Phellinus linteus Extract Regulates Macrophage Polarization in Human THP-1 Cells

Sang-Yull Lee2†, Sul-Gi Park†, Sun-Nyoung Yu1, Ji-Won Kim1, You-Lim Hwang1, Dong-Seob Kim3 and Soon-Cheol Ahn1*†

1Department of Microbiology & Immunology, Pusan National University School of Medicine, Yangsan 50612, Korea
2Department of Biochemistry, Pusan National University School of Medicine, Yangsan 50612, Korea
3Department of Food Science & Technology, College of National Resources & Life Science, Pusan National University, Milyang 50463, Korea

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Macrophages are initiators for regulating a host’s defenses to eliminate pathogens and trigger tissue repair. Macrophages are classified into two types: classically (M1) activated macrophages and alternatively (M2) activated macrophages. M1-phenotype macrophages directly or indirectly kill infectious organisms and tumor cells via pro-inflammatory responses, whereas M2-phenotype macrophages remodel wounded tissue through anti-inflammatory responses. In this paper, we investigated how Phellinus linteus hot water extract passed from Diaion HP-20 resin (PLEP) regulates polarization of M1-like or M2-like macrophages in human THP-1 cells. PLEP did not have cytotoxicity at a high concentration of 300 μg/ml. We observed morphological alteration of the THP-1 cells, which are stimulated by PLEP, LPS/INF-γ (M1 stimulators) or IL-4/IL13 (M2 stimulators). PLEP exposure induced morphology contiguous with LPS/INF-γ, qPCR was also performed to determine whether PLEP influences M1 or M2 polarization-related genes. M1-phenotype macrophage-specific genes, such as TNF-α, IL-1β, IL-6, IL-8, CXCL10 and CCR7, were enhanced by PLEP in a dose-dependent manner similar to LPS/INF-γ. Conversely, M2-phenotype-specific genes, such as MRC-1, DC-SIGN, CCL17 and CCL22, were suppressed by PLEP. PLEP also significantly up-regulated secretory inflammation cytokines related to M1 polarization of macrophages, including TNFα, IL-1β and IL-6, which was similar to the gene expression. Further, MAPK and NF-κB signaling were increased by treatment with PLEP, resulting in enhancement of cytokine secretion. PLEP might therefore be used as a promising booster of pro-inflammatory responses through M1 polarization of human THP-1 cells.

Key words: Human THP-1 cells, MAPK signaling, NF-κB signaling, Phellinus linteus extract, polarization of macrophage

Introduction

Macrophages are leukocyte-derived cells which are well known for eliminating pathogens via phagocytosis. In the past, macrophages were categorized by means of organs, however, current classification has been changed from organ-specific macrophages to M1- and M2-polarized macrophages [6]. M1-polarized (classically activated) macrophages are usually activated by lipopolysaccharides (LPS) and interferon-γ (INF-γ) and directly kill contagious organisms and tumor cells against pathological status through secretion of cytokines and chemokines or indirectly attract other immune cells to remove them. M2-polarized (alternatively activated) macrophages are stimulated by interleukin-4 (IL-4) and interleukin-13 (IL-13) and repair injured-tissue and build extracellular matrix [13]. M1-polarized macrophages are inclined to produce not only pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), but also representing chemokine receptors (CCL21, CCL24, CCR7 and CXCL10) [26]. On the other hand, M2-polarized macrophages tend to restrain a secretion of pro-inflammatory cytokines and produce anti-inflammatory cytokines, such as IL-10, CCL17, CCL18 and CCL22 [24]. Also, they express various receptors, such as mannose receptor (MRC-1; CD206), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN; CD209) and scavenger receptor (CD163) on their surface [15].

Mitogen-activated protein kinases (MAPKs) are mainly
related with the transduction of externally originated signals. The kinases regulate cellular homeostasis which is involved in cell growth, differentiation and death [18]. Interestingly, the extracellular-signal regulated protein kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAPK signaling pathways are activated in macrophages by lipopolysaccharide (LPS), which is constituent of Gram-negative bacterial outer membrane [23]. And also other modulator, INF-γ, activates MAPKs signaling pathway in a numerous cellular models including macrophages [22]. It has been demonstrated that the production of TNF-α and IL-1β is inhibited by suppression of phosphorylation of ERK in macrophages [21]. And expression of other chemokines and inducible NO synthase is substantially up-regulated by this kind of phosphorylation [22].

NF-κB is involved in several cellular responses in an immune cell such as regulation of survival, activation and differentiation. It is crucial mediator of pro-inflammatory responses and regulates induction of cytokine and chemokine [12]. This family consists of five members, containing homo- and heterodimers of p50 (also known as NF-κB1), p52 (also known as NF-κB2), p65 (also known as RelA), RelB and c-Rel, and controls transcriptional activities of target genes [19]. Inhibitors-of-kappaB (IκB) disrupts the nucleus translocation and transcriptional activity of Rel/ NF-κB [3, 25]. Also, other researchers have been demonstrated that degradation of IκB is important factor to activate NF-κB [9].

Many therapeutic drugs have been developed from natural sources mostly like plants. Medicinal plants, which are widely used in Asian countries, have various compounds as potential pharmaceutical agents and have been used as a resources to discover the novel drugs [2]. Also, Javed Iqbal et al. summarized the anti-cancer materials which are derived from plants [10]. Phellinus linteus is one of the popular medicinal products and consists of several bioactive complements like polysaccharides, triterpenoids, polyphenols and furans [4]. Many researchers have reported that extract of P. linteus have pharmaceutical activities such as anti-oxidation, anti-microbe, anti-cancer, immunomodulatory effect, anti-diabetes and neuroprotection [4]. Most published papers have consistently asserted that extracts from the fruiting body of P. linteus have anti-inflammatory activity [11, 14, 17]. Our previous research has shown that extract of P. linteus induced pro-inflammatory responses in murine RAW 264.7 cells [20]. Therefore, we further explored whether extract of P. linteus promotes inflammatory or anti-inflammatory responses and which signals are related to immune response in human THP-1 cells through their macrophage polarization.

Materials and Methods

Chemicals and Materials

Phellinus linteus hot water extract passed from Diaion HP-20 resin (PLEEP) was used to research the polarization of human THP-1 cells. Procedure of extraction and purification was described in our previous paper [20]. Briefly, hot water extract of P. linteus was passed through a Diaion HP-20 resin, which binds the lipophilic elements, to remove hydrophobic elements and active anti-inflammatory components. Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). To differentiate monocytic THP-1 cells to macrophagic THP-1 cells, phorbol 12-myristate 13-acetate (PMA, Cayman Chemical, Ann Arbor, MI, USA) was used. And other immune stimulators, INF-γ, IL-4 and IL-13, were purchased from Peprotech (Seoul, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Duchefa Biochemie, Haarlem, Netherland) was used to examine cell viability. Antibodies against p-ERK, p-JNK, p-p38, p-NF-κB and p-IκBα were purchased from Cell Signaling Technology (Beverly, MA, USA) and goat-anti-rabbit or -mouse IgG secondary antibodies from Enzo Life Science (Farmingdale, NY, USA). Western blot was performed by using AmershamTM ECL™ Prime western blotting detection reagents (GE Healthcare, Chicago, IL, USA). Total RNA was purified by RiboEx solution purchased from GeneAll® (Seoul, Korea).

Cell line and culture

Human monocytic cell line THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum and 100 unit/ml of penicillin and streptomycin. All of culturing stuff were purchased from Welgene (Gyeongsan, Korea). Fig. 1 shows how to stimulate the differentiation and polarization of THP-1 cells, modified referring to Marie Genin et al [7]. Briefly, THP-1 cells were treated with PMA for 24 hr to differentiate monocytes to macrophages. To polarize into M1-like phenotype, 10 ng/ml of LPS and 20 ng/ml INF-γ (LPS/INF-γ) were treated for 24 hr. For polarization of M2, 20 ng/ml
Fig. 1. Scheme of the process of differentiation and polarization on human THP-1 cells. To differentiate from monocytic THP-1 cells to M0-like macrophages, 50 nM of PMA was treated for 24 hr. To polarize from M0-like macrophages to M1-like macrophages, 10 ng/ml of LPS and 20 ng/ml of INF-γ were treated for 24 hr. And to polarize from M0-like macrophages to M2-like macrophages, 20 ng/ml of IL-4 and 20 ng/ml of IL-13 were treated for 24 hr.

Analysis of cell viability

The cell viability was measured using MTT assay. Human monocytic THP-1 cells were seeded and treated with PMA for 24 hr. Then, PMA-derived (M0) THP-1 cells, were exposed by several concentrations of PLEP for 24 and 48 hr. 0.5 mg/ml MTT solution was added and incubated at 37 ℃ for 1.5 hr. The supernatant was discarded by suction and the precipitated formazan was dissolved in dimethyl sulfoxide (DMSO, Junsei Chemical, Chuo-ku, Japan). The optical density was carried out at 570 nm using by SpectraMAX M2e (Molecular Devices, San Jose, CA, USA).

Analysis of quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted with RiboEx solution, followed by modified TRIZOL method [5]. cDNA synthesis and qPCR were performed by using TOPscriptTM RT DryMIX (Enzymomics, Daejeon, Korea) and TOPreal ™ qPCR 2X PreMIX (Enzymomics), respectively, according to manufacturer’s instructions. Primer sequences were as follows: TNFa-ss, 5'-CAGGCAGTCAGATTCCTTCATTCGAGAAGTTAT-3' and IL-6-as, 5'-GTTGTTAGGTGTGTGTTAT-3'; IL-8-ss, 5'-GCAGGTTACCTGTGAGAGATA-3' and IL-8-as, 5'-CCCT ACAAACAGTCACCA-3'; CCR7-ss, 5'-TGTGTTATGCCTTTCCTGA TGTAAT-3' and CCR7-as, 5'-TGTGTTATGCCTTTCCTGA TGTAAT-3'; CXCL10-ss, 5'-GAAAGAGCTTTGAGAGAGATA GAGAGATA-3' and CXCL10-as, 5'-ATGTGAAAGGTGATTTG GAGAGATA-3'; MRC-1-ss, 5'-AGAAGGCTTTGAGAGAGATA GAGAGATA-3' and MRC-1-as, 5'-TACCCCTGCTCCTGGTTTT-3'; DC- SIGN-ss, 5'-GAAAGGCTTTGAGAGAGATA GAGAGATA-3' and DC- SIGN-as, 5'-CAGGAGGCTTGGAGACTTTTT-3'; CCL17-ss, 5'- CCGGACTACCTGGGACCC-3' and CCL17-as, 5'-TCTCTCTC CCAGATCCG-3'; CCL22-ss, 5'-TCTCCTCTCCTCAGATCCG-3' and CCL22-as, 5'-CAGGAGGCTTGGAGACTTTTT-3'; GAPDH-ss, 5'-CAGGAGGCTTGGAGACTTTTT-3' and GAPDH-as, 5'-TCTCCTCTCCTCAGATCCG-3'. First qPCR step was pre-incubation at 95 ℃ for 600 s and the amplification step was performed at 95 ℃ for 20 s, at 58 ℃ for 20 s and 72 ℃ for 20 s, followed by 40 cycles. And last step of melt curve was processed at 95 ℃ for 10 s, at 65 ℃ for 30 s, at 97 ℃ for 1 s and finally a total of 45 cycles. All reactions were carried out in triplicate and normalization of mRNA expression was used to GAPDH genes.

Western blotting

THP-1 cells were seeded into 6-well plate at a density of 1×10⁵ cells/well. After 24 hr, monocytic THP-1 cells were incubated with PMA to differentiate into macrophages. Before immune stimuli exposes, cells were washed twice with 1xPBS to remove PMA effects. Various stimuli were treated to M0-like macrophages for 24 hr; LPS/INF-γ, IL-4/IL-13 and PLEP exposure. Cells were lysed in PRO-PREP solution (iNtRON Biotechnology, Seongnam, Korea) with phosphatase inhibitor cocktail (Sigma-Aldrich Co) following manufacturer’s specifications. Bradford method was performed to quantify proteins from cell lysates. Proteins were separated using 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare). The membranes were blocked with 5% skim milk in TBS-T buffer [150 mM NaCl, 20 mM Tris (pH 7.4), 0.1% Tween 20] at room temperature for 1 hr and probed with specific primary or secondary antibodies. Finally, ECL solution was used to detect specific proteins on the membranes. Intensities of each bands were measured by using a fluorescence scanner (Amersham Imager 680, GE Healthcare) and analyzed with its software (GE Healthcare).
Enzyme-linked immunosorbent assay (ELISA)

Immuno-modulators were treated to THP-1 cells for various time points (3, 6, 12 and 24 hr). Supernatant from stimulated-THP-1 cells was collected to examine secretory cytokines related with pro-inflammation. ELISA was performed using human TNF-α, IL-1β and IL-6 ELISA kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s guidelines.

Statistical analysis

All experiments were repeated at least three times and the results were expressed as means ± SEM. Unless otherwise stated, the data are expressed as means ± SEM. Each experimental results were verified by one-way ANOVA, followed by Tukey-Kramer multiple comparison tests. Results were statistically significant at *p<0.05, **p<0.01 and ***p<0.001.

Results

PLEP did not affect cell viability but induced morphological changes of THP-1 cells

At first, we carried out to confirm whether PLEP itself have a cytotoxic effect on macrophage-like (M0) THP-1 cells which are stimulated with PMA. Monocytic THP-1 cells were incubated with PMA for 24 hr to differentiate to macrophage nature. Various concentration of PLEP from 10 to 300 μg/ml was treated to cells for 24 or 48 hr. As shown in Fig. 2, PLEP did not affect cell viability of THP-1 cells up to 300 μg/ml.

Morphological change indicates a sign of cellular alteration procedure. Monocytic THP-1 cells basically presented a round shape and a suspended culture property, whereas M0 THP-1 cells had an attached culture property. When M0 THP-1 cells were stimulated with LPS/INF-γ (M1 stimulators) or IL-4/IL13 (M2 stimulators), they displayed characteristic flat, ramose and amoeboid shapes. PLEP-stimulated THP-1 cells showed some morphological changes, compared to M0 THP-1 cells. The flatted and branched shapes like typical M1 morphologies, were increased in PLEP-stimulated THP-1 cells as pointed by a red arrow (Fig. 3). Taken together, those results represented that PLEP had no effect on cytotoxicity, but induced morphological alteration from M0-like phenotype to M1-like phenotype in THP-1 cells.

PLEP influenced macrophage polarization of THP-1 cells

Previously we confirmed that PLEP promotes gene expressions, which are involved in pro-inflammation, of RAW 264.7 cells [20]. We further explored whether PLEP induces pro-inflammatory or anti-inflammatory responses in macrophage-like THP-1 cells through their polarization. Macrophages secrete inflammatory cytokines and chemokines to regulate their host defense against external factors. Real-time PCR was performed to quantify gene expressions related

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**Fig. 3.** Morphological changes of THP-1 cells after exposure of several immune stimuli. Morphological changes were observed via microscopy (100x and 400x). Cells were treated with 50 nM of PMA for 24 hr and then another 24 hr incubated with immunomodulators such as 10 μg/ml of LPS and 20 ng/ml of INF-γ, IL-4 and IL-13 or PLEP. Various stimuli-induced morphological changes were observed in PMA-derived human THP-1 cells. Some morphological alterations were accentuated by red arrows. M0, PMA-derived macrophages; M1, LPS/INF-γ-stimulated macrophages; M2, IL-4/IL-13-stimulated macrophages; PLEP, PLEP-stimulated macrophages.
Fig. 4. Expression of M1 macrophages-related genes. After being stimulated with immune modulators (LPS/INF-γ or PLEP), cells were harvested and subjected to Trizol method to analyze M1 type inflammatory-related genes. The levels of mRNA expression were performed using quantitative real-time PCR and normalized to GAPDH. Data are presented as mean ± SEM (n=3 in each group). **p<0.01, ***p<0.001 vs. M0 group. M0, PMA-derived macrophages; M1, LPS/INF-γ-stimulated macrophages; PLEP, PLEP-stimulated macrophages.
Fig. 5. Expression of M2 macrophages-related genes. After being stimulated with immune modulators (IL-4/IL-13 or PLEP), cells were harvested and implemented using Trizol method to analyze M2-like nature-related genes. The levels of mRNA expression were determined by quantitative real-time PCR and normalized to GAPDH. Data are presented as mean ± SEM (n=3 in each group). *p<0.05, **p<0.01, ***p<0.001 vs. M0 group. M0, PMA-derived macrophages; M2, IL-4/IL-13-stimulated macrophages; PLEP, PLEP-stimulated macrophages.

Fig. 6. The level of inflammatory cytokines in M1-type macrophage THP-1 cells. After cells were exposed to immune modulators (LPS/INF-γ or PLEP), the supernatants were assayed to analyze pro-inflammatory cytokines. The levels of secretory protein expression were examined at various time points (3, 6, 12 and 24 hr). Data are presented as mean ± SEM (n=3 in each group). *p<0.05, **p<0.01, ***p<0.001 vs. M0 group. M0, PMA-derived macrophages; M1, LPS/INF-γ-stimulated macrophages; PLEP, PLEP-stimulated macrophages.

Discussion

Our previous research has demonstrated that Phellinus linteus has a paradoxical effect of immune responses in murine RAW 264.7 cell line. Flow-through fraction of a Diaion HP-20 resin (PLEP) showed pro-inflammatory activity through up-regulation of nitric oxide (NO) and inflammatory cytokine, whereas fraction eluted from Diaion HP-20 resin
Kines, such as TNF-α, confirmed up-regulation of secreted pro-inflammatory cytokines, such as CXCL10, IL-8, CCL22, and anti-inflammatory cytokines (CCL17 and CCL22) were suppressed by PLEP in a dose-dependent manner (Fig. 5).

In conclusion, PLEP has potential activity of enhancement of pro-inflammation via strengthening M1 polarization and suppressing M2 polarization on THP-1 cells.

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**The Conflict of Interest Statement**

The authors declare that they have no conflicts of interest with the contents of this article.

**References**


4. Chen, W., Tan, H., Liu, Q., Zheng, X., Zhang, H., Liu, Y. and Xu, L. 2019. A Review: The bioactivities and pharmacological activities of PLEP displayed anti-inflammatory activity, including suppression of NO production [20]. Thus, it was assumed that pro-inflammatory and anti-inflammatory agents must be separated from Diaion HP-20 resin. To further identify the role of PLEP in human THP-1 system, we carried out and characterized THP-1 cells after stimulating with several immunomodulators. Our finding proposed that PLEP has accelerated property of pro-inflammation unlike other published papers that extract of *P. linteus* has anti-inflammatory activity. As shown in Fig. 4, PLEP increased pro-inflammatory genes, such as TNF-α, CCR7, IL-β, IL-6, IL-8 and CXCL10, like as positive control LPS/INF-γ (M1 stimulators), similar with expressions in RAW 264.7 cells of our previous paper [20].

Furthermore, we carried out ELISA and western blot assay to confirm the secretory protein level, which signaling pathway contributes under the PLEP treatment. We confirmed up-regulation of secreted pro-inflammatory cytokines, such as TNF-α, IL-β and IL-6, from THP-1 cells exposed with M1 modulators or PLEP (Fig. 6). And inflammation related-MAPK signaling was extremely increased by PLEP in a dose-dependent manner (Fig. 7). Once MAPKs are activated, they stimulate phosphorylation of various factors, including ETS like-1 protein, c-Jun, activating transcription factor 2 and cAMP-response element binding protein, and consequently control macrophage activity, such as cell growth, differentiation, polarization and inflammatory responses through regulation of gene expression [16]. NF-κB is a major manipulator of the inflammatory responses to eliminate external pathogens and tumors. It is controlled by the IκB kinase, which facilitates the phosphorylation of IκB, causing degradation mediated by proteasome [1]. When IκB is degraded, the NF-κB is unconstrained to move into the nucleus, after then transcribing the pro-inflammatory genes [8]. Biologically active NF-κB was promoted by PLEP, whereas phosphorylation of IκBα was diminished (Fig. 6).

To identify whether PLEP affect an anti-inflammatory response, expression of M2 markers were observed using qPCR. Surface markers of M2 polarized macrophage (MRC-1 and DC-SIGN) and anti-inflammatory cytokines (CCL17 and CCL22) were suppressed by PLEP in a dose-dependent manner (Fig. 5).

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**References**


초록: 상황버섯 추출물의 인간 유래 THP-1 단핵구 세포주의 분극화 조절

이상률†, 박슬기††, 유선녕†, 김지원†, 황유림†, 김동섭†, 안순철†*
(1부산대학교 의학전문대학원 미생물학 및 면역학 교실, 2부산대학교 의학전문대학원 생화학 교실, 3부산대학교 생명자원과학대학 식품공학과)

대식세포는 특성에 따라 크게 classically activated macrophages (M1-phenotype macrophages)와 alternatively activated macrophages (M2-phenotype macrophages) 두 가지의 형태로 나눌 수 있다. M1 대식세포의 경우 직접, 간접적으로 병원체, 감염된 조직 및 암세포 등을 제거하는 능력을 가진 반면, M2 대식세포의 경우 항염증 반응을 동반한 손상된 세포 조직의 복구 및 세포외기질의 생성에 관여하고 있다. 본 연구에서는 상황버섯 열수 추출물을 합성 흡착제인 Diaion HP-20에 통과시켜 소수성 물질을 제거한 시료 (PLEP)를 이용하여 인간 유래 THP-1 단핵구 세포주의 염증성 혹은 항염증성 분극화 특성을 알아보았다.

먼저 PLEP 자체의 단핵구 세포에 대한 세포독성을 확인한 결과, 고농도의 300 μg/ml에서 세포독성이 확인되지 않았다. 한편 세포의 형태학적 변화를 확인한 결과, PLEP의 농도가 증가함에 따라 M1-phenotype 대식세포와 유사한 flatted and branched 형태가 증가하였다. 대식세포로 분화시킨 THP-1 세포주에 PLEP를 처리한 후, M1 분극화 관련 유전자인 TNFα, IL-1β, IL-6, CXCL10, CCR7과 M2-분극화 관련 유전자인 MRC-1, DC-SIGN, CCL17, CCL22의 유전자 발현량을 조사하여 분극화 양상을 판정하였다. 그 결과, M1-분극화 관련 유전자들은 PLEP 농도 의존적으로 증가하였지만, M2-분극화 관련 유전자들은 반대로 감소하였다. 또한 ELISA assay를 통하여 M1 분극화 관련 cytokine인 TNFα, IL-1β, IL-6의 발현량이 유전자 발현량과 동일하게 증가하였다. 이러한 cytokine들의 분비를 촉진시키는 MAPK signaling 또한 PLEP의 농도가 증가함에 따라 촉진되었고 염증성 cytokine과 관련된 전사인자 NF-κB의 활성화도 증가하였다. 따라서 PLEP는 인간 유래 THP-1 세포주에서의 M1 대식세포 분극화를 통해 염증 반응을 유발하는 것으로 확인되어 염증을 촉진하는 천연물질로 이용할 수 있을 것으로 전망된다.