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Purification, Identification and Functional Characterization of an Immunomodulatory Protein from *Pleurotus eryngii*.

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Abstract: *Pleurotus eryngii* contains bioactive compounds that can activate the immune system. Here we report the identification, purification, and functional characterization of the bioactive *P. eryngii* protein (PEP) 1b. PEP 1b was discovered to be a 21.9 kDa protein with the ability to induce M1-polarization of macrophages cell line, Raw 264.7 cells. Biochemical measurements showed that PEP 1b stimulated nitric oxide (NO), IL-1β, IL-6 and TNF-α production and regulated inducible NO synthase. Phosphorylation and inhibitor studies revealed that PEP 1b promoted the translocation of NF-kB from the cytosol to the nucleus allowing the induction of target gene expression and NO production. Phosphorylation of JNK and ERK1/2 was found necessary for NO production. Each phosphorylation pathway was found to require a Toll-like receptor (TLR) 4 as a prerequisite for PEP1b-induced NO production. This study suggests that PEP 1b is an immunomodulatory protein that can boost cellular immune responses through activation of the TLR4-NF-κB and MAPK signaling pathways.

Keyword: Immunomodulatory activity; Macrophage activation; MAPK; NF-κB; *Pleurotus eryngii* protein; TLR4.

1. INTRODUCTION

Pleurotus eryngii (*P. eryngii*), more commonly known as the king oyster mushroom, possesses both nutritional and pharmaceutical value. The king oyster mushroom originated in Europe, but is now widely consumed in other parts of the world which include Asia and Africa [1]. *P. eryngii* contains bioactive constituents (e.g., polysaccharides, polyphenols, sterols, dietary fiber, and proteins) that can confer a variety of health benefits such as hepatoprotective and hypolipidemic effects [2], immunopotentiation [3], antioxidant [4], anti-inflammation [5], and antitumor properties [6]. The activation and regulation of the immune system by polysaccharides that are present in fruiting body have received considerable attention and extensive studies[7-9]. It has been documented that edible fungi produce a huge number of biologically active proteins such as lectins, fungal immunomodulatory proteins (FIP), and ribosome inactivating proteins (RIP) [10] , however, the effect of bioactive proteins on stimulating the immune system and modulating specific cellular responses by interfering in particular transduction pathways remains to be fully understood [11].

The immune boosting activity of mushroom proteins, a new research concern, appears to be mediated through the stimulation of macrophages and dendritic cells which are essential components of the innate immune system in animals[12]. Macrophages are known to engulf exogenous pathogens and secrete inflammatory mediators and cytokines in an effort to maintain optimal health [13]. Depending upon the identity of the activating factor, the exposed macrophages will differentiate into either classically-activated macrophages or alternatively-activated macrophages, designated as M1 and M2, respectively [14, 15]. For example, M1 macrophages activated by interferon-γ (IFN-γ) or lipopolysaccharide (LPS) will produce high levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12, etc.),

chemokines (CCL2, CXCL1, CXCL10, etc.), and iNOS expression[16]. M2 macrophages activated by cytokines (IL-4, IL-13) will produce high amounts of cytokines (IL-8, IL-10) [17, 18]. Some of bioactive proteins from *P. eryngii* have been reported to have diversified functional activities, such as immunoregulation [19], anti-inflammation [5] and anti-cancer [20], which has revealed the potential for the application of *P. eryngii* protein in the development of health foods and pharmaceutical products, thereby providing the new insight into functional food for human health and well-being. In our previous studies[3, 19], the immunostimulatory effects of *P. eryngii* proteins has been discovered preliminarily, but, however, the understanding of the molecular mechanism(s) by which these proteins mediate activation of this immunomodulatory activity as well as the identity of the bioactive proteins remains incomplete.

As a step toward filling this gap in our knowledge, we report here on the purification, identification, and functional characterization of *P. eryngii* protein 1b (PEP 1b). A coupling of biochemical and biological methodologies led to the identification and purification of this novel bioactive protein, PEP 1b, which was capable of inducing M1-polarized activation of macrophages. In addition, utilizing an array of biochemical, cellular, and molecular technologies, it was determined that this M1-polarized activation of macrophages by PEP 1b occurred through the TLR4-NF-kB and MAPK signaling pathways.

2. MATERIALS AND METHODS

2.1 Materials and Chemicals

Fresh fruiting bodies of *Pleurotus eryngii* were supplied by Jiangsu Tianfeng Biological Technology Co., Ltd. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and LPS were obtained from Solarbio

Technologies (Beijing, China). A SDS-PAGE gel preparation kit and cell counting kit-8 (CCK-8) were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Anti-Toll like receptor 2 (TLR2) and anti-Toll like receptor 4 (TLR4) antibodies were purchased from Beijing biosynthesis biotechnology Co., Ltd (Beijing, China). NF-κB inhibitors (SC75741 and EVP4593), MAPK inhibitors (SP600125, SB203580, and PD98059) and polymyxin B sulfate were purchased from Targetmol (Boston, MA, USA). Nitric oxide assay kits, phenylmethanesulfonyl fluoride (PMSF), BeyoECL star, nitrocellulose membrane, TBS, TBST, and nonfat dry milk were procured from Beyotime Biotechnology (Shanghai, China). All Western-blot antibodies (β-actin, PARP, iNOS, p65, IκB-α, p-IκB-α, IKK, p-IKK, JNK3, ERK1/2, p38, p-JNK3, p-Erk1/2, p-p38, IgG-HRP) were supplied by Cell Signaling Technology (MA, USA). PureLink® RNA mini kits, high capacity cDNA reverse transcription kits, and Power UpTM SYBRTM green master mix were obtained from Thermo Fisher Scientific Inc. (CA, USA). All other chemicals were purchased from Sinopharm Chemical Reagent (Beijing, China).

2.2 Extraction and Purification of PEP

PEP was extracted from fruiting bodies according to the methods reported by Jeurink et al. [21] and Zhang et al. [22] with some modifications. In brief, fresh fruiting bodies were lyophilized, ground into powder, and sieved through a No.100 mesh screen. An ice-cold 5% (v/v) acetic acid solution containing 0.1% (v/v) *β*-mercaptoethanol was added to the freeze-dried powder in a 1:15 ratio (w/v) and the mixture homogenized with stirring for 5 h at 4°C. The homogenate then was cleared by centrifugation at 15,500 x g for 15 min at 4°C. Ammonium sulfate was added to the cleared supernatant to 75% (w/v) saturation with stirring at 4°C for 12 h. The precipitant was collected by centrifugation at 25,000 x g for 20 min at 4°C, redissolved in deionized water, and dialyzed (3500 Da) for 24 h at 4 °C.

The deionized water was replaced every three hours. The dialyzed fraction then was freeze-dried generating a crude PEP powder, and approximately 18.74 g crude protein could be prepared from 100 g of fruiting body powder. The crude PEP powder was redissolved in 10 mM Tris-HCl buffer (pH 8.2) at a 1:20 (w/v) ratio and fractionated using ultra-filtration membranes (Millipore Co., Ltd.). The 10-100kDa ultrafiltrate fraction was collected and chromatographed on a DEAE-52 column. The protein fractions were eluted with a step gradient of 0.0 M, 0.1 M, 0.3 M and 0.5 M NaCl in 10 mM Tris-HCl buffer (pH 8.2). Elution of the proteins was monitored at 280 nm using a HD-3 UV detector (Shanghai, China). Subsequently, the DEAE fraction that possessed the best immunoregulatory activity (i.e. induction of NO production) was further purified over a Sephadex G75 column.

2.3 Endotoxin determination

ToxinSensor™ chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA) was used to measure the level of the endotoxin in PEP samples. Endotoxin standards, samples and Limulus amebocyte lysate (LAL) reagent water were dispensed into endotoxin-free vials, adjusting the pH at 7.0. LAL reagent was added into each vial and swirled gently, and then incubated at 37 °C for 15 min. After incubation, chromogenic substrate solution was added to each vial and incubated at 37 °C for 6 min. Stop solution, color-stabilizer #2 and #3 were added into each vial successively and mixed thoroughly. The absorbance at 545 nm was determined by microplate reader, and the concentration of endotoxin (EU/ml) of sample was calculated with the standard curve obtained by standard solution (ranging from 0.1 EU/ml to 1 EU/ml).

2.4 Homogeneity and Molecular Weight

The homogeneity and molecular weight of PEP 1b was determined by SDS-PAGE,

HPLC (1200, Agilent Technologies, Waldbronn, Germany) and MALDI-TOF-MS (5800, AB SCIEX, Framingham, USA). The HPLC was fitted with a ZORBAX 300 SB-C18 column (4.6 × 250 mm, 5μm). Samples were dissolved in 6M urea including 5% acetic acid solution, filtered through a 0.45 μm filter, and injected for HPLC analysis. HPLC analysis was run using mobile phase generated from buffer A (water and 0.05% TFA) and buffer B (water, 80% acetonitrile and 0.09% TFA), a flow rate of 1.0 ml/min, and a 60°C column temperature; linear gradient program: from 100% A to 30% A + 70% B in 40 min. Matrix of PEP 1b was prepared as a saturated solution in sinapic acid. Matrix solution (6 μl) was pipetted onto the sample target and allowed to air dry at room temperature. Then analyte solution (1μl) was pipetted onto the sample target to form a thin layer and allowed to air dry. Linear method was selected to determine the molecular weight of PEP 1b in positive ion mode for further analysis by MALDI-TOF-MS.

2.5 Macrophage Cell Culture

Murine macrophage cell line RAW 264.7 (FuDan IBS Cell Center, Shanghai, China) was cultured in DMEM including 1% penicillin, 1% streptomycin and 10% FBS at 37 °C in 5% CO_2. Cells were treated with medium for 48h and then stimulated with various concentrations (0, 50, 100, or 200 μg/ml) of PEP 1b or LPS (10 ng/ml). After 24 h of incubation (unless otherwise noted), the culture medium was collected to evaluate for NO and cytokines (TNF-α, IL-1β, IL-6 and IL-8) production and/or harvested for Western blot and qRT-PCR analysis.

2.6 Cell Viability Assay

Macrophage cell suspension (100 μl) was loaded into 96-well plates with each well containing 1×10^4 cells. After 24 h of preincubation, the medium was discarded and replaced with 100 μl of fresh medium containing various concentrations (25, 50, 75, 100,

150 150, 200 and 300 μg/ml) of PEP 1b and incubated for 48 h. During the cell viability assay
151 the medium was removed and replaced with media containing 10 μl of CCK-8 solution.
152 After a 3 h treatment the absorbance of each well was measured at 450 nm using a
153 microplate reader (Spectra MAX 190, Molecular Devices Inc., CA, U.S.A.). The
154 experiment was preformed three times with five replicates for each sample. Absorbance of
155 untreated cells was considered 100%.

156 **2.7 NO and cytokine measurements**

157 NO concentrations were quantified using a nitric oxide assay kit (Beyotime
158 Biotechnology, Shanghai, China). Briefly, cell culture supernatants (50 μl) and standards
159 of $NaNO_2$ (50 μl; 0, 1, 2, 5, 10, 20, 40, 60, 100 μM) were dispensed into individual wells
160 of a 96-well plate. Fifty microliters of Griess reagent I and Griess reagent II then were
161 dripped into each well successively. After a 3 min incubation time, the absorbance was
162 measured at 540 nm utilizing a microplate reader, and the concentrations of NO
163 determined.

164 Extracellular cytokine (TNF-α, IL-1β, IL-6 and IL-8) levels were determined using
165 ELISA kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the
166 manufacturer's instructions. In short, diluted standard (50 μl) and culture supernatant (50
167 μl) were added into 96-well plate. Then the standard-sample diluent (50 μl) and HRP
168 conjugate reagent (50 μl) were added into each well, consecutively. After 60 min
169 incubation at 37°C, the plate was washed five times and patted dry. Then 50 μl of
170 chromogenic solution A and B were added to each well and incubated for 10 min with
171 gentle shaking. The reaction was stopped through the addition of 50 μl of stop solution
172 and the absorbance measured at 450 nm. Known concentrations of TNF-α, IL-1β, IL-6 and
173 IL-8 were used to generate a standard curve for use in the assay. Determinations of NO

concentration and cytokine levels were made in quintuplicate, and the cell cultivated by regular culture medium served as the blank control.

2.8 Western Blot

Macrophage nuclear and cytoplasmic proteins were extracted according to the manufacturer's instructions (Beyotime Biotechnology, Shanghai, China). In brief, cells were incubated in lysis buffer containing 1mM PMSF for 15 min on ice. Following lysis, the mixture was centrifuged at 14,000 x g for 5 min at 4 °C. The protein concentration of lysate was determined by Bradford assay [23]. Thirty μg of the protein sample was prepared in SDS sample loading buffer, fractionated by SDS-PAGE (10% gel) and transferred onto a nitrocellulose (NC) membrane. NC-blots were blocked with blocking solution (5% nonfat dry milk in 1x TBST buffer) for 2 h at room temperature. The blocked-membranes then were incubated overnight at 4 °C with the appropriate primary antibody in 5% BSA, 1x TBST with gentle shaking. The next day the membrane was rinsed five times with 1x TBST buffer followed by a 2 h incubation with a secondary antibody prepared in 1x TBST with 5% dry milk. After this incubation, the blots were washed five times as before, treated using enhanced luminol chemiluminescence reagent (Beyotime Biotechnology, Shanghai, China). The protein bands were visualized by enhanced chemiluminescence detection system (Tanon 5200, Shanghai, China) and quantified by the imaging analyzer software of Tanon. Determinations of western blot were made in triplicate for each antibody.

2.9 Quantitative Real-Time PCR (qRT-PCR)

The mRNA expression of cytokine genes was analyzed by qRT-PCR. Total RNA was extracted according to the instruction provided in the PureLink® RNA mini kit manual. Using the total RNA as template, cDNA was prepared utilizing the high capacity cDNA

reverse transcription kits (Thermo Fisher Scientific, Waltham, USA). For qRT-PCR, 2μl of cDNA was amplified in a reaction volume of 20 μl containing 10 μl PowerUp™ SYBR™ Green master mix (Thermo Fisher Scientific, Waltham, USA), 2 μl of the appropriate primers, and 4 μl of nuclease-free water. The primer sequences for qRT-PCR amplification were: iNOS, 5'-CATGCTACTGGAGGTGGGTG-3', 5'-CATTGATCTCCG TGACAGCC-3'; TNF-α, 5'-AGCACAGAAAGCATGATCCG-3', 5'-CTGATGAGAGG GAGGCCATT-3'; IL-1β, 5'-TCGTGCTGTCGGACCCATAT-3', 5'-GTCGTTGCTTGGT TCTCCTTGT-3'; IL-6, 5'-GAGGATACCACTCCCAACAGACC-3', 5-AAGTGCATCA TCGTTGTTCATACA-3'; IL-8 5'-TTGCCTTGACCCTGAAGCCCCC-3', 5'-GGCACAT CAGGTACGATCCAGGC-3'; β-actin 5'-ATCACTATTGGCAACGAGCG-3', 5'-TCAG CAATGCCTGGGTACAT-3'. The qRT-PCR reactions were performed on 7500 Real Time PCR System (AB Applied Biosystems, MA, USA). The PCR reaction (20 μl) was carried out using the following conditions: UDG activation at 50°C for 2 min and Dual-Lock™ DNA polymerase at 95 °C for 2 min in the holding stage; denaturation at 95°C for 15 s, anneal at 60°C for 30 s, and extension at 72 °C for 1 min in the cycling stage for 40 cycles. The mean Ct of the gene was calculated from triplicate measurements and the relative mRNA levels of iNOS, TNF-α, IL-1β, IL-6 and IL-8 in each sample was normalized with the content of control gene, β-actin.

2.10 TLR signaling assay

HEK-Blue™ mTLR4 cells (InvivoGen, San Diego, CA, USA) are designed for studying the stimulation of mouse TLR4 by monitoring the activation of NF-κB. The cells were cultivated in DMEM supplemented with 10% (v/v) fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. After resuspending 1×10^5 cells in HEK-Blue™ Detection media (InvivoGen, San Diego, CA, USA), cells were treated with either PEP 1b

(50, 100 and 200 μg/ml), LPS as a positive control (10 ng/ml) or sterile endotoxin-free PBS as the negative control. After incubation at 37°C in 5% CO_2 for 16 h, secreted embryonic alkaline phosphatase (SEAP) was determined at 655 nm using a spectrophotometer.

2.11 Statistical Analysis

Results were expressed as the mean values ± standard deviation (SD) of triplicate experiments. Statistically significant values were compared using ANOVA and Dunnett's test, and *p*-values of less than 0.05 were considered statistically significant. In figures, the symbol */** represent statistically significant difference between treatment group and negative control group, and symbol #/## show the statistical significance between PEP group and LPS group. (*$p < 0.05$, **$p < 0.01$; # < 0.05, ##$p < 0.01$.)

3. RESULTS AND DISCUSSION

3.1 Purification of PEP 1b macrophage activating activity

In order to elucidate the activation mechanism of *P. eryngii* protein-mediated immunity, protein extracts were prepared by homogenizing freeze-dried *P. eryngii* fruiting body powders. After centrifugation, the proteins were concentrated by ammonium sulfate precipitation and then subjected to ultrafiltration fractionation. NO production has been widely used as a quantitative indicator of macrophage activation and has been shown to be the key factor produced by macrophages for defense against pathogen infection and tissue injury during autoimmune responses[24]. The bioactivity of three protein fractions (200 μg/ml), 0-10 kDa, 10-100 kDa and above 100 kDa, were assessed for the ability to stimulate NO production in RAW264.7 macrophages. The 10-100kDa fraction induced the highest NO production among the three fractions (data not shown), that is, the 10-100kDa fraction contained the main active protein. This bioactive fraction was further purified by

column chromatography on DEAE-52 cellulose and eluted into four fractions (Fig. 1A) and the ability of each fraction to induce NO production was determined (Fig. 1B).

The PEP 1 fraction induced the highest NO production among the four protein fractions, thereby exhibiting the highest immunomodulatory activity (Fig. 1B). This fraction was further purified by Sephadex G75 column chromatography. The elution profile of the PEP 1 fraction consisted of two peaks referred to as PEP 1a and PEP 1b (Fig. 1C). The activity of each fraction (200 μg/ml) was assessed and PEP 1b showed the highest NO-inducing activity (Fig. 1D). The purification table for PEP 1b is shown in Table 1. Thus, the primary immunomodulatory activity from *P. eryngii* fruiting bodies resides in fraction PEP 1b.

3.2 Characterization of PEP 1b

A total yield of 7.48±2.71 mg PEP 1b was separated from 100 mg of crude protein. Visual analysis of the PEP 1b fraction by SDS-PAGE revealed a single polypeptide (Fig. 2A). HPLC analysis confirmed the presence of a single purified polypeptide. (Fig. 2B) of a molecular weight 21.9 kDa as determined by MALDI-TOF-MS (Fig. 2C).

3.3 PEP 1b induces NO and iNOS expression in a dose dependent manner

To gain insight into the amount of PEP 1b required to induce NO production, RAW 264.7 cells were treated with varying concentrations of PEP 1b (50, 100, and 200 μg/mL). The control cells treated with culture media generated 2.638±0.319 μM of NO, whereas cells stimulated with 10 ng/mL of a strong macrophage activator, LPS, as a positive control, generated 29.430±0.279 μM of NO. NO production appeared to significantly increase in response to increasing doses of PEP 1b compared to the control (Fig. 3A). In

cell assays, residual LPS in the samples could give misleading results. To verify that the observed increase in NO production occurred in response to PEP 1b exposure rather than residual endotoxin, cultures containing Polymyxin B (Poly B) in addition to PEP 1b were tested (Fig. 3A). Poly B is a LPS inhibitor, which binds to the lipid A of LPS and restrains LPS effects [25]. When Poly B was added, NO production was significantly inhibited in the cells treated with LPS but had no effect on NO production in cells treated with PEP 1b (Fig. 3A). To further clarify whether any contaminated trace of endotoxins including LPS in PEP protein preparations affects NO production in our assay, the traces of endotoxins in PEP samples (200 μg/ml) were determined as shown in Table 2. A series of LPS solutions (LPS-1, LPS-2, LPS-3, LPS-4, LPS-1a and LPS-1b) were prepared, that mirrored the amount of LPS found in the PEP samples. Individual aliquots of RAW 264.7 cells were treated with each LPS sample series and the amount of induced NO was determined. NO production in cells induced by these solutions were ranged from 1.974±0.701 μM to 2.132±0.617 μM (Fig. 3B). Compared with the induction effect of PEP 1b (Fig. 3A), the induction of NO production (2.072±0.924 μM) by any of the residual LPS in the PEP 1b fraction was significantly less than that by PEP 1b (16.594±0.597 μM). The similar results were also reported in previous studies, such that the LPS contamination were not attributed to the immunomodulatory responses induced by bioactive compounds from edible fungi [26-28]. Overall, the NO-inducing activity of PEP 1b does not appear to result from endotoxin contamination.

To determine if PEP 1b induced NO was mediated through the iNOS pathway, the expression of iNOS was examined in response to PEP 1b exposure. Analysis of iNOS mRNA expression, using qRT-PCR, revealed a general trend of increasing transcript

abundance in cells exposed to increasing concentrations of PEP 1b (Fig. 3C). This increase in iNOS expression was more evident at the protein level using an immunoblot assay (Fig. 3D). With the treatment of PEP 1b, especially at 200 μg/ml of PEP 1b, mRNA and protein levels of iNOS were increased significantly compared with the control. In further support, CCK-8 assays showed that the lower concentration of PEP 1b samples implemented in the assay had little cytotoxic effects on RAW 264.7 cells and the higher concentration of PEP 1b did have some cytotoxicity to RAW 264.7 cells. However, it did not reach the IC_{50} level, and thus, would not be expected to cause severe toxicity (Fig. 3E), which indicated that the treatment concentrations chosen in the study were suitable for Raw 264.7 cells. Taken together, these findings suggest that PEP 1b induces NO production and that this induction occurs through an enhancement in iNOS expression rather than through some cytotoxicity response pathway.

3.4 PEP 1b induces secretion of selective cytokines

Activated macrophages develops immunomodulatory effects by secreting pro-inflammatory cytokines, such as interleukins (IL-1β, IL-6, IL-8, IL-12, etc.) and TNF-α, assisting the eliminations of abnormal cells and exogenous pathogens [29]. In non-inflammatory models, the levels of cytokines in macrophages act as an index to evaluate the activator's capacity of stimulating immune response [25]. Previous reports have revealed that edible fungi proteins were capable of stimulating the cytokines involved in human or murine immune cells in *vitro*, such as, TNF-α, IL-5, IL-8, IL-10 and IL-12 p40 [30].

To probe the influence of PEP 1b on cytokines secretion, RAW 264.7 cells were cultured with medium that contained different concentrations of PEP 1b (0, 50, 100 and 200 μg/ml). LPS treated cells were utilized as a positive control (10 ng/ml). Cell culture

supernatants were collected to measure the production of IL-1β, IL-6, IL-8, and TNF-α. The concentrations of IL-1β, IL-6, and TNF-α were increased with treatment of PEP 1b in a dose dependent manner (Fig. 4A, B, and D), and were significantly higher compared with control (cell medium treatment). No significant changes in IL-8 concentrations were measured under the tested PEP 1b doses (Fig. 4C). In addition, qRT-PCR analysis revealed that the transcript abundance profile for each cytokine (Fig. 5A-D) mirrored their respective cytokine abundance profile (Fig. 4A-D) in response to the different PEP 1b doses. Since the cytokines IL-1β, IL-6, and TNF-α are typically produced by M1 macrophage polarization [16] while IL-8 is secreted during M2 macrophage polarization [18] our results suggest that PEP 1b is an activator of M1 macrophage polarization.

3.5 PEP 1b induces activation of NF-κB signaling pathway

It has been shown that M1 macrophage polarization can occur through the activation of NF-κB signaling pathway. In this pathway, the NF-κB transcription factor plays a critical role in regulating the genes involved in innate immune and inflammatory responses. Such responses often result in the production of TNF-α, IL-12, NO, and ROS in macrophages[31]. Normally, NF-κB binds to IκB in the cytoplasm, which inhibits the translocation of NF-κB to the nucleus. Once the process of NF-κB signaling has been activated, IKK phosphorylates IκB resulting in the release NF-κB. Once released, NF-κB is free to translocate to the nucleus where it can induce transcription of target genes and induce the production of pro-inflammatory mediators [32]. In our previous study, a 40 kDa PEP protein exerted the anti-inflammatory bioactivity through inhibiting NF-κB signaling in LPS-stimulated RAW 264.7 cells [5].

To determine if this pathway is responsible for PEP 1b-induced macrophage activation, immunoblot analysis was utilized to assess the phosphorylation state of both

IKK and IκB-α. As shown in Fig. 6A and 6B, PEP 1b significantly induced both the phosphorylation of IKK and IκB-α. To verify the nuclear translocation of the NF-κB, the intracellular location of p65 subunit was monitored by immunoblot assay. Cellular fractionation of PEP 1b induced RAW264.7 cells showed increasing concentrations of p65 within the nuclear fraction in response to increasing PEP 1b concentrations (Fig. 6C). A corresponding decrease in p65 was measured within the cytoplasmic compartment utilizing this same set of PEP 1b treated samples (Fig. 6C). To provide further support of PEP 1b-activated NF-κB pathway in M1-polarization, RAW264.7 macrophages were pretreated with the NF-κB inhibitors, SC75741 and EVP4593, and their effect on NO production assessed. As shown in Fig. 6D, each inhibitor resulted in a significant decrease in the amount of NO produced by PEP 1b-induced RAW264.7 cells. Overall, these findings suggest that the PEP 1b induces NO and cytokine production through activation of the NF-κB signaling pathway.

3.6 PEP 1b induces activation of MAPK signaling pathway

In addition to the NF-κB transcription factor, MAP kinases (JNK, p38 and ERK) can also impact the production of cytokines and NO. The phosphorylation of MAPK is a prerequisite for NO and cytokine production in stimulated macrophages [33, 34]. Crosstalk can occur between the different signaling pathways leading to M1-macrophage polarization [35]. As shown in Fig. 7A~C, JNK3 and ERK1/2 were phosphorylated in RAW 264.7 cells in response to PEP 1b exposure in a dose-dependent manner. In contrast, p38 did not show a significant change in phosphorylation with the addition of PEP 1b. To verify the involvement of the MAPK signaling pathway in PEP 1b-dependent induction of NO, MAPK inhibitors SP600125, SB203580, and PD98059 were used to block the phosphorylation of JNK3, p38 and ERK1/2, respectively. As illustrated in Fig 7D, NO

production significantly decreased in PEP 1b-treated RAW264.7 cells in the presence of SP600125 and PD98059 but not SB203580. These findings demonstrate that the MAPK JNK3 and ERK1/2 are also involved in the production of NO in PEP 1b-induced RAW264.7 cells.

3.7 PEP 1b activity requires TLR4 receptors

Studies have shown, in some cases, that immunomodulators are capable of stimulating the innate immune system through pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) [36, 37]. A growing number of studies have shown that TLR2 and TLR4 recognize several ligand molecules, in addition to LPS and lipopeptide [31, 38, 39]. To determine if PEP 1b-induced macrophage activation utilizes toll-like receptors, RAW264.7 cells were pretreated with anti-TLR2 and anti-TLR4 antibodies to neutralize each receptor before exposure to PEP 1b. The typical ligands of TLR2 and TLR4, peptidoglycan (PGN) and LPS, were used as positive controls. As showed in Fig. 8A, PEP 1b-induced NO production was significantly reduced in cells pretreated with anti-TLR4, while no significant reduction was detected in cells pretreated with anti-TLR2. Moreover, TLR4 signaling assay (Fig. 8B) revealed that the levels of SEAP secreted by HEK-Blue™ mTLR4 cells were significantly increased after the cells treated with PEP 1b (100 μg/ml and 200 μg/ml) in a dose dependent manner. The results indicate that PEP 1b is capable of activating TLR4 signaling and sequentially acts on NF-κB. TLR4 mediated intracellular signaling pathways activate NF-κB p65, ultimately resulting in the production of cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-12, TNF-α, IFN-γ and iNOS, thereby initiating and regulating innate and adaptive immunity [40, 41]. Our findings support a role for PEP 1b in TLR4-induced macrophage activation and related immunomodulatory effect.

In this study, a novel *P. eryngii* protein, PEP 1b, was identified, purified, and found to be capable of inducing activation and M1-polarization of macrophages. M1-polarization of macrophages is an essential step in the activation of the immune system in animals. In addition, it was determined that this induction of macrophages occurred via the TLR4-NF-κB and MAPK signaling pathways. In recent years, animal studies were used gradually to demonstrate the biological functions of immunostimulatory proteins from edible fungi. Tsao et al. illuminated the immunostimulatory properties and antitumor effects of a novel protein from *Grifola frondosa* by model of tumor bearing mice [42]. Moreover, Zhou et al. reported the immunoregulatory effect of a recombinant *Ganoderma lucidum* immunoregulatory protein on mouse models of cyclophosphamide-induced leukopenia, which increased the number of neutrophils, lymphocytes, monocytes and the percentage of $CD4^+$ T cells and the levels of secreted IL-3 and IL-4, contributing to the cyclophosphamide-induced immune dysfunction and immune system imbalance [43]. In this case, it is feasible to explore the potentials biological function of PEP 1b in animal study in the follow-up study.

It is our hope that the discovery of such new bioactive proteins, coupled with knowledge into their mechanism(s) of function, will lead to the development of new functional foods and/or pharmaceutical products that find use in the promotion of optimal human health and well-being.

ABBREVIATIONS UESD

CCK-8, Cell counting kit-8; **ERK1/2**, Extracellular regulated protein kinases; **IKK**, Inhibitor of nuclear factor kappa B kinase; **IκB**, inhibitor of NF-κB; **iNOS**, Inducible nitrogen oxide synthase; **JNK**, Jun N-terminal kinase; **LPS**, Lipopolysaccharides; **MAPK**, Mitogen-activated protein kinase; **NF-κB**, Nuclear factor kappa B; **NO**, Nitric oxide;

PARP, Poly ADP-ribose polymerase; **PEP**, *Pleurotus eryngii* protein; **PGN**, Peptidoglycan; **SEAP**, Secreted embryonic alkaline phosphatase; **TLR**, Toll like receptor; **TNF-α**, Tumor necrosis factor alpha.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. R. Shenbhagaraman, L. K. Jagadish, K. Premalatha and V. Kaviyarasan, Optimization of extracellular glucan production from Pleurotus eryngii and its impact on angiogenesis, *International journal of biological macromolecules*, 2012, **50**, 957-964.
2. J. Chen, D. Mao, Y. Yong, J. Li, H. Wei and L. Lu, Hepatoprotective and hypolipidemic effects of water-soluble polysaccharidic extract of Pleurotus eryngii, *Food Chemistry*, 2012, **130**, 687-694.
3. A. M. Mariga, F. Pei, W. J. Yang, L. Y. Zhao, Y. N. Shao, D. K. Mugambi and Q. H. Hu, Immunopotentiation of Pleurotus eryngii (DC. ex Fr.) Quel, *Journal of ethnopharmacology*, 2014, **153**, 604-614.
4. A. Zhang, X. Li, C. Xing, J. Yang and P. Sun, Antioxidant activity of polysaccharide extracted from Pleurotus eryngii using response surface methodology, *International journal of biological macromolecules*, 2014, **65**, 28-32.

5. B. Yuan, L. Zhao, K. Rakariyatham, Y. Han, Z. Gao, B. M. Kimatu, Q. Hu and H. Xiao, Isolation of a novel bioactive protein from an edible mushroom Pleurotus eryngii and its anti-inflammatory potential, *Food & Function*, 2017, **8**, 2175-2183.
6. Z. Yang, J. Xu, Q. Fu, X. Fu, T. Shu, Y. Bi and B. Song, Antitumor activity of a polysaccharide from Pleurotus eryngii on mice bearing renal cancer, *Carbohydrate polymers*, 2013, **95**, 615-620.
7. X. Meng, H. Liang and L. Luo, Antitumor polysaccharides from mushrooms: a review on the structural characteristics, antitumor mechanisms and immunomodulating activities, *Carbohydrate research*, 2016, **424**, 30-41.
8. A. C. Ruthes, F. R. Smiderle and M. Iacomini, Mushroom heteropolysaccharides: A review on their sources, structure and biological effects, *Carbohydrate polymers*, 2016, **136**, 358-375.
9. A. Zong, H. Cao and F. Wang, Anticancer polysaccharides from natural resources: A review of recent research, *Carbohydrate polymers*, 2012, **90**, 1395-1410.
10. X. Xu, H. Yan, J. Chen and X. Zhang, Bioactive proteins from mushrooms, *Biotechnology advances*, 2011, **29**, 667-674.
11. S. P. Wasser, Medicinal mushroom science: history, current status, future trends, and unsolved problems, *International Journal of Medicinal Mushrooms*, 2010, **12**.
12. J. H. Wong, T. B. Ng, R. C. Cheung, X. J. Ye, H. X. Wang, S. K. Lam, P. Lin, Y. S. Chan, E. F. Fang, P. H. Ngai, L. X. Xia, X. Y. Ye, Y. Jiang and F. Liu, Proteins with antifungal properties and other medicinal applications from plants and mushrooms, *Applied microbiology and biotechnology*, 2010, **87**, 1221-1235.
13. G. A. Duque and A. Descoteaux, Macrophage cytokines: involvement in immunity and infectious diseases, *Frontiers in immunology*, 2014, **5**.

14. C. Liu, J. Chen, L. Chen, X. Huang and P. C. Cheung, Immunomodulatory Activity of Polysaccharide–Protein Complex from the Mushroom Sclerotia of Polyporus rhinocerus in Murine Macrophages, *Journal of agricultural and food chemistry*, 2016, **64**, 3206-3214.
15. N. Wang, H. Liang and K. Zen, Molecular mechanisms that influence the macrophage m1–m2 polarization balance, *Frontiers in immunology*, 2014, **5**.
16. H. Qin, A. T. Holdbrooks, Y. Liu, S. L. Reynolds, L. L. Yanagisawa and E. N. Benveniste, SOCS3 deficiency promotes M1 macrophage polarization and inflammation, *Journal of immunology*, 2012, **189**, 3439-3448.
17. L. Van Tits, R. Stienstra, P. Van Lent, M. Netea, L. Joosten and A. Stalenhoef, Oxidized LDL enhances pro-inflammatory responses of alternatively activated M2 macrophages: a crucial role for Krüppel-like factor 2, *Atherosclerosis*, 2011, **214**, 345-349.
18. A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi and M. Locati, The chemokine system in diverse forms of macrophage activation and polarization, *Trends in immunology*, 2004, **25**, 677-686.
19. A. M. Mariga, W. J. Yang, D. K. Mugambi, F. Pei, L. Y. Zhao, Y. N. Shao and Q. Hu, Antiproliferative and immunostimulatory activity of a protein from Pleurotus eryngii, *Journal of the science of food and agriculture*, 2014, **94**, 3152-3162.
20. B. Yuan, N. Ma, L. Zhao, E. Zhao, Z. Gao, W. Wang, M. Song, G. Zhang, Q. Hu and H. Xiao, In vitro and in vivo inhibitory effects of a Pleurotus eryngii protein on colon cancer cells, *Food & function*, 2017, **8**, 3553-3562.
21. P. V. Jeurink, C. L. Noguera, H. F. Savelkoul and H. J. Wichers, Immunomodulatory capacity of fungal proteins on the cytokine production of human peripheral blood

mononuclear cells, *International immunopharmacology*, 2008, **8**, 1124-1133.

22. Y. Zhang, Z. Liu, T. B. Ng, Z. Chen, W. Qiao and F. Liu, Purification and characterization of a novel antitumor protein with antioxidant and deoxyribonuclease activity from edible mushroom Pholiota nameko, *Biochimie*, 2014, **99**, 28-37.
23. N. J. Kruger, The Bradford method for protein quantitation, *The protein protocols handbook*, 2009, 17-24.
24. T. Teruya, S. Takeda, Y. Tamaki and M. Tako, Fucoidan isolated from Laminaria angustata var. longissima induced macrophage activation, *Bioscience, biotechnology, and biochemistry*, 2010, **74**, 1960-1962.
25. K.-L. Cheong, L.-Z. Meng, X.-Q. Chen, L.-Y. Wang, D.-T. Wu, J. Zhao and S.-P. Li, Structural elucidation, chain conformation and immuno-modulatory activity of glucogalactomannan from cultured Cordyceps sinensis fungus UM01, *Journal of Functional Foods*, 2016, **25**, 174-185.
26. V. C. Castro-Alves, D. Gomes, N. Menolli, M. L. Sforça and J. R. O. do Nascimento, Characterization and immunomodulatory effects of glucans from Pleurotus albidus, a promising species of mushroom for farming and biomass production, *International journal of biological macromolecules*, 2017, **95**, 215-223.
27. S. Bastiaan-Net, W. Chanput, A. Hertz, R. D. Zwittink, J. J. Mes and H. J. Wichers, Biochemical and functional characterization of recombinant fungal immunomodulatory proteins (rFIPs), *International immunopharmacology*, 2013, **15**, 167-175.
28. Y.-J. Chen, P.-C. Cheng, C.-N. Lin, H.-F. Liao, Y.-Y. Chen, C.-C. Chen and K.-M. Lee, Polysaccharides from Antrodia camphorata mycelia extracts possess immunomodulatory activity and inhibits infection of Schistosoma mansoni,

International immunopharmacology, 2008, **8**, 458-467.

29. H. Luan, Q. Zhang, L. Wang, C. Wang, M. Zhang, X. Xu, H. Zhou, X. a. Li, Q. Xu and F. He, OM85-BV induced the productions of IL-1β, IL-6, and TNF-α via TLR4-and TLR2-mediated ERK1/2/NF-κB pathway in RAW264. 7 cells, *Journal of Interferon & Cytokine Research*, 2014, **34**, 526-536.
30. X. Xu, H. Yan, J. Chen and X. Zhang, Bioactive proteins from mushrooms, *Biotechnology advances*, 2011, **29**, 667-674.
31. C. L. Hung, A. J. Chang, X. K. Kuo and F. Sheu, Molecular cloning and function characterization of a new macrophage-activating protein from Tremella fuciformis, *Journal of agricultural and food chemistry*, 2014, **62**, 1526-1535.
32. Y. L. Lin, Y. C. Liang, Y. S. Tseng, H. Y. Huang, S. Y. Chou, R. S. Hseu, C. T. Huang and B. L. Chiang, An immunomodulatory protein, Ling Zhi-8, induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and MAPK pathways, *Journal of leukocyte biology*, 2009, **86**, 877-889.
33. D.-J. Yang, J.-T. Lin, Y.-C. Chen, S.-C. Liu, F.-J. Lu, T.-J. Chang, M. Wang, H.-W. Lin and Y.-Y. Chang, Suppressive effect of carotenoid extract of Dunaliella salina alga on production of LPS-stimulated pro-inflammatory mediators in RAW264. 7 cells via NF-κB and JNK inactivation, *Journal of Functional Foods*, 2013, **5**, 607-615.
34. Y. Liu, E. G. Shepherd and L. D. Nelin, MAPK phosphatases—regulating the immune response, *Nature Reviews Immunology*, 2007, **7**, 202-212.
35. D. Zhou, C. Huang, Z. Lin, S. Zhan, L. Kong, C. Fang and J. Li, Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways, *Cellular signalling*, 2014, **26**, 192-197.
36. T. Kawai and S. Akira, The role of pattern-recognition receptors in innate immunity:

update on Toll-like receptors, *Nature immunology*, 2010, **11**, 373-384.

37. L. A. O'neill, D. Golenbock and A. G. Bowie, The history of Toll-like receptors [mdash] redefining innate immunity, *Nature Reviews Immunology*, 2013, **13**, 453-460.
38. H. Kumar, T. Kawai and S. Akira, Toll-like receptors and innate immunity, *Biochemical and biophysical research communications*, 2009, **388**, 621-625.
39. M. R. Dasu, S. Devaraj, S. Park and I. Jialal, Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects, *Diabetes care*, 2010, **33**, 861-868.
40. S. Akira, K. Takeda and T. Kaisho, Toll-like receptors: critical proteins linking innate and acquired immunity, *Nature immunology*, 2001, **2**.
41. Y. Liu, H. Yin, M. Zhao and Q. Lu, TLR2 and TLR4 in autoimmune diseases: a comprehensive review, *Clinical reviews in allergy & immunology*, 2014, **47**, 136-147.
42. Y. W. Tsao, Y. C. Kuan, J. L. Wang and F. Sheu, Characterization of a novel maitake (Grifola frondosa) protein that activates natural killer and dendritic cells and enhances antitumor immunity in mice, *Journal of Agricultural & Food Chemistry*, 2013, **61**, 9828.
43. H. Zhou, F. Sun, H. Li, S. Zhang, Z. Liu, J. Pei and C. Liang, Effect of recombinant Ganoderma lucidum immunoregulatory protein on cyclophosphamide-induced leukopenia in mice, *Immunopharmacol Immunotoxicol*, 2013, **35**, 426-433.

Figure captions:

Figure 1. Purification of PEP 1b macrophage activating activity. (A) DEAE-52 fractionation of PEPs; (B) Induction of NO production (macrophage activating activity) of RAW 264.7 cells in response to PEP fractions, and in the culture medium without treatment of PEP proteins served as a control (the concentration of each PEP fraction was 100 μg/ml). (C) Sephadex G75 fractionation of PEP 1; (D) Induction of NO production (macrophage activating activity) in response to PEP 1a and 1b fractions (the concentration of each PEP 1 fraction was 200 μg/ml). ^{a,b,c,d} Values not sharing a common superscript letter differ significantly at $p < 0.05$.

Figure 2. Characterization of PEP 1b. (A) SDS-PAGE of the purified PEP 1b fraction stained with Coomassie blue; (B) HPLC analysis of PEP 1b fraction; (C) MALDI TOF-TOF analysis of PEP 1b.

Figure 3. PEP 1b induces NO and iNOS expression in a dose-dependent manner. (A) NO production by cells pre-treated with either cell culture media (negative control) or polymyxin B (10 μM) before stimulated with increasing concentrations of PEP 1b. LPS served as a positive control; (B) NO production by cells treated with a series of LPS solutions in the same concentration as the LPS level detected in PEP samples; (C) qRT-PCR analysis of iNOS transcript levels in cells exposed to increasing concentrations of PEP 1b. LPS served as a positive control. (D) Western-blot analysis of iNOS protein levels in cells exposed to increasing concentrations of PEP 1b. LPS served as a positive control and β-Actin as an internal loading control. (E) CCK-8 assay to assess cytotoxic effects of increasing concentrations of the PEP 1b fraction. Results are showed as the means ± SDs of three independent experiments done in triplicate. */** represent

574 statistically significant difference between treatment group and negative control group,
575 and #/## represent statistical significance between PEP group and LPS group (* $p < 0.05$,
576 ** $p < 0.01$; # $p < 0.05$, ## $p < 0.01$). [a,b] Values not sharing a common superscript letter
577 differ significantly at $p < 0.05$.

578 Figure 4. PEP 1b induces secretion of selective cytokines. (A) Secreted IL-1β
579 concentrations in response to cellular exposure to increasing PEP 1b concentrations; (B)
580 Secreted IL-6 concentrations in response to cellular exposure to increasing PEP 1b
581 concentrations (C) Secreted IL-8 concentrations in response to cellular exposure to
582 increasing PEP 1b concentrations and (D) Secreted TNF-α concentrations in response to
583 cellular exposure to increasing PEP 1b concentrations. LPS served as a positive control.
584 Results are showed as the means ± SDs of three independent experiments with five
585 replicates per sample. */** represent statistically significant difference between treatment
586 group and negative control group, and #/## represent statistical significance between PEP
587 group and LPS group (* $p < 0.05$, ** $p < 0.01$; # $p < 0.05$, ## $p < 0.01$).

588 Figure 5. PEP 1b induces expression of selective cytokine transcripts. (A) IL-1β transcript
589 abundance in response to cellular exposure to increasing PEP 1b concentrations; (B) IL-6
590 transcript abundance in response to cellular exposure to increasing PEP 1b concentrations;
591 (C) IL-8 transcript abundance in response to cellular exposure to increasing PEP 1b
592 concentrations; and (D) TNF-α transcript abundance in response to cellular exposure to
593 increasing PEP 1b concentrations. LPS served as a positive control. Results are presented
594 as the means ± SDs of three independent experiments. */** represent statistically
595 significant difference between treatment group and negative control group, and #/##

represent statistical significance between PEP group and LPS group (* $p < 0.05$, ** $p < 0.01$; # $p < 0.05$, ## $p < 0.01$).

Figure 6. PEP 1b activation of the NF-κB signaling pathway. (A) Immunoblot analysis to assess the native and phosphorylated state of IKK in response to increasing concentrations of PEP 1b. LPS served as a positive control and β-actin as an internal loading control. (B) Immunoblot analysis to assess the native and phosphorylated state of IκB-α in response to increasing concentrations of PEP 1b. LPS served as a positive control and β-actin as an internal loading control. (C) Immunoblot analysis to assess the changes in the intracellular localization of p65 in response to increasing concentrations of PEP 1b. LPS served as a positive control while β-actin and PARP as an internal loading controls. C, cytoplasm; N, nuclear. (D) NO production in response to PEP 1b in cells pretreated with inhibitors of NF-κB activation. LPS served as a positive control. Results are presented as the means ± SDs of three independent experiments. */** represent statistically significant difference between treatment group and negative control group, and #/## represent statistical significance between PEP group and LPS group (* $p < 0.05$, ** $p < 0.01$; # $p < 0.05$, ## $p < 0.01$).

Figure 7. PEP 1b activation of MAPK signaling pathway. (A-C) Immunoblot analysis to assess the native and phosphorylated state of JNK (A), p38 (B), and ERK (C) in response to increasing concentrations of PEP 1b. β-actin served as an internal load control; (D) Induction of NO production in cells pretreated with inhibitors of MAPK (SP600125, SB203580, and PD98059) activation in response to PEP 1b. LPS served as a positive control. Results are presented as the means ± SDs of three independent experiments. */** represent statistically significant difference between treatment group and negative control

group, and #/## represent statistical significance between PEP group and LPS group (* $p <$ 0.05, ** $p <$0.01; # $p <$ 0.05, ## $p <$0.01).

Figure 8. PEP 1b recognition by TLR4. (A) Induction of NO production in cells pretreated with antibodies directed toward TLR2 or TLR4. PGN and LPS served as positive controls for TLR2 and TLR4, respectively. (B) HEK-Blue™ mTLR4 cells were stimulated with a series of PEP 1b solutions. LPS treatment (10 ng/ml) served as a positive control and sterile, endotoxin-free PBS treatment was utilized as a negative control. Results are presented as the means ± SDs of three independent experiments. */** represent statistically significant difference between treatment group and negative control group, and #/## represent statistical significance between PEP group and LPS group (* $p <$ 0.05, ** $p <$0.01; # $p <$ 0.05, ## $p <$0.01).

630 Table 1 Purification table of PEP 1b summarizing the protein recovery at each step in the
631 isolation procedure.

Steps	Total Protein (mg)	Total activity * (U)	Specific activity (U/mg)	Yield （%）	Purification fold
Crude	50.00±0.12	8.78±1.24	0.18	100	1
Ultrafiltration	14.70±1.43	7.71±0.82	0.52	87.81	2.89
DEAE-52	5.26±0.87	7.18±1.53	1.37	81.78	7.61
Sephadex G75	3.74±0.41	6.94±0.77	1.86	79.04	10.33

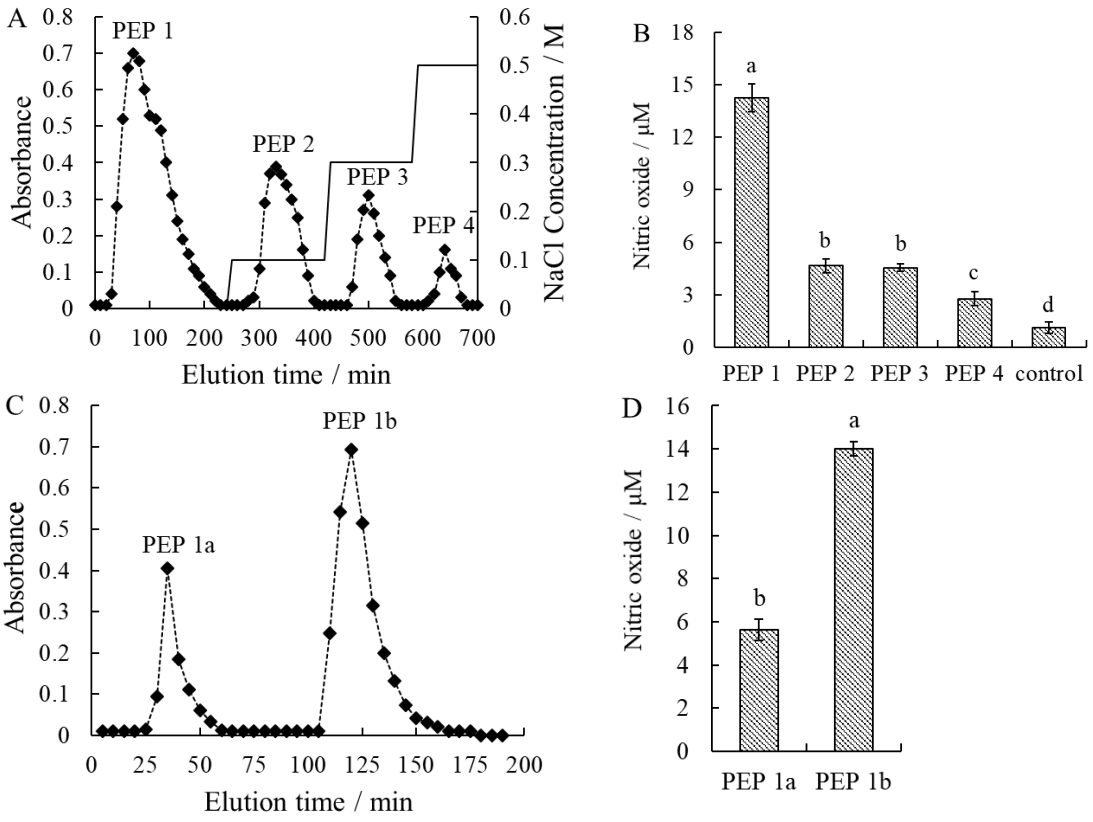
632 * Total activity (μM/h) is defined as production of NO (μM) per unit time (hour).

633 Table 2 Endotoxin content in PEP fractions

Endotoxin concentration	PEP 1	PEP 2	PEP 3	PEP 4	PEP 1a	PEP 1b
EU/ml	0.475±0.058	0.540±0.078	0.649±0.043	0.614±0.038	0.530±0.074	0.570±0.085

634

635 Figure 1



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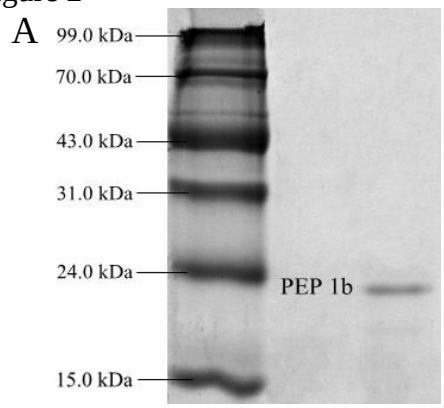
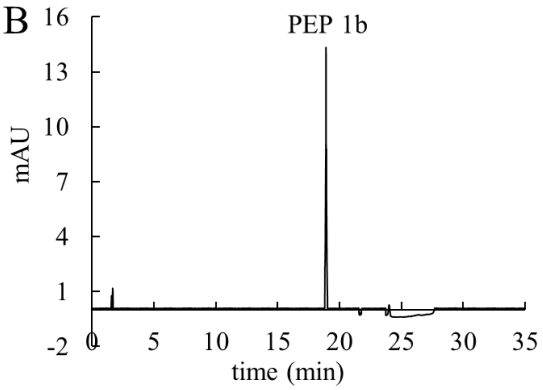
638 Figure 2





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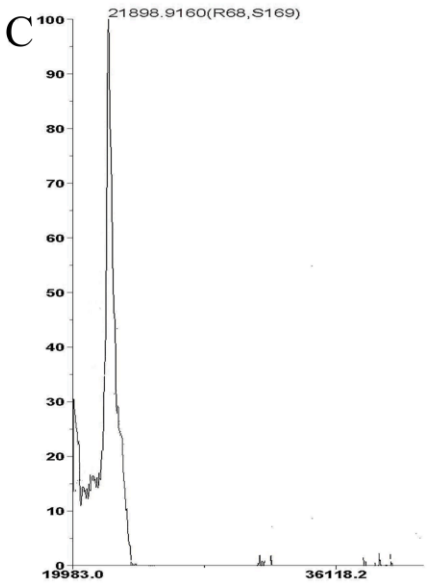

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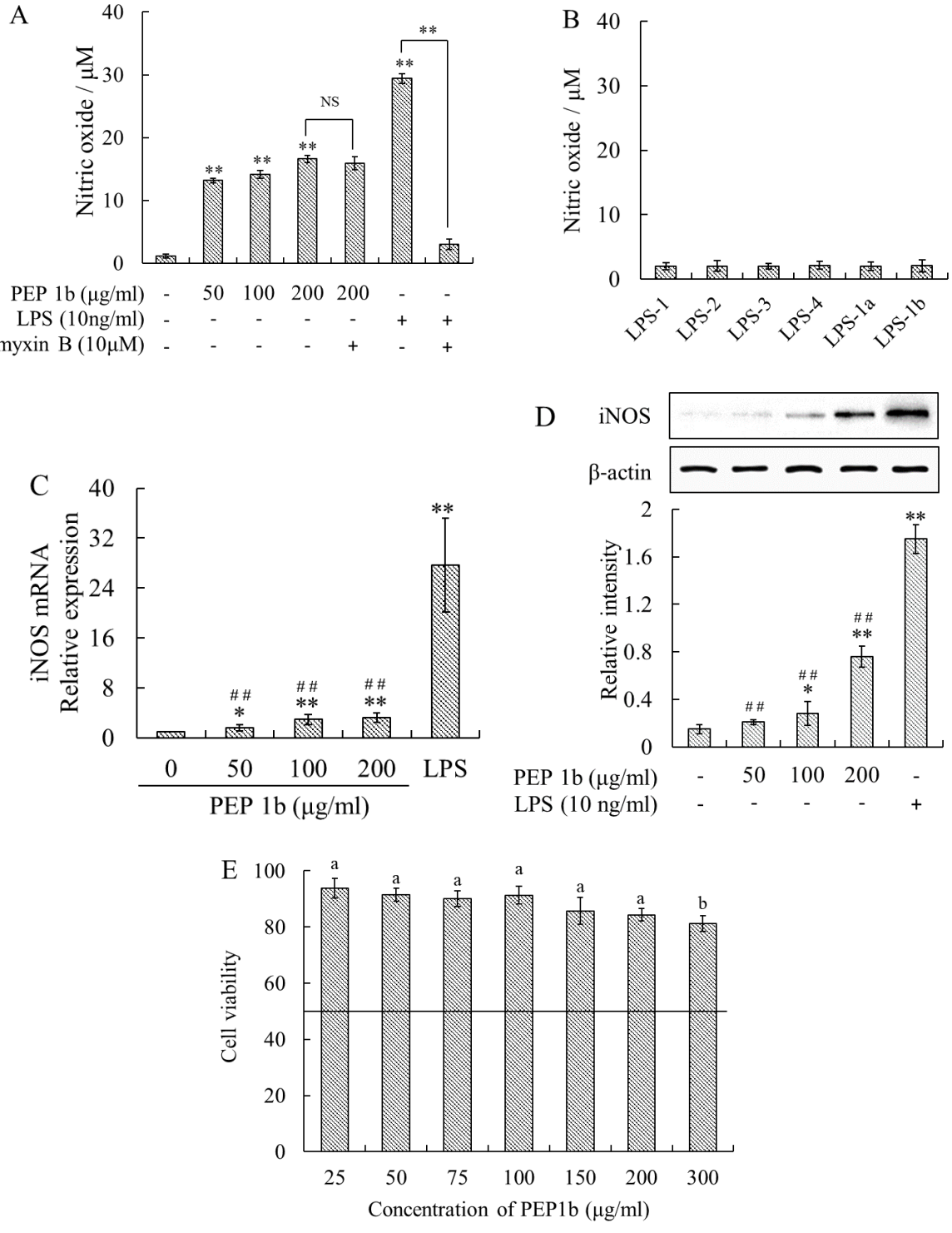
641 Figure 3



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645 Figure 4

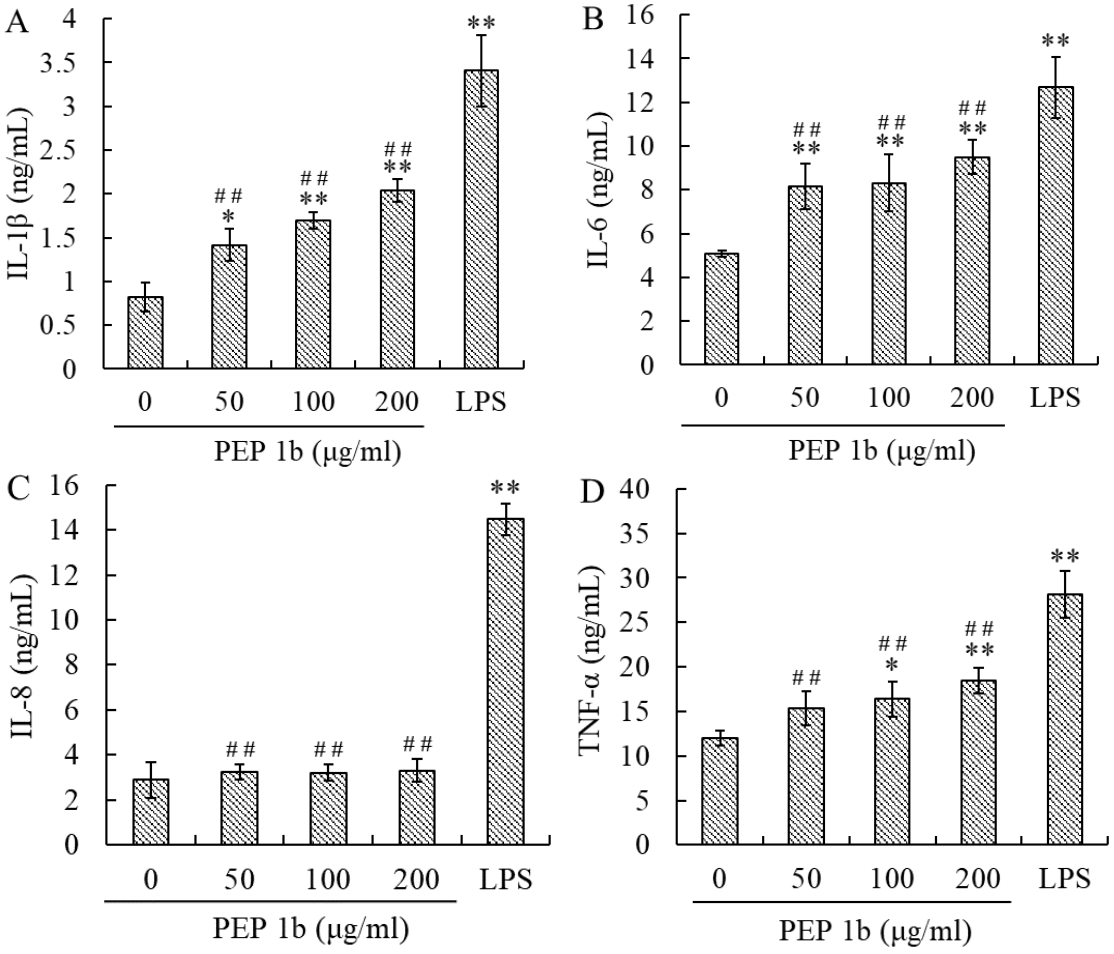

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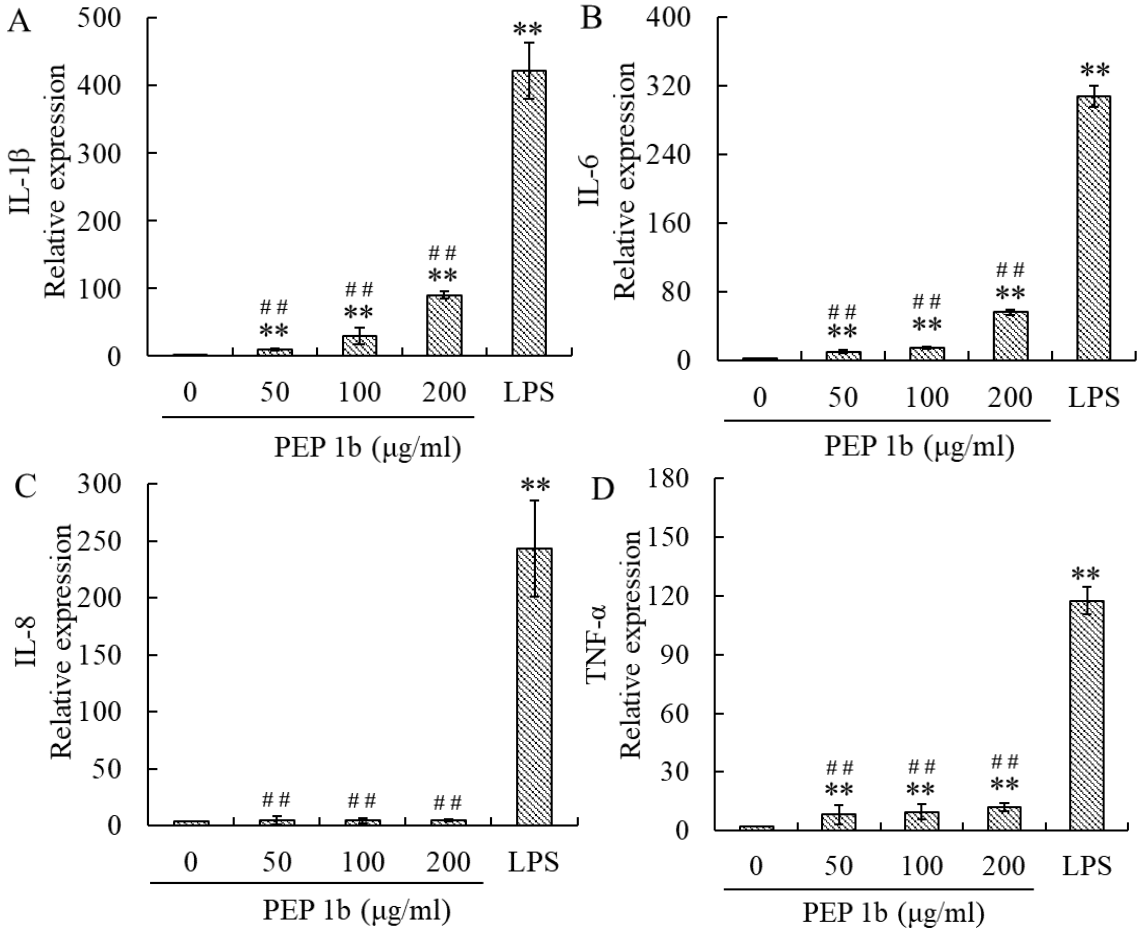
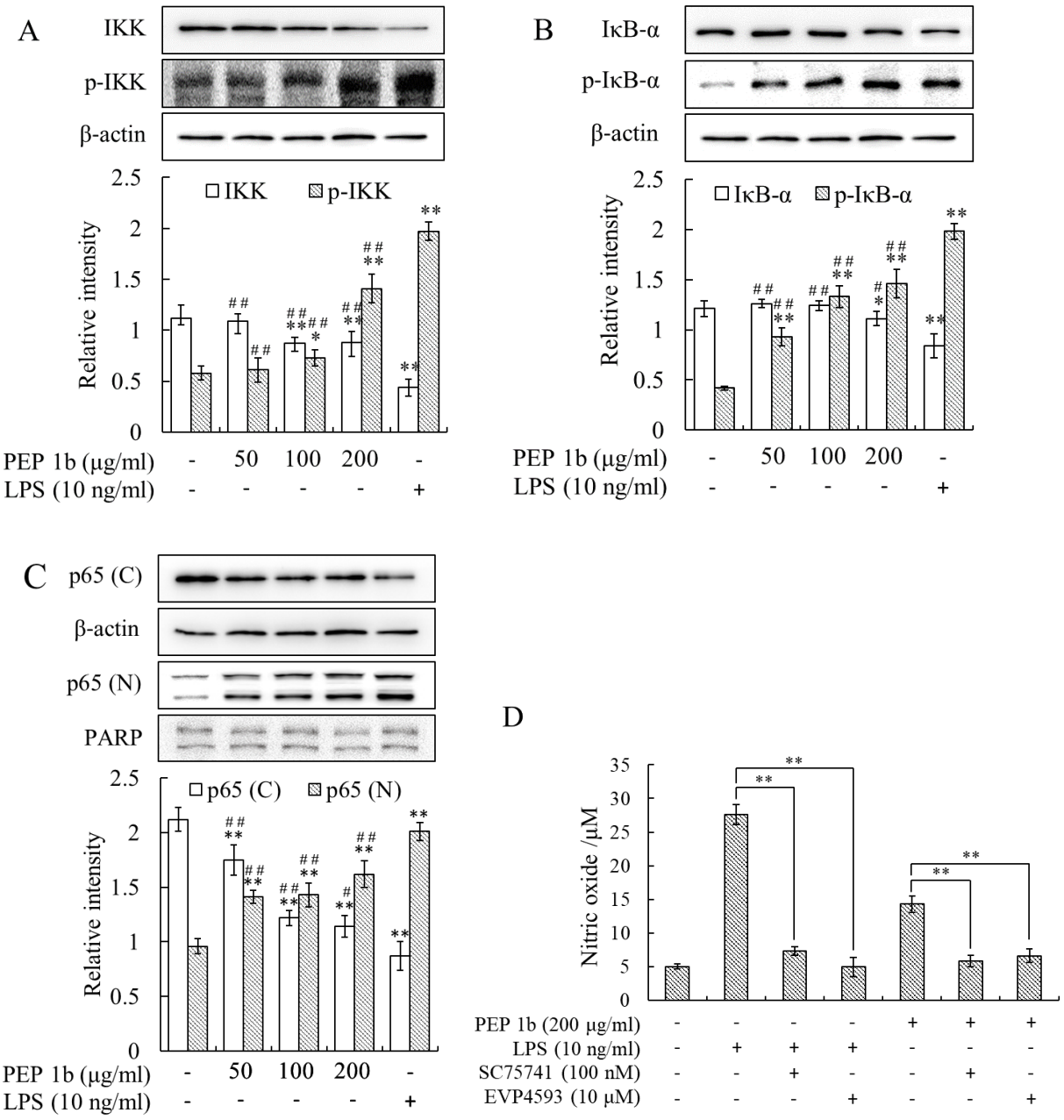
Figure 5


A
IL-1β
Relative expression
500
400
300
200
100
0
B
IL-6
Relative expression
400
320
240
160
80
0
C
IL-8
Relative expression
300
250
200
150
100
50
0
D
TNF-α
Relative expression
180
150
120
90
60
30
0
0
50
100
200
LPS
PEP 1b (μg/ml)
**
##

651 Figure 6



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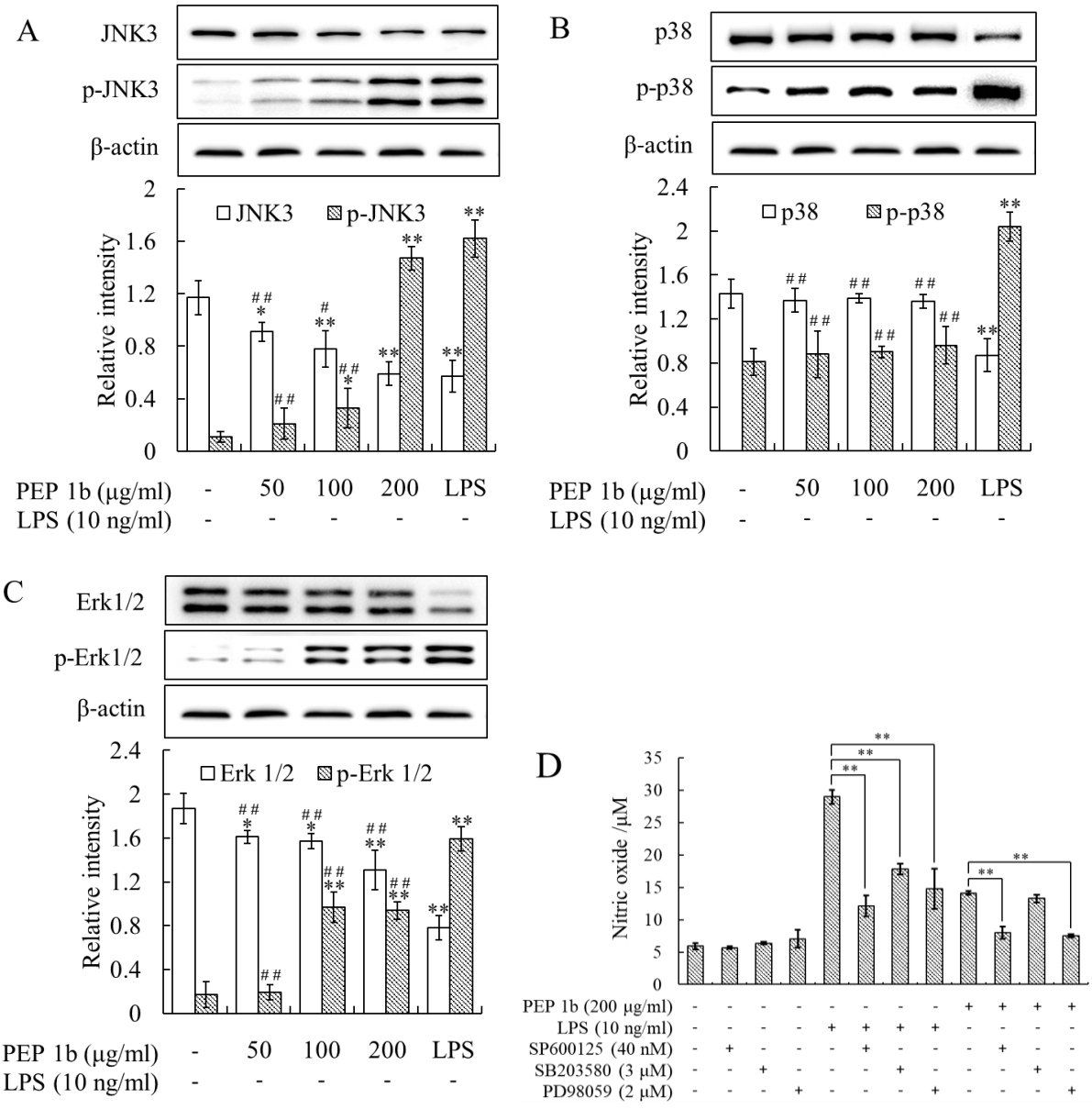
654 Figure 7



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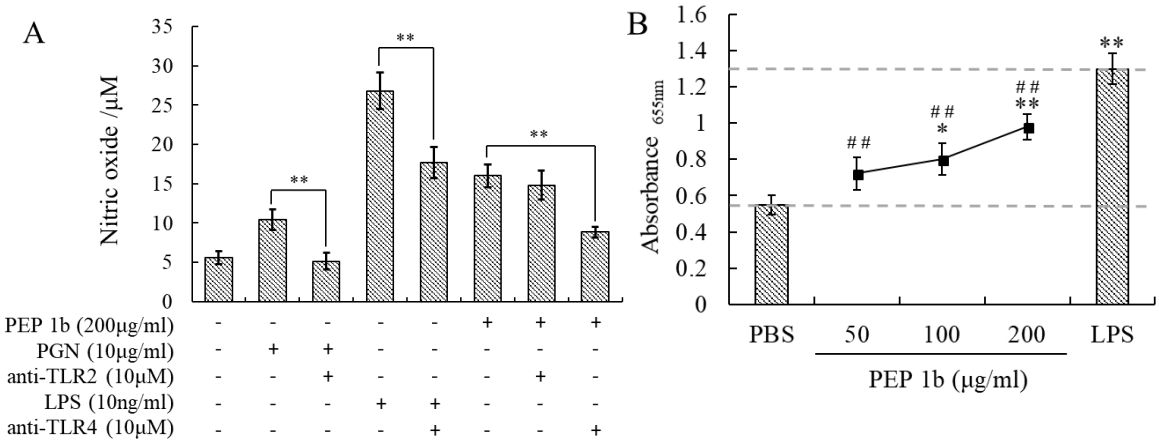
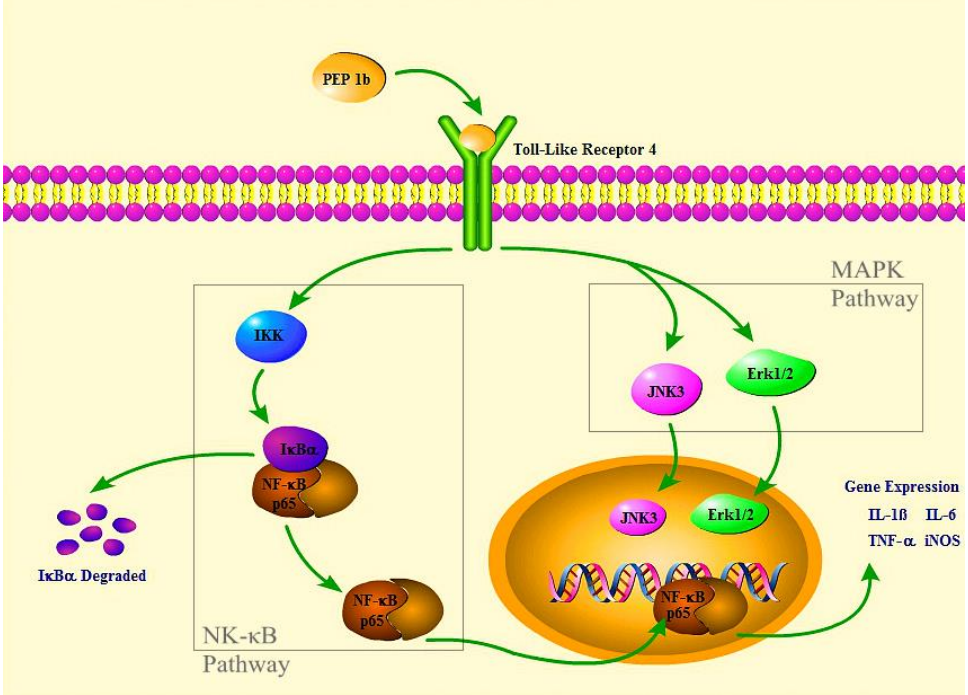
657 Figure 8



658

659 **Image of graphical abstract**



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