Immunopharmacol.* **2003**, *3*, 1051−1059.

(3) Yuan, H. M.; Song, J. M.; Li, X. G.; Li, N.; Dai, J. C. Immunomodulation and antitumor activity of κ-carrageenan oligosaccharides. *Cancer Lett.* **2006**, *243*, 228−234.

(4) Forabosco, A.; Bruno, G.; Sparapano, L.; Liut, G.; Marino, D.; Delben, F. Pullulans produced by strains of *Cryphonectria parasitica*—I. Production and characterisation of the exopolysaccharides. *Carbohydr. Polym.* **2006**, *63*, 535−544.

(5) Schepetkin, I. A.; Quinn, M. T. Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. *Int. Immunopharmacol.* **2006**, *6*, 317−333.

(6) Wang, J.; Zuo, G.; Li, J.; Guan, T.; Li, C.; Jiang, R.; Xie, B.; Lin, X.; Li, F.; Wang, Y.; Chen, D. Induction of tumoricidal activity in mouse peritoneal macrophages by ginseng polysaccharide. *Int. J. Biol. Macromol.* **2010**, *46*, 89−95.

(7) Lin, Z. B.; Zhang, H. N. Anti-tumor and immunoregulatory activities of *Ganoderma lucidum* and its possible mechanisms. *Acta. Pharmacol. Sin.* **2004**, *11*, 1387−1395.

(8) Kim, G. Y.; Han, M. G.; Song, Y. S.; Shin, B. C.; Park, Y. M. Proteoglycan Isolated from *Phellinus linteus* induces Toll-like receptors

2- and 4-mediated maturation of murine dendritic cells via activation of ERK, p38, and NF-κB. *Biol. Pharm. Bull.* **2004**, *27*, 1656−1662.
(9) Rock, F. L.; Hardiman, G.; Timans, J. C.; Kastelein, R. A.; Bazan, J. F. A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 588−593.
(10) Takeuchi, O.; Hoshino, K.; Kawai, T.; Sanjo, H.; Takada, H.; Ogawa, T.; Takeda, K.; Akira, S. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* **1999**, *11*, 443−451.
(11) Kawai, T.; Adachi, O.; Ogawa, T.; Takeda, K.; Akira, S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **1999**, *1*, 15−22.
(12) Akashi, S.; Shimazu, R.; Ogata, H.; Nagai, Y.; Takeda, K.; Kimoto, M.; Miyake, K. Cutting edge: cell surface expression and lipopolysaccharide signaling via the Toll-like receptor 4−MD-2 complex on mouse peritoneal macrophages. *J. Immunol.* **2000**, *164*, 3471−3475.
(13) Ando, I.; Tsukumo, Y.; Wakabayashi, T.; Akashi, S.; Miyake, K.; Kataoka, T.; Nagai, K. Safflower polysaccharides activate the transcription factor NF-κB via Toll-like receptor 4 and induce cytokine production by macrophages. *Int. Immunopharmacol.* **2002**, *2*, 1155−1162.
(14) Han, S. B.; Yoon, Y. D.; Ahn, H. J.; Lee, H. S.; Lee, C. W.; Yoon, W. K.; Park, S. K.; Kim, H. M. Toll-like receptor-mediated activation of B cells and macrophages by polysaccharide isolated from cell culture of *Acanthopanax senticosus*. *Int. Immunopharmacol.* **2003**, *3*, 1301−1312.
(15) Yun, T. K. Update from Asia. Asian studies on cancer chemoprevention. *Ann. N.Y. Acad. Sci.* **1999**, *889*, 157−192.
(16) Lin, Z. B. Cellular and molecular mechanisms of immuno-modulation by *Ganoderma lucidum*. *J. Pharmacol. Sci.* **2005**, *99*, 144−153.
(17) Sliva, D. *Ganoderma lucidum* (Reishi) in cancer treatment. *Integr. Cancer Ther.* **2003**, *2*, 358−364.
(18) Boh, B.; Berovic, M.; Zhang, J.; Zhi-Bin, L. Ganoderma lucidum and its pharmaceutically active compounds. *Biotechnol. Annu. Rev.* **2007**, *13*, 265−301.
(19) Wasser, S. P. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 258−274.
(20) Chen, Y.; Xie, M. Y.; Nie, S. P.; Li, C.; Wang, Y. X. Purification, composition analysis and antioxidant activi,ty of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. *Food Chem.* **2008**, *107*, 231−41.
(21) Li, W. J.; Nie, S. P.; Xie, M. Y.; He, M.; Zhang, S. S.; Zhu, K. X. *Ganoderma atrum* polysaccharide induces anti-tumor activity via the mitochondrial apoptotic pathway related to activation of host immune response. *J. Cell Biochem.* **2011**, *112*, 860−871.
(22) Li, W. J.; Nie, S. P.; Yi, C.; Wang, Y. X.; Chang, L.; Xie, M. Y. Enhancement of cyclophosphamide-induced antitumor effect by a novel polysaccharide from *Ganoderma atrum* in sarcoma 180-bearing mice. *J. Agric. Food Chem.* **2011**, *59*, 3707−3716.
(23) Ghaffar, A.; Cullen, R. T.; Woodruff, M. A. Further analysis of the anti-tumour effect in vitro of peritoneal exudate cells from mice treated with *Corynebacterium parvum*. *Br. J. Cancer* **1975**, *31*, 15−24.
(24) Zhou, S.; Liu, X. Z.; Guo, Y.; Wang, Q.; Peng, D. Y.; Cao, L. Comparison of the immunological activities of arabinoxylans from wheatbran with alkali and xylanase-aidedextraction. *Carbohydr. Polym.* **2010**, *81*, 784−789.
(25) Dai, Z. Y.; Zhang, H.; Zhang, Y. P.; Wang, H. H. Chemical properties and immunostimulatory activity of a water-soluble polysaccharide from the clam of *Hyriopsis cumingii* Lea. *Carbohydr. Polym.* **2009**, *77*, 365−369.
(26) Chen, X. M.; Lu, J. X.; Zhang, Y. D.; He, J. T.; Guo, X. Z.; Tian, G. Y.; Jin, L. Q. Studies of macrophage immuno-modulating activity of polysaccharides isolated from *Paecilomyces tenuipes*. *Int. J. Biol. Macromol.* **2008**, *43*, 252−256.
(27) Andreesen, R.; Scheibenbogen, C.; Brugger, W.; Krause, S.; Meerpohl, H. G.; Leser, H. G.; Engler, H.; Löhr, G. W. Adoptive transfer of tumor cytotoxic macrophages generated in vitro from circulating monocytes: a new approach to cancer immunotherapy. *Cancer Res.* **1990**, *50*, 7450−7456.
(28) Werling, D.; Jungi, T. W. Toll-like receptors linking innate and adaptive immune response. *Vet. Immunol. Immunopathol.* **2003**, *91*, 1−12.
(29) Moynagh, P. N. The NF-κB pathway. *J. Cell Sci.* **2005**, *118*, 4389−4392.
(30) D'Acquisto, F.; May, M. J.; Ghosh, S. Inhibition of nuclear factor kappa B (NF-κB): an emerging theme in anti-inflammatory therapies. *Mol. Interv.* **2002**, *2*, 22−35.
(31) Vermeulen, L.; Wilde, G. D.; Damme, P. V.; Berghe, W. V.; Haegeman, G. Transcriptional activation of the NF-κB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO J.* **2003**, *22*, 1313−1324.
(32) Borchers, A. T.; Stern, J. S.; Hackman, R. M.; Keen, C. L.; Gershwin, M. E. Mushrooms, tumors, and immunity. *Proc. Soc. Exp. Biol. Med.* **1999**, *221*, 281−293.
(33) Zhang, S. S.; Nie, S. P.; Huang, D. F.; Li, W. J.; Xie, M. Y. Immunomodulatory effect of *Ganoderma atrum* polysaccharide on CT26 tumor bearing mice. *Food Chem.* **2013**, *136*, 3−4.
(34) Jiao, L. L.; Li, X.; Li, T. B.; Jiang, P.; Zhang, L. X.; Wu, M. J.; Zhang, L. P. Characterization and anti-tumor activity of alkali-extracted polysaccharide from *Enteromorpha intestinalis*. *Int. Immunopharmacol.* **2009**, *9*, 324−329.
(35) Marciani, D. J.; Press, J. B.; Reynolds, R. C.; Pathak, A. K.; Pathak, V.; Gundy, L. E.; Farmer, J. T.; Koratich, M. S.; May, R. D. Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. *Vaccine* **2000**, *18*, 3141−3151.
(36) Palmer, R. M.; Ashton, D. S.; Moncada, S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **1988**, *333*, 664−666.
(37) Lejeune, F. J.; Liénard, D.; Matter, M.; Rüegg, C. Efficiency of recombinant human TNF in human cancer therapy. *Cancer Immun* **2006**, *6*, 6−16.
(38) Baugh, J. A.; Bucala, R. Mechanisms for modulating TNF α in immune and inflammatory disease. *Curr. Opin. Drug Discovery* **2001**, *4*, 635−650.
(39) Feng, X.; Zhou, Y. Z. The effect of tea oil, corn oil and fish oil on the immune function. *Acta Nutr. Sin.* **1996**, *18*, 412−416.
(40) Rowinsky, E. K. Update on the antitumor activity of paclitaxel in clinical trials. *Ann. Pharmacother.* **1994**, *28*, 18−22.
(41) Ozinsky, A.; Underhill, D. M.; Fontenot, J. D.; Hajjar, A. M.; Smith, K. D.; Wilson, C. B.; Schroeder, L.; Aderem, A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13766−137671.
(42) Ninomiya-Tsuji, J.; Kishimoto, K.; Kiyama, A.; Inoue, J.; Cao, Z.; Matsumoto, K. The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* **1999**, *398*, 252−256.
(43) Pan, M. H.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. H.; Lin, J. K. Suppression of lipopolysaccharide-induced nuclear factor-κB activity by theaflavin-3,3′-digallate from black tea and other polyphenols through down-regulation of IκB kinase activity in macrophages. *Biochem. Pharmacol.* **2000**, *59*, 357−367.
(44) Baeuerle, P. A.; Baltimore, D. IκB: a specific inhibitor of the NF-κB transcription factor. *Science* **1988**, *242*, 540−546.
(45) Guha, M.; Mackman, N. LPS induction of gene expression in human monocytes. *Cell Signal.* **2001**, *13*, 85−94.
(46) Garrington, T. P.; Johnson, G. L. Organization and regulation of mitogenactivated protein kinase signaling pathways. *Curr. Opin. Cell Biol.* **1999**, *11*, 211−218.
(47) Ruland, J.; Mak, T. W. Transducing signals from antigen receptors to nuclear factor κB. *Immunol. Rev.* **2003**, *193*, 93−100.