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博士（スポーツ科学）

Characterization of exercise-induced inflammation and anti-inflammatory effects of polyphenols on lipopolysaccharide-induced inflammation

運動誘発性炎症とリポ多糖による炎症に対するポリフェノールの抗炎症作用の解析

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早稲田大学大学院　スポーツ科学研究科

洪　永豊

HUNG, Yung-Li

研究指導教員：　鈴木　克彦　教授

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Chapter 1. Background and purpose

1.1 Inflammatory response of innate immune system

Introduction of inflammation

The redness, swelling, fever, pain and loss of function are triggered by inflammation. The increasing recruitment of leukocytes and production of pro-inflammatory mediators and cytokines contribute to the redness, swelling, fever, pain and dysfunction of inflammation. The inflammatory response is initiated by harmful conditions and stimuli such as microbial infection, tissue damage and dysfunction. Inflammation is a pivotal contributor in process of host defense and tissue repair [1]. The inflammatory response of innate immune cells is the primary response to microorganism infection and damaging cells. The dendritic cells (DCs) and macrophages not only produce pro-inflammatory mediators and cytokines against microbial pathogens, but also provide antigen presentation to initiate adaptive immune system [2]. Inflammatory response of innate immune cells is triggered by germline-encoded pattern recognition receptors (PRRs) which recognize molecules of pathogens and damaged cells. The pathogen-associated molecular patterns (PAMPs) are regarded to be pathogen-specific molecules including bacteria lipopolysaccharides (LPS), flagellins, fungal glucans, CpG-DNA and viral DNA/RNA. The damage-associated molecular patterns (DAMPs)

are considered to be molecular components representing endogenous alarms. DAMPs are released forms of damaged cells such as advanced glycation end products (AGEs) and mitochondrial and nuclear DNA. A part of DAMPs are considered to be danger signals which induce inflammatory responses including heat-shock proteins (HSPs), high mobility group box 1 (HMGB1), fibrinogen and S100 proteins [3, 4]. Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), receptor for advanced glycation end products (RAGE) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) are PRRs which play important roles in elimination of microbial pathogens infection and damaging cells [5, 6].

The pattern recognition receptors

The TLRs are central PRRs in host cell recognition and response to several microbial pathogens such as bacteria, mycobacteria, viruses, parasites and fungi. Toll/interleukin-1 receptor (TIR) and leucine-rich repeat (LRR) are central components of TLR. TIR is cytosolic domain to transduce signaling pathways, and LRR is extracellular domain to recognize pathogens [7]. TLRs, including transmembrane receptor TLR1, TLR2, TLR4, TLR5, TLR6, cytoplasmic receptor TLR3, TLR7, TLR8, and TLR9, broadly recognize

PAMPs and DAMPs. The ligands of transmembrane receptor TLR1 are lipopeptides, TLR2 (lipopeptides/lipoprotein), TLR4 (LPS, HSPs, fibrinogen and HMGB1), TLR5 (flagellins), and TLR6 (lipopeptides), whereas cytoplasmic receptor TLR3 recognizes double-stranded RNA, TLR7 (single-stranded RNA), TLR8 (singe-stranded RNA) and TLR9 (CpG-DNA) [8, 9]. At present, the ligand of TLR10 remains unclear.

The NLRs are cytoplasmic receptors that recognize PAMPs (bacterial peptidoglycans, viral RNA and microbial toxins) and DAMPs (adenosine triphosphate (ATP) and monosodium urate (MSU) crystals). NLRs include four subfamilies NLRA/Class II transactivator (CIITA), NLRB/neuronal apoptosis inhibitor proteins (NAIPs), NLRC, and NLRP [10]. In addition, NLRs comprise a C-terminal series of LRR and an N-terminal homotypic protein-protein interaction domain. The interaction domain of NLRAs is acid transactivation domain. NLRBs contain baculovirus inhibitor of apoptosis protein repeat (BIR). NLRCs contain a caspase-recruitment domain (CARD), and pyrin domain (PYD) [11]. The NOD1 and NOD2 trigger inflammatory response by recognizing Gram-negative and Gram-positive bacterial peptidoglycan. NLRPs are key regulators of inflammasomes which are a group of protein complexes and produce pro-inflammatory cytokine interleukin-1β (IL-1β) and IL-18 [12]. Especially, NLRP3

inflammasome is a regulator in pathogenesis of diabetes, atherosclerosis and several immune-mediated diseases [12]. The NLRP3 inflammasome complex is composed of NLRP3, apoptosis-associated speck-like protein containing a caspase-activation and -recruitment domain (ASC) and caspase-1. The activation of NLRP3 inflammasome in macrophages is triggered by two steps (priming and activation). TLRs or tumor necrosis factor-α (TNF-α) receptor-mediated nuclear factor-κB (NF-κB) produces NLRP3, pro-IL-1β and pro-IL-18 in the priming steps, and then PAMPs or DAMPs activate NLRP3, and increase ASC oligomerization in the activation step. Subsequently, ASC converts caspase-1 into the active form, and activated caspase-1 cleaves the pro-IL-1β and pro-IL-18 [13].

The CLRs such as Dectin-1, Dectin-2, mannose receptor (MR), Dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and macrophage-inducible C-type lectin (Mincle) recognize fungal, viral and bacterial carbohydrates, and initiate to antifungal immune response directly [14]. Dectin-1 recognizes β-glucan (a component of fungal cell wall) and transduces the spleen tyrosine kinase (Syk) signaling. Dectin-2 and MR contribute to recognition of high mannose-type carbohydrates. DC-SIGN binds high mannose and fucose to recognize

fungi and virus. In particular, Mincle not only binds glycolipids and α-mannose, but also recognizes spliceosome-associated protein 130 (SAP130) which is ribonucleoprotein of death cells [15].

The RLRs are the cytoplasmic receptors that recognize RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology gene 2 (LGP2). RIG-I and MDA5 are activated by viral double-stranded RNA. Activated RIG-I and MDA5 bind with RLRs, and transduce the essential adaptor protein mitochondrial antiviral signaling (MAVS) to initiate antiviral immune response. In contrast, LGP2 negatively regulates RIG-I and MDA5 [16]. Thus, the RLRs are crucial contributors to recognize RNA virus and exert antiviral effects.

The RAGE is a transmembrane receptor of DAMPs such as AGEs, S100 proteins, phosphatidylserine and advanced oxidation protein products (AOPPs) and HMGB1. Interestingly, RAGE also recognizes LPS and triggers inflammatory response [6]. RAGE regulates inflammation and cell migration by recognition of different ligands. The engagement of HMGB1 stimulates monocyte migration, and binding with S100 proteins induces synthesis of pro-inflammatory cytokines [4].

Taken together, the TLRs are presented on several immune cells (DCs, macrophages, B cells and T cells), fibroblasts and epithelial cells, and wildly recognize various microbial pathogens and endogenous molecules [3]. TLRs are central contributors to recognition of PAMPs and DAMPs. It is considered that TLRs initiate several signaling transduction pathways to trigger inflammation [7].

TLR4 signaling transduction pathways

Among several TLRs, TLR4 is a crucial receptor for recognition of bacterial LPS and leading to inflammatory response. Myeloid differentiation 2 (MD2) assists TLR4 to recognize LPS, and CD14 and LPS-binding protein (LBP) enhance LPS binding to TLR4/MD2 receptor complex. Subsequently, TIR-like domains are recruited, and transduce signal to other adaptor proteins. The downstream adaptor proteins include myeloid differentiation primary response protein 88 (MyD88), MyD88 adaptor-like (Mal) as known as TIR domain containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing interferon-β (TRIF, also known as TIR-containing adapter molecule-1 (TICAM-1)), TRIF related adaptor molecule (TRAM, also called TICAM-2) and sterile-α and armadillo motif containing protein (SARM) [17].

MyD88-dependent pathway and TRIF-dependent pathway (MyD88-independent pathway) have been defined in TLR4 signaling transduction pathways. The MyD88-dependent pathway regulates the early stage of acute inflammation. In contrast, the TRIF-dependent pathway contributes to the late stage of acute inflammation [18]. In the MyD88-dependent pathway, the recruitment of MyD88 activates IL-1 receptor-associated kinases 1 (IRAK1), IRAK2, IRAK4 and TNF receptor-associated factor 6 (TRAF6). Subsequently, transforming growth factor β-activated kinase 1 (TAK1)-binding protein 2 (TAB2) and TAK1-binding protein 3 (TAB3) are transduced, and then TAB2 and TAB3 activate TAK1. TAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) that regulates activity of MAPKs. The MAPKs including c-JUN N-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinases (ERK) crucially regulate downstream transcription factors such as activator protein-1 (AP-1) and NF-κB [8]. These transcription factors regulate production of pro-inflammatory mediators and cytokines. Moreover, the Mal (TRIF) regulates activation of NF-κB through the bridging of MyD88-dependent pathway.

On the other hand, the TRIF-dependent pathway is initiated by recruitment of TRIF and

TRAM, and activates TRAF3, tank-binding kinase 1 (TBK1) and inhibitor-κB kinase ε (IKKε). These signaling transduction factors induce nuclear translocation of interferon regulatory factor-3 (IRF-3) and IRF-7. Subsequently, interferon inducible protein-10 (IP-10), type I interferon (IFN) and regulated on activation, normal T cell expressed and secreted (RANTES) are produced [9]. TRIF and TRAM also regulate activation of NF-κB without MyD88 [19]. Furthermore, the SARM downregulates IRF-3, IRF-7 and NF-κB by TRAM [18].

Taken together, the LPS-induced TLR4 signaling is regulated by these five adaptor proteins (MyD88, Mal, TRIF, TRAM and SARM), and activated transcription factor AP-1, NF-κB, IRF-3 and IRF-7 to produce pro-inflammatory mediators and cytokines. The MyD88-dependent pathway is a pivotal regulator of inflammatory host response to LPS. Thus, MyD88-dependent pathway signaling transduction factors are critical contributors for acute inflammation such as MAPK and NF-κB.

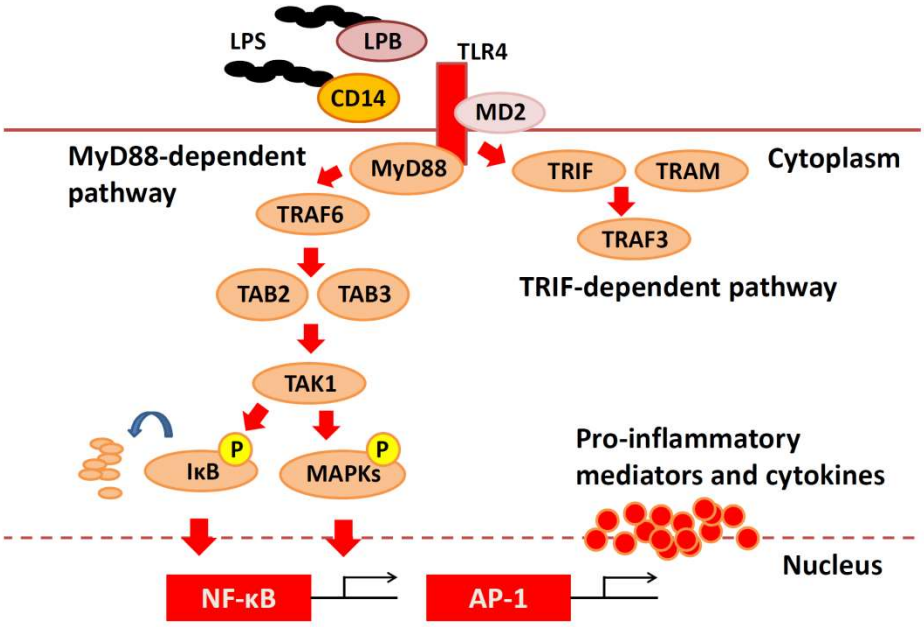

Fig. 1. The TLR4 signaling transduction pathways.

LPS-induced pro-inflammatory mediators and cytokines

The LPS-induced TLR4 signaling transduction factors lead to produce several pro-inflammatory mediators and cytokines by LPS-activated macrophages. The major pro-inflammatory mediators including nitric oxide (NO) and prostaglandin E_2 (PGE_2) cause redness, swelling, fever and pain. Inducible NO synthase (iNOS, also called NOS2) synthesizes NO from L-arginine. The production of iNOS is regulated by NF-κB after immunological stimulation [20]. NO is transformed to cytotoxic free radical peroxynitrite ($ONOO^-$) by superoxide (O_2^-), and kills microorganism by free radical reaction. The overproduction of NO contributes cell death (necrosis and apoptosis) and

tissue destruction by cytotoxicity and oxidative stress [21]. Additionally, iNOS is a harmful enzyme involved in development of inflammatory diseases [22].

Another major pro-inflammatory mediator, PGE_2 is a lipid pro-inflammatory mediator. The cell membrane phospholipid and phospholipase A_2 (PLA_2) release arachidonic acid (AA), and cyclooxygenase-2 (COX-2) converts AA to PGH_2 through cyclooxygenase and peroxidase activity. Subsequently, PGH_2 is transformed to PGE_2 by PGE synthase [23]. The PGE_2 contributes to vascular hyperpermeability, immune cell infiltration and plasma leakage in acute inflammation. Therefore, COX-2 inhibitors suppress prostanoid biosynthesis, and decrease vasodilatation, vascular permeability and recruitment of immune cells in inflammation [23]. On the other hand, NO causes vascular dysfunction characterized by vascular hyperpermeability due to endothelial cell death [21]. Interestingly, it has been reported that there is a cross-talk between iNOS and COX-2. NO elicits prostaglandin synthesis via upregulation of COX-2 activity. In contrast, COX-2 inhibitor does not affect iNOS expression [24].

TNF-α, IL-1β and IL-6 are crucial pro-inflammatory cytokines produced by LPS-activated macrophages and lymphocytes. IL-1β and TNF-α contribute to activate

immune cells, and induce fever and coagulation in inflammation. Particularly, TNF-α is a master regulator of pro-inflammatory cytokine production. Both IL-1β and TNF-α augment inflammatory cascades, and induce severe inflammatory reaction in an autocrine and paracrine manner by activated macrophages. IL-6 activates B and T lymphocytes, and also contributes fever [25]. Additionally, IL-6 and IL-12 are family cytokines that share subunit and receptor by structural relation. IL-12 is a critical contributor to connect innate immune system to adaptive immune system [26]. Especially, IL-12p40 shares the p40 subunit with IL-23, and induces inflammation together with IL-17 [27].

1.2 LPS-induced systemic inflammation (sepsis and exercise-induced endotoxemia)

Introduction of endotoxemia

The high level of LPS (endotoxin) in blood leads to systemic inflammation such as endotoxemia and sepsis. The endotoxemia is characterized that immoderate LPS into the bloodstream, which triggers production of pro-inflammatory mediators and cytokines by activation and recruitment of leukocytes and macrophages. Subsequently, augmentation of these immune responses causes harmful systemic inflammation that attributed systemic inflammatory response syndrome (SIRS). The SIRS is defined by

two or more clinical finding (1. body temperature <36°C or >38°C, 2. heart rate >90 beats/min, 3. respiratory rate >20 times/min or $PaCO_2$ <32 mmHg and 4. white blood cell (WBC) count >12000 cells/μL or <4000 cells/μL, or >10% immature (band) forms) [25].

The pathogenesis of sepsis

The infection of gram-negative bacteria and other pathogens contributes to pathogenesis of sepsis. The gram-negative bacterial LPS and other microbial component initiate harmful systemic host response. The overproduction of pro-inflammatory mediators and cytokines by activated innate immune cells lead to cell apoptosis and necrosis. The inflammatory cascades contribute to multiple organ damage, sepsis and septic shock [28]. TLR4/MD2 receptor complex is central contributor to initiate systemic inflammation of endotoxemia, and transduce signaling to produce pro-inflammatory mediators and cytokines. The hypercytokinemia (known as cytokine storm) is overspilling cytokines in blood. TNF-α, IL-1β, IL-6, IL-12 and IL-17 have been found that are elevated in sepsis. These pro-inflammatory cytokines are considered that are main cytokines in pathogenesis of sepsis [29]. On the other hand, HMGB1 as a pro-inflammatory mediator contributes to late stage of sepsis [30]. Especially, HMGB1

also binds to TLR4, and initiates TLR4-mediated inflammatory response. The hypercytokinemia causes endothelial cell necrosis and dysfunction, and then leads to the vascular hyperpermeability and plasma leakage. Concurrently, pyrogenic cytokine IL-1β and TNF-α contribute to fever and coagulation, and IL-6 also contributes to fever. In addition, these pro-inflammatory cytokines induce production of PGE_2 and NO. The hypercytokinemia contributes to fever, vasodilation, coagulopathy and capillary leak. Taken together, the hypercytokinemia causes to multiple organ damage, sepsis, and septic shock [28].

The exercise-induced endotoxemia and systemic inflammation

The endotoxemia is not only triggered by pathogenic bacterial infection, but also initiated by intestinal gram-negative bacterial translocation. The hyperpermeability of intestine increases translocation of intestinal gram-negative bacteria. It has been found that prolonged and strenuous exercise causes the hyperpermeability of intestine. It increases the blood supply of muscle and cardiopulmonary system, but reduces the blood supply of gastrointestinal (GI) system by the prolonged and strenuous exercise-induced redistribution of blood flow. It leads to gastrointestinal ischemia during exercise, and the perfusion of gastrointestinal evolves inflammation after

exercise [31]. The exercise-induced redistribution of blood flow contributes to hyperpermeability of intestine through intestinal barrier dysfunction and gastrointestinal ischemia [31]. Concurrently, the prolonged and strenuous exercise increases core body temperature that leads to hyperthermia, and exercise-induced hyperthermia also augments intestinal hyperpermeability [32]. The intestinal gram-negative bacteria translocate into blood, and increase circulatory bacterial LPS. It leads to endotoxemia during prolonged and strenuous exercise.

On the other hand, exercise-induced endotoxemia triggers production PGE_2, TNF-α, IL-1β and IL-6 by activated macrophages. These pro-inflammatory mediator and cytokines contribute to fever by pyrogenic response, and even result in exertional heat stroke. The pathogenesis of heat stroke is not only associated with hyperthermia, but also involved in endotoxemia [33]. Lim and Suzuki suggested that the pathogenesis of heat stroke is explained by dual-pathway model (DPM) of hyperthermia and endotoxemia [34].

Non-steroidal anti-inflammatory drugs (NSAIDs) reduces core body temperature through inhibitory effect of COX-2, but a part of NSAIDs (aspirin and ibuprofen)

enhance permeability of intestine [35]. Although the probiotic supplementation decreases exercise-induced intestinal barrier dysfunction, it does not affect exercise-induced pro-inflammatory cytokine production [36, 37]. On the other hand, the ascorbic acid supplementation suppresses exercise-induced endotoxemia [38], and also reduces sepsis-induced organ damage [39]. Many polyphenols which derived from herbs, fruits, and vegetables exhibit anti-inflammatory effects, and attenuate LPS-induced inflammation [40, 41].

1.3 The anti-inflammatory effects of *Camellia sinensis* (L.) O. Kuntze

The introduction of Camellia sinensis (L.) O. Kuntze

Tea is produced from the leaves of *Camellia sinensis* (L.) O. Kuntze, and exhibits various health benefits. Tea has been found that contains abundant polyphenols, and has potential beneficial effects such as reduction of cardiovascular diseases risk [42], anti-obesity [43], inhibition of tumorigenesis [44] and improvement of type II diabetes [45]. The three major categories of tea, green tea, oolong tea and black tea are all produced from the *Camellia sinensis*, but are produced with different process of fermentation. The green tea is produced without fermentation, oolong tea is fermented partially, and black tea is fermented fully. The variety of polyphenols of tea is associated

with process of fermentation. The green tea contains the most amount of (-)-epigallocatechin 3-gallate (EGCG) and total catechin (flavan-3-ols) [46]. After enzymatic fermentation process, the amount of EGCG and total catechin of black tea become lower than green tea, and polyphenol oxidase converts into theaflavins and thearubigins [44]. Since green tea is produced without fermentation, the bioactivity compounds which are isolated from green tea are the nearest the bioactivity compounds which are isolated from leaves of *Camellia sinensis* among black tea, oolong tea and others.

The major bioactivity compounds of green tea *(Camellia sinensis)*

The major bioactivity compounds of green tea are flavonoids include (+)-catechin (C), (-)-epicatechin (EC), (+) gallocatechin (GC), (-)-epigallocatechin (EGC), (+)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG) and EGCG. The EGCG (48~55%) is the most abundant component derived from green tea, and other compounds are ECG (9~12%), EGC (9~12%) and EC (5~7%) [47]. According to the chemical structure of polyphenols, catechin contain a C-ring which is a dihydropyran heterocycle with a hydroxyl group on carbon 3, and C-ring connects two or more aromatic rings (A- and B-rings) which bears one or more aromatic hydroxyl. This

chemical structure is called flavan-3-ol. Moreover, GCG, ECG and EGCG are with a gallate D-ring. The B-ring has been defined two moiety types which are catechol B-ring and pyrogallol B-ring. C, EC, GC and EGC contain catechol B-ring. In contrast, GCG and EGCG contain pyrogallol B-ring (Fig. 2). The chemical structure A-ring is near a resorcinol moiety [47].

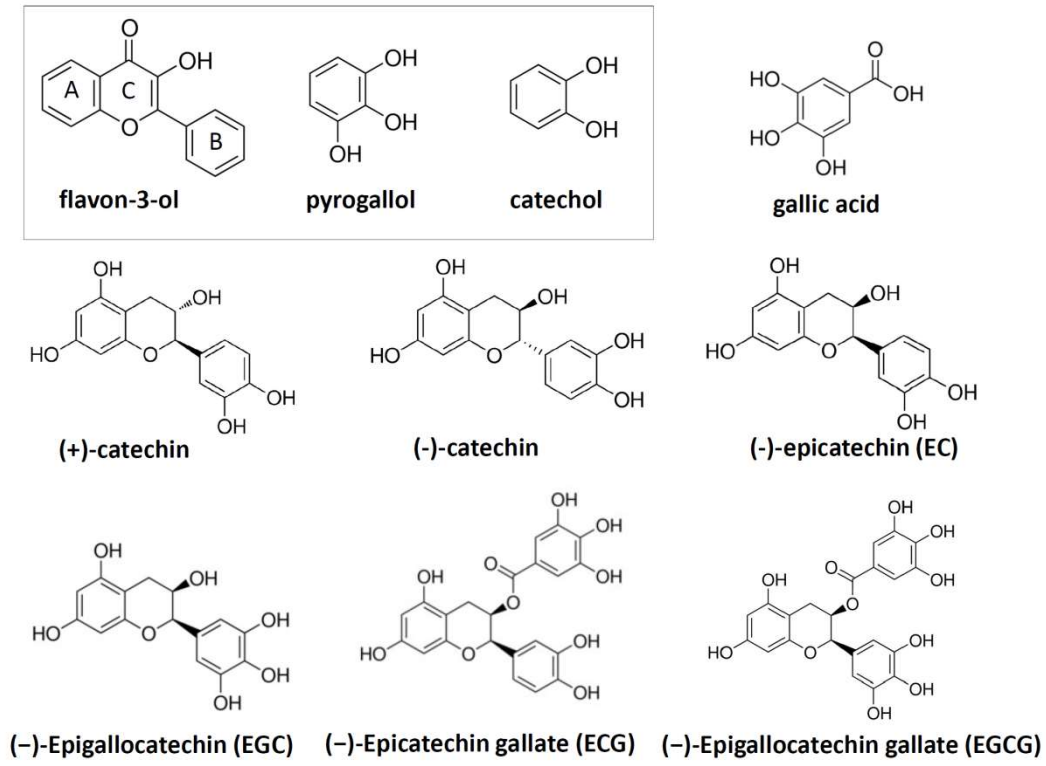

Fig. 2. The chemical structure of polyphenols in green tea (*Camellia sinensis*).

Reference： https://www.sigmaaldrich.com

The several epidemiological evidences have indicated that green tea consumption might

reduce inflammation and prevent lifestyle-related diseases such as cardiovascular diseases and cancers [44, 48]. The green tea polyphenols suppress the production of pro-inflammatory mediator NO and cytokines [49]. Notably, EGCG has been demonstrated that exerts anti-inflammatory properties on LPS-induced inflammation *in vitro* and *in vivo* [50, 51]. These polyphenols derived from green tea (*Camellia sinensis*) have been considered that contribute to the anti-inflammatory effects of *Camellia sinensis*, and might be potential compounds for treating LPS-induced inflammation.

1.4 The anti-inflammatory effects of *Psoralea corylifolia* L. (Fabaceae)

The introduction of Psoralea corylifolia L. (Fabaceae)

An annual herb*, Psoralea corylifolia* L. (Fabaceae) is a medicinal plant which has been used in Ayurvedic medicine and traditional Chinese medicine, as well as applied in traditional herbal medicine in other Asian countries. *Psoralea corylifolia* L. has been used as a traditional medicine treatment for a variety of diseases and symptoms such as cardiovascular diseases, osteoporosis, psoriasis, leukoderma, and inflammatory skin diseases [52]. It is also a tonic food in Asian countries. The whole plant of *Psoralea corylifolia* L., especially the seed or fruit, has significant medicinal value in traditional medicine. The dried fruit of *Psoralea corylifolia* L. is called Buguchi or Buguzhi in

Chinese. In the previous studies, the extract of *Psoralea corylifolia* L. suppresses the pro-inflammatory gene expression in live by high-fat diet-induced obese mice [53], and exerts antitumor properties and immunomodulatory effects [54].

The flavonoids of Psoralea corylifolia L.

The major active constituents of *Psoralea corylifolia* L. are polyphenolic compounds including coumarins, flavonoids, and meroterpenes [55]. In the previous studies, the flavonoids of *Psoralea corylifolia* L exert anti-inflammatory effects. Bavachin attenuates chemokine production and cell migration by IL-1β-stimulated THP-1 monocytic cells [56]. Neobavaisoflavone decreases production of pro-inflammatory cytokines by LPS plus IFN-γ-stimulated RAW264.7 macrophages [57]. Furthermore, bavachin, bavachinin, corylin, corylifol, neobavaisoflavone, and isobavachalcon exert inhibitory effects of IL-6-triggered signal transducer and activator of transcription 3 (STAT3) in Hep3B hepatoma cells [58]. The flavonoids which are derived from *Psoralea corylifolia* L. exhibit potentially inhibitory effects on inflammation. Nevertheless, the anti-inflammatory effects of flavonoids of *Psoralea corylifolia* L. in LPS-induced inflammation are unclear.

1.5 Research purpose

The LPS-induced inflammation is widely associated with pathogenesis of several diseases. Especially, it might cause systemic inflammatory response syndrome, sepsis, multiple organ failure, and even death. At present, there are no specific therapeutic agents or supplementation on treatment of exercise-induced endotoxemia. A part of NSAIDs (aspirin and ibuprofen) improve exercise-induced hyperthermia, but increase permeability of intestine [35]. On the other hand, the probiotic supplementation reduces exercise-induced intestinal barrier dysfunction, but does not affect exercise-induced inflammation [36, 37]. In previous studies, the polyphenols which derived from herbs, fruits, and vegetables inhibit LPS-induced production of pro-inflammatory mediators and cytokines, as well as exert protection against sepsis [40, 41]. Thus, the polyphenols which are isolated from *Camellia sinensis* (L.) O. Kuntze and *Psoralea corylifolia* L. might exert anti-inflammatory effects on LPS-induced inflammation.

The research purpose of this thesis is to investigate the anti-inflammatory effects of polyphenols which are isolated from *Camellia sinensis* (L.) O. Kuntze and *Psoralea corylifolia* L. by LPS-activated macrophages (murine peritoneal macrophages, RAW264 cells, J774A.1 cells and RAW264.7 cells) (*ex vivo* and *in vitro*), and then select

candidate compounds. The candidate compounds are supposed to confirm the anti-inflammatory effects on LPS-challenged mice (*in vivo*).

Chapter 2. The anti-inflammatory effects of green tea polyphenols in LPS-induced inflammation

2.1 Introduction of green tea polyphenols (*Camellia sinensis* (L.) O. Kuntze)

It has been reported that polyphenols of green tea (*Camellia sinensis*) have health benefits in preventing lifestyle-related diseases such cardiovascular diseases and cancers [59-61]. The polyphenols are isolated from green tea are (+)-catechin (C), (-)-epicatechin (EC), (+) gallocatechin (GC), (-)-epigallocatechin (EGC), (+)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG) and EGCG. Additionally, (-)-catechin is the negative form of (+)-catechin. Furthermore, gallic acid is phenolic acid whose structure is similar to pyrogallol, and also isolated from green tea. Among major green tea polyphenols, EGC, ECG, and EGCG are polyphenols with pyrogallol-type structure. In contrast, (+)-catechin, (-)-catechin and (-)-epicatechin are polyphenols without pyrogallol-type structure. It has been found that the pyrogallol-type polyphenols increase the macrophage phagocytic activity [62], and are recognized by cell-surface receptor, 67-kDa laminin receptor [63, 64].

In previous studies, EGCG decreased the production of NO, TNF-α and IL-6 through suppressing the activation of MAPKs and NF-κB by LPS-activated macrophages [50,

65, 66]. However, the anti-inflammatory effects of other polyphenols of green tea remain unclear, especially, the gallic acid and polyphenols without pyrogallol-type structure ((-)-catechin, (+)-catechin, and (-)-epicatechin). The structure characters of catechin (flavon-3-ol) with pyrogallol-type structure (EGCG), catechin without pyrogallol-type structure ((-)-catechin, (+)-catechin, and (-)-epicatechin) and pyrogallol-type structure (gallic acid) were investigated in this chapter.

2.2 Methods

Reagents

The gallic acid (monohydrate) and (-)-catechin were purchased from Wako Chemicals (Osaka, Japan). The (+)-catechin (hydrate), (-)-epicatechin, EGCG and LPS (from *Escherichia coli* O55: B5) were obtained from Sigma-Aldrich (St. Louis, USA). Phosphate-buffered saline (PBS) was purchased from Gibco-BRL (Life Technologies, Grand Island, USA). The p38 MAPK, phospho-p38 MAPK and β-actin antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, USA).

Cell culture

Murine macrophage cell line, RAW264 cells were purchased from European Collection

of Cell Culture (ECACC, Salisbury, UK) and were cultured in DMEM with 10% FBS (HyClone, Logan, USA) in humidified incubator containing 5% CO_2 at 37 ˚C and maintained growth by passaged every other day.

Cytokine measurement

Cells were seeded on at a concentration of 1.45 × 10^6 cells/mL in 6-well plates, and were allowed to acclimate. After 24 h, cells were treated with or without gallic acid, (+)-catechin, (-)-catechin, (-)-epicatechin and EGCG (1 and 10 μM) for 4 h. After 4 h, cell culture supernatant was removed, and cells were washed with PBS prior to LPS (50 ng/mL) treatment. After 24 h, the supernatant was collected. The production of TNF-α and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). The supernatant was used for the quantification of cytokine concentration by ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's instruction.

Total protein analysis

The total protein of the cells was used to detect the effects of green tea polyphenols on cell growth/viability [67]. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Rockford, USA), and the cell lysates were centrifuged at

16,000 g for 20 min at 4 ˚C, and the supernatant was collected as whole cell protein. The whole cell protein was used for the determination of protein concentration using the micro-bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, USA), according to the manufacturer's instruction.

Western blotting

The whole cell proteins were separated by 10% SDS-PAGEs, and transferred onto PVDF membranes. The protein expression levels were analyzed using antibodies against phospho-p38 MAPK, and p38 MAPK (Cell Signaling, Beverly, USA). The protein bands were developed by using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, U.K.), and intensities were quantified using ImageJ software (NIH, Bethesda, USA).

Statistical analysis

All data are expressed as means ± standard error of the mean (SEM). Each value is the mean of three independent experiments. SPSS V22.0 (IBM, Tokyo, Japan) was used to determine the p values using two-way ANOVA followed by Bonferroni post-hoc tests. The p values of <0.05 were considered statistically significant.

2.3 Results and figures

Gallic acid and EGCG increased LPS-repressed cell growth/viability by RAW264 cells

RAW264 cells were treated with or without gallic acid, (+)-catechin, (-)-catechin, (-)-epicatechin and EGCG (1 and 10 μM) for 4 h prior to LPS (50 ng/mL) treatment for 24 h. As shown in Fig. 3, the total protein of cells was reduced by LPS treatment for 24 h. The treatment of 10 μM gallic acid and EGCG significantly prevented the repression in total protein of LPS-activated RAW264 cells ($p<0.05$) (Fig. 3). The treatment with 10 μM (-)-catechin also exhibited the similar effect, but it was not significant ($p=0.07$). The treatment of 10 μM EGCG was significantly more effective than the treatment with 10 μM (+)-catechin for preserving cell viability ($p<0.05$) (Fig. 3).

Gallic acid, (-)-catechin and EGCG decreased LPS-induced production of TNF-α and IL-6 by RAW264 cells

RAW264 cells were treated with or without gallic acid, (+)-catechin, (-)-catechin, (-)-epicatechin and EGCG (1 and 10 μM) for 4 h prior to LPS (50 ng/mL) treatment for 24 h. Both treatment with EGCG (1 and 10 μM) decreased the production of TNF-α by LPS-activated RAW264 cells significantly as shown in Fig. 4A ($p<0.05$). The treatment

with (-)-catechin (1 and 10 μM) also significantly reduced the production of TNF-α (p<0.05). Furthermore, the treatment with gallic acid (10 μM) significantly inhibited the production of TNF-α (p<0.05). At the concentration of 10 μM, production of TNF-α was greater in response to treatment with (+)-catechin and epicatechin compared with EGCG (p<0.05). The treatment with EGCG (1 and 10 μM) and (-)-catechin (1 and 10 μM) suppressed the production of IL-6 by LPS-activated RAW264 cells (p<0.05) (Fig. 4B). The treatment with gallic acid (10 μM) also suppressed IL-6 production (p<0.05) (Fig. 4B).

The polyphenols with a flavan-3-ol structure affected p38 MAPK

RAW264 cells were treated with or without gallic acid, (+)-catechin, (-)-catechin, (-)-epicatechin and EGCG (10 μM) for 4 h prior to LPS (50 ng/mL) treatment for 45 min and 2 h. LPS-induced production and phosphorylation of p38 MAPK was increased at 45 min (early stage), but after LPS treatment for 2 h (late stage) was decreased compared with LPS treatment for 45 min. As shown in Fig. 5, the treatment with (+)-catechin, (-)-catechin, (-)-epicatechin and EGCG enhanced phosphorylation and total expression of p38 MAPK after LPS treatment for 45 min. By contrast, phosphorylation and total expression of p38 MAPK were suppressed after LPS

treatment for 2 h. After LPS treatment for both 45 min and 2 h, gallic acid neither enhanced nor suppressed production and phosphorylation of p38 MAPK.

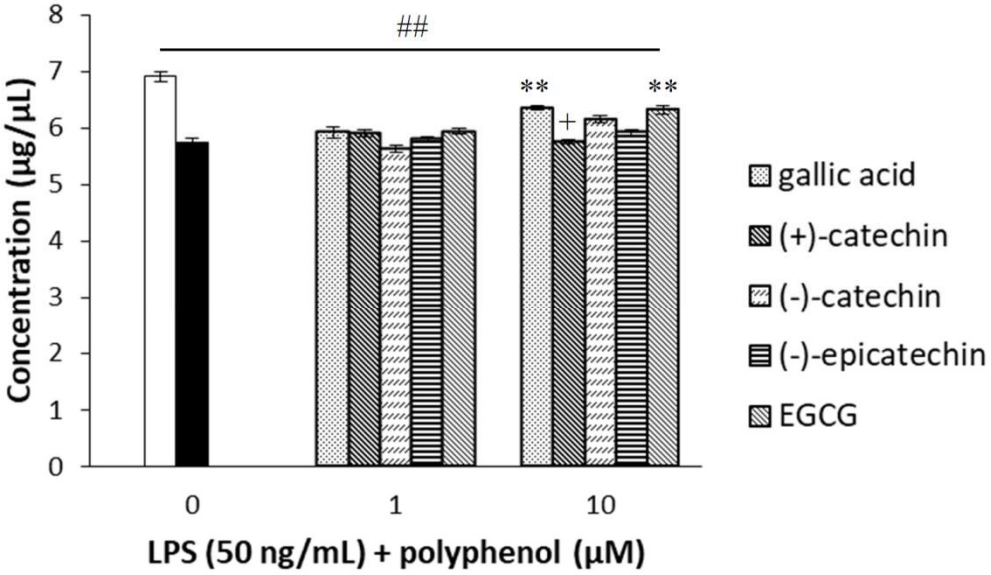

Fig. 3. The effects of green tea polyphenols on cell growth/viability by RAW264 cells. Cells were treated with polyphenols of green tea for 4 h, and then washed cells with PBS prior to LPS (50 ng/ml) treatment for 24 h. The open bar: without treatment of polyphenols and LPS challenge. The close bar: without treatment with polyphenols. Each column represents the mean ± SEM from three independent experiments. ##: $p<0.01$ versus the negative control. **: $p<0.01$ versus the pre-treatment without polyphenol of green tea. +: $p<0.05$ versus the same concentration of EGCG.

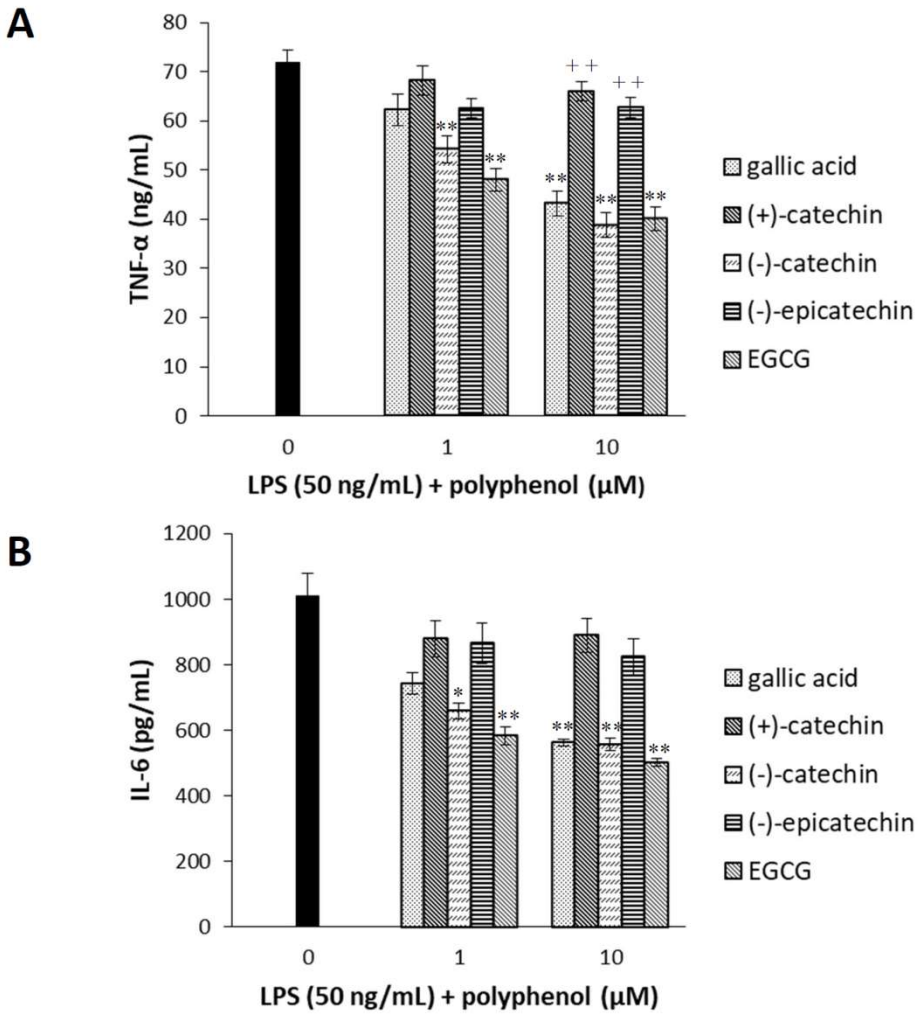

Fig. 4. The effects of green tea polyphenols on LPS-induced TNF-α and IL-6 production by RAW264 cells. Cells were treated with polyphenols of green tea for 4 h, and then washed cells with PBS prior to LPS (50 ng/ml) treatment for 24 h. The concentration of (A) TNF-α and (B) IL-6 in the supernatant. The close bar: without pretreatment with polyphenols. Each column represents the mean ± SEM from three independent experiments. *: $p<0.05$, **: $p<0.01$ versus the pre-treatment without polyphenol of green tea. ++: $p<0.01$ versus the same concentration of EGCG.

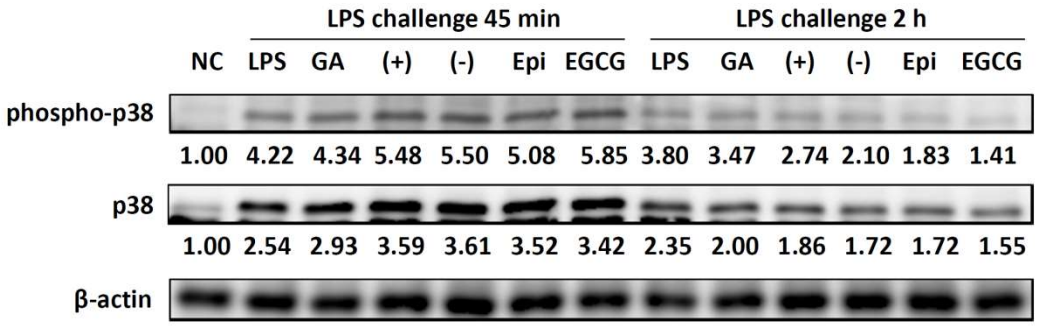

Fig. 5. The effects of green tea polyphenols on p38 MAPK by LPS-activated RAW264 cells. NC, negative control; LPS, stimulated with LPS (50 ng/ml) and without treatment of polyphenol; GA, treatment with gallic acid 4 h; (+), treatment with (+)-catechin 4 h; (-), treatment with (-)-catechin 4 h; Epi, treatment with (-)-epicatechin 4 h; EGCG, treatment with EGCG 4 h.

2.4 Discussion

The cell death and apoptosis of macrophages are increased by LPS stimulation [68-70]. Our results indicated that LPS decreased cell growth/viability (whole cell protein expression). The treatment with EGCG and gallic acid increased cell growth/viability compare with LPS alone. Interestingly, the treatment with (+)-catechin, (-)-catechin and (-)-epicatechin did not affect LPS-induced reduction of cell growth/viability. Taken together, these results suggest that pyrogollol-type polyphenols might protect the cell growth/viability against the effects of LPS stimulation in macrophages.

In a previous study, EGCG exerted anti-inflammatory effects on LPS-activated macrophages [50]. Our results also demonstrated that EGCG had the most powerful anti-inflammatory effects compared with other green tea polyphenols. The polyphenols of green tea with pyrogallol-type structure, such as gallic acid and EGCG exerted anti-inflammatory effect on LPS-activated RAW264 cells. Interestingly, (-)-catechin is a polyphenol in green tea without pyrogallol-type structure, but also repressed the production of TNF-α and IL-6 of LPS-stimulated RAW264 cells. In the previous study, Singh et al. demonstrated that catechin attenuated the production of TNF-α by LPS-activated human monocytic cell line THP-1 cells [66]. The form of catechin

includes (-)-catechin, (+)-catechin, and (±)-catechin hydrate. The form of catechin which was investigated in this previous study was not explained. It might be (-)-catechin or (±)-catechin hydrate, not (+)-catechin.

LPS increased both phosphorylation and total expression of p38 MAPK in the early stage, whereas it was diminished in the late stage [71, 72]. Our results demonstrated that polyphenols with a flavan-3-ol structure regulated phosphorylation and total expression of p38 MAPK by LPS-activated macrophages, but gallic acid neither enhanced nor attenuated phosphorylation and total expression of p38 MAPK. It suggests that the anti-inflammatory effects of gallic acid on LPS-stimulated RAW264 cells are not through regulation of p38 MAPK.

Chapter 3. The anti-inflammatory effects of bavachin in LPS-induced inflammation

3.1 Introduction of bavachin (*Psoralea corylifolia* L. (Fabaceae))

Bavachin is a flavonoid which is derived from the seed and fruit of *Psoralea corylifolia* L. [55, 73]. Bavachin is a selective estrogen-receptor modulator which regulates the activation of estrogen receptors ERα and ERβ [74]. Accordingly, bavachin improves estrogen deficiency-induced osteoporosis and activates proliferation of osteoblasts [75]. Bavachin enhances glucose uptake of adipocyte 3T3-L1 cells [76]. Notably, bavachin suppresses IL-6-triggered activation and phosphorylation of STAT3 in hepatoma Hep3B cells [58], reduces IL-1β-induced NF-κB activity and chemokine production in chondrocyte CHON-002 cells, and inhibits IL-1β-induced migration in monocyte THP-1 cells [56]. However, the anti-inflammatory effects of bavachin on LPS-induced inflammation are unclear.

3.2 Methods

Ethics statement

The experiments in this chapter were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the

National Institutes of Health. The animal care and experimental protocols were accepted by the Committee on the Ethics of Animal Experiments of the Kaohsiung Medical University (Permit Number: 104092).

Reagents

Bavachin was obtained from ChemFaces (Wuhan, China), and confirmed the endotoxin levels using limulus amebocyte lysate single test (Associates of Cape Cod, Inc., Falmouth, USA). The endotoxin levels in bavachin were fewer than 0.03 EU/mL. All cell culture reagents were obtained from Gibco-BRL (Life Technologies, Grand Island, USA). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), phosphatase inhibitor cocktail, protease inhibitor cocktail, Griess reagent, trypan blue, thioglycollate medium, LPS (from *Escherichia coli* O111:B4), RIPA buffer and PBS were purchased from Sigma Aldrich (St. Louis, USA). BCA assay, ECL chemiluminescence substrate and stripping buffer were purchased from Thermo Scientific (Waltham, USA). For Western blotting, rabbit antibodies against mouse phospho-JNK 1/2, JNK 1/2, phospho-ERK 1/2, ERK 1/2, phospho-p38 MAPK, p38 MAPK and NLRP3 were purchased from Cell Signaling (Farmingdale, USA). Mouse antibody against caspase-1 (p20) was purchased from Adipogen (San Diego, USA).

Mouse antibody against β-tubulin was purchased from Thermo Scientific (Waltham, USA). Rabbit antibodies against mouse ASC, IL-1β, iNOS, COX-2, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Rabbit antibody against mouse mPGES-1 and PGE_2 ELISA kit were purchased from Cayman Chemical Company (Ann Arbor, USA). IL-1β, IL-6 and IL-12p40 ELISA kits were purchased from eBioscience (San Diego, USA).

Cell culture

Murine macrophage cell line J774A.1 cells were obtained from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan), and were cultured in RPMI-1640 medium with 10% FBS, penicillin (100 units/mL) and streptomycin (100 units/mL) and passaged every other day to maintain growth at 37 ˚C in humidified incubator containing 5% CO_2. Murine peritoneal macrophages were harvested from C57BL/6 mice. The specific pathogen-free female C57BL/6 mice were obtained from the BioLASCO Taiwan., Co. Ltd (Taipei, Taiwan). The female C57BL/6 mice (6~8 week of age) were intraperitoneally injected with 1 mL sterile 3% thioglycollate medium and peritoneal macrophages were harvested after 4 d. The peritoneal macrophages were confirmed 95% viability using trypan blue dye

exclusion assay, and were re-suspended in RPMI-1640 medium. The cells were seeded at a concentration of 1×10^6 cells/mL in 96-well plates to allow macrophage adherence at 37 ˚C in a humidified incubator containing 5% CO_2.

NO determination and cell viability

J774A.1 cells and murine peritoneal macrophages were seeded at a concentration of 1×10^6 cells/mL in 96-well plates. The cells were treated with bavachin (0, 10, 20, 30 and 40 μM) for 1 h prior to LPS (1 μg/mL) treatment. After 24 h, the supernatant was harvested and the NO concentration was measured using the Griess reagent according to the manufacturer's instruction (Sigma Aldrich, St. Louis, USA). The cells were incubated with MTT at 37 ˚C for 4 h, and then lysed with isopropanol. The cell viability was analyzed by estimating the OD at 570 nm.

Cytokine measurement

J774A.1 cells and murine peritoneal macrophages were seeded at a concentration of 1×10^6 cells/mL in 96-well plates, and were allowed to acclimatize overnight. The cells were treated with of bavachin (0, 10, 20, 30 and 40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. The supernatant was used for quantification of PGE_2, IL-6 and

IL-12p40 concentrations by ELISA according to the manufacturer's protocols (PGE_2: Cayman Chemical Company, Ann Arbor, USA) (IL-6 and IL-12p40: eBioscience, San Diego, USA).

NF-κB promoter reporter assay

J774A.1 cells were stably transfected with pNiFty2-SEAP plasmids which carry a reporter gene induced by NF-κB, and secrete embryonic alkaline phosphatase (SEAP). These cells are called J-Blue cells [77]. J-Blue cells were cultured in RPMI-1640 medium supplemented with Zeocin (200 μg/mL) (InvivoGen, San Diego, USA). Cells were seeded at a concentration of 1×10^6 cells/mL in 96-well plates, and were allowed to acclimatize overnight, and then treated with bavachin (0, 20 and 40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. The supernatant was mixed with QUANTI-Blue medium (InvivoGen, San Diego, USA) and incubated at 37 ˚C for 45 min. SEAP activity was assessed by measuring the OD at 655 nm.

NLRP3 inflammasome experiment

J774A.1 cells were seeded at a concentration of 1×10^6 cells/mL in 6-well plates, and were allowed to acclimatize overnight. The cells were treated with LPS (1 μg/mL) for 5

h, and then treated with bavachin (0, 10, 20, 30 and 40 μM) for 1 h following treatment of ATP (5 mM) for 30 min. The supernatant was harvested and the concentration of IL-1β was measured using ELISA according to the manufacturer's instructions (eBioscience, San Diego, USA), and the cell lysate was used for analysis of NLRP3 inflammasome protein levels by Western blotting.

Western blotting

The whole cell proteins were separated on 8~15% SDS-PAGEs and were transferred onto PVDF membranes. The protein expression levels were detected using antibodies against phospho-JNK 1/2, JNK 1/2, phospho-ERK 1/2, ERK 1/2, phospho-p38 MAPK, p38 MAPK, iNOS, mPGES-1, COX-2, NLRP3, ASC, IL-1β, caspase-1 (p20) and β-tubulin. The protein band signals were developed by ECL chemiluminescence substrate and quantified by Alphaview SA software (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis

All data are expressed as means ± SD. Each value is the mean of three independent experiments. GraphPad Prism 5 (San Diego, CA, USA) was used to determine the *p*

values using one-way ANOVA followed by Tukey post-hoc test, and the significant difference was set at *$p < 0.05$; **$p < 0.01$.

3.3 Results and figures

Bavachin suppressed the LPS-induced production of NO and PGE_2 by J774A.1 cells

To confirm the effect of bavachin on the cell viability of J774A.1 cells, the cells were treated with bavachin (0~40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. The treatment of bavachin (0~40 μM) did not affect the cell viability (Fig. 6A). To avoid the cytotoxicity of bavachin, the maximum concentration of bavachin used in the following experiments was 40 μM. To examine the effects of bavachin on NO and PGE_2, in LPS-activated J774A.1 cells, the cells were treated with bavachin (0~40 μM) for 1 h, and then treated with LPS (1 μg/mL) for 24 h. These results indicated that bavachin significantly decreased the production of NO and PGE_2 by LPS-stimulated J774A.1 cells in a concentration-dependent manner (Fig. 6B and C).

Bavachin reduced LPS-induced the expression of iNOS and mPGES-1, but did not affect the expression of COX-2

NO is synthesized by iNOS, and PGE_2 is converted by COX-2 and mPGES-1 in

LPS-activated macrophages. For the reason that bavachin decreased LPS-induced NO and PGE_2 production, the effects of bavachin on the expression levels of iNOS, COX-2 and mPEGS-1 in LPS-activated J774A.1 cells were treated with bavachin (0, 20 and 40 μM) for 1 h prior to with or without LPS (1 μg/mL) treatment for 16 h. LPS increased the expression levels of iNOS, COX-2 and mPGES-1 dramatically. Additionally, bavachin suppressed the LPS-induced expression of iNOS and mPGES-1 concentration-dependently, but did not affect the expression of COX-2 (Fig. 7 A and B).

Bavachin decreased LPS-induced production of IL-6 and IL-12p40 by J774A.1 cells

To examine the effects of bavachin on LPS-induced production of IL-6 and IL-12p40, J774A.1 cells were treated with bavachin (0~40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. The treatment of bavachin at 40 μM significantly decreased the production of IL-6 and IL-12p40 production (Fig. 8A and B).

Bavachin suppressed LPS-stimulated phosphorylation of JNK 1/2 and ERK 1/2, but did not affect the phosphorylation of p38 MAPK

To investigate the effect of bavachin on LPS-induced MAPKs activation, cells were treated with bavachin (0, 20 and 40 μM) for 1 h prior to with or without LPS (1 μg/mL)

treatment for 1 h. LPS markedly increased the phosphorylation levels of JNK 1/2, ERK 1/2 and p38 MAPK. Furthermore, bavachin significantly inhibited LPS-induced phosphorylation of JNK 1/2 and ERK 1/2, but not p38 MAPK (Fig. 9A and B).

Bavachin suppressed LPS-induced activation of NF-κB by J-Blue cells

To confirm the effect of bavachin on NF-κB activity by detection of SEAP activity, J-Blue cells were treated with bavachin (0, 20 and 40 μM) for 1 h prior to with or without LPS (1 μg/mL) treatment for 24 h. LPS increased approximately 3-fold NF-κB activity. Additionally, bavachin significantly suppressed LPS-induced NF-κB activity at both 20 μM and 40 μM (Fig. 9C).

Bavachin inhibited NLRP3 inflammasome-derived IL-1β production and caspase-1 activation

To investigate the effects of bavachin on NLRP3 inflammasome-derived IL-1β production and NLRP3 inflammasome complex formation, cells were treated with or without of LPS (1 μg/mL) for 5 h, and then treated with bavachin (0~40 μM) for 1 h following treatment of ATP (5 mM) for 30 min. The experimental results showed that the production of IL-1β was markedly increased by ATP-activated NLRP3

inflammasome. Bavachin significantly reduced NLRP3 inflammasome-primed production of IL-1β concentration-dependently (Fig. 10A). In addition, ATP-activated NLRP3 inflammasome increased the active form of caspase-1 p20 protein expression level. Bavachin decreased the expression of caspase-1 p20 and mature IL-1β protein (Fig. 10B).

Bavachin reduced the production of NO, IL-6 and IL-12p40 by murine peritoneal macrophages

To examine the effects of bavachin in LPS-stimulated murine peritoneal macrophages, cells were treated with bavachin (0~40 μM) for 1 h prior to with or without LPS (1 μg/mL) treatment for 24 h. The experimental results indicated there was no toxic effect of bavachin treatment on the cell viability (Fig. 11A). Subsequently, bavachin significantly suppressed LPS-induced production of NO, IL-6 and IL-12p40 by LPS-activated murine peritoneal macrophages concentration-dependently (Figure 11B, C and D).

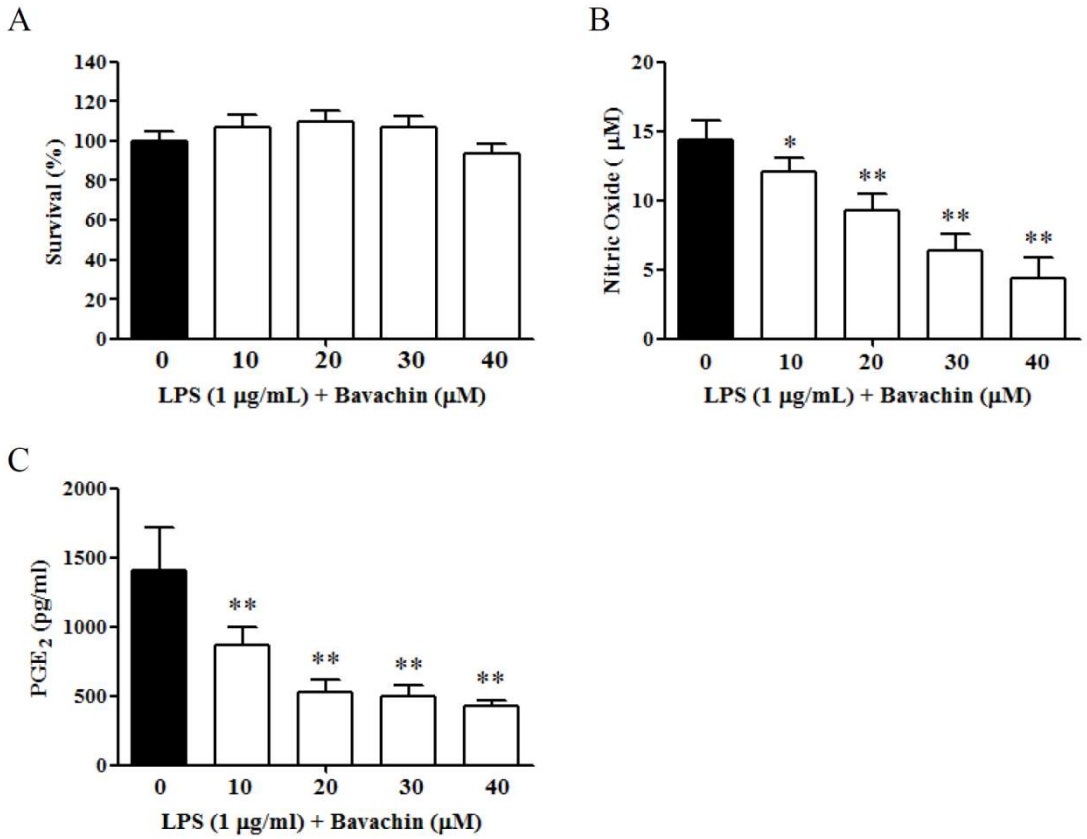

Fig. 6. The effects of bavachin on the cell viability and the production of NO and PGE_2 by LPS-stimulated J774A.1 cells. Cells were treated with bavachin (0~40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. (A) The cell viability. (B) The concentration of NO and (C) PGE_2 in the supernatant. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ and ** $p< 0.01$ vs LPS alone.

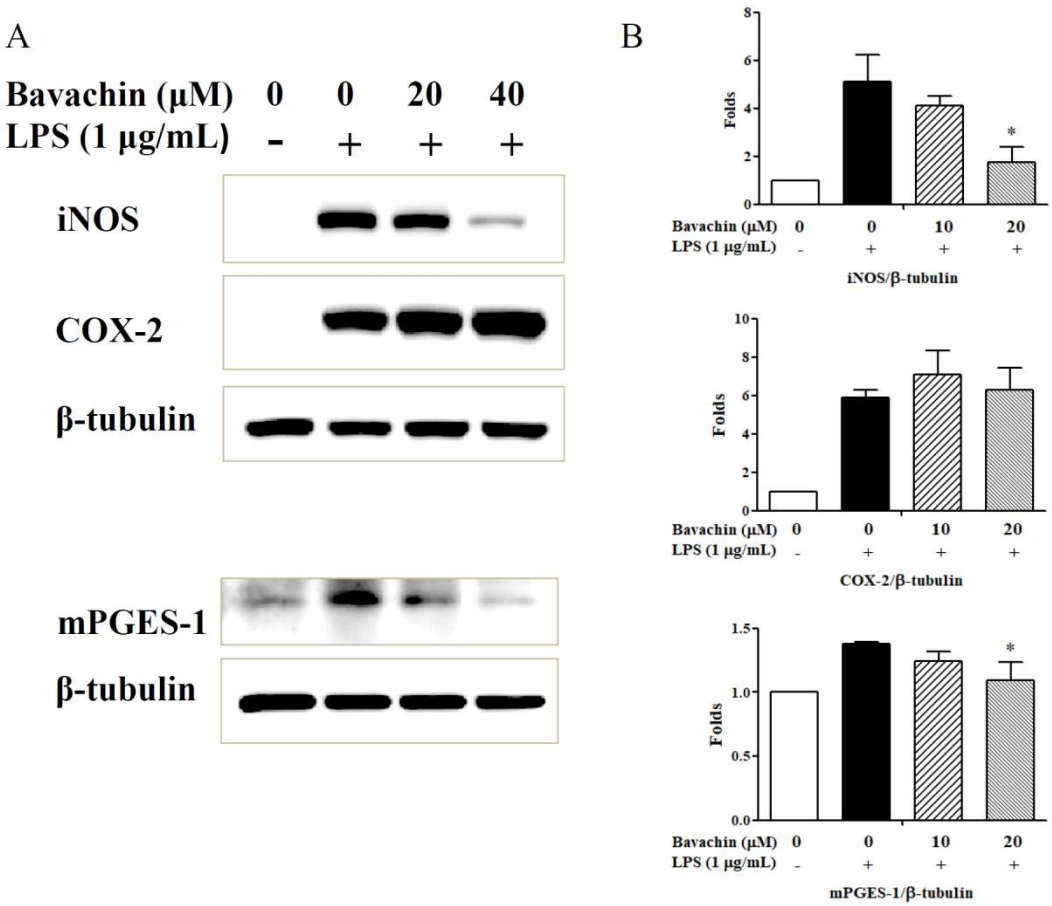

Fig. 7. The effects of bavachin on the expression of iNOS, COX-2 and mPGES-1 by LPS-activated J774A.1 cells. Cells were treated with different bavachin (0, 20 and 40 μM) for 1 h prior to LPS (1 μg/mL) treatment. After 16 h, cell lysates were harvested. (A) The representative of Western blotting results was obtained in three separate experiments. (B) The intensities of bands were quantified from three separate experiments and normalized to untreated samples. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ vs LPS alone.

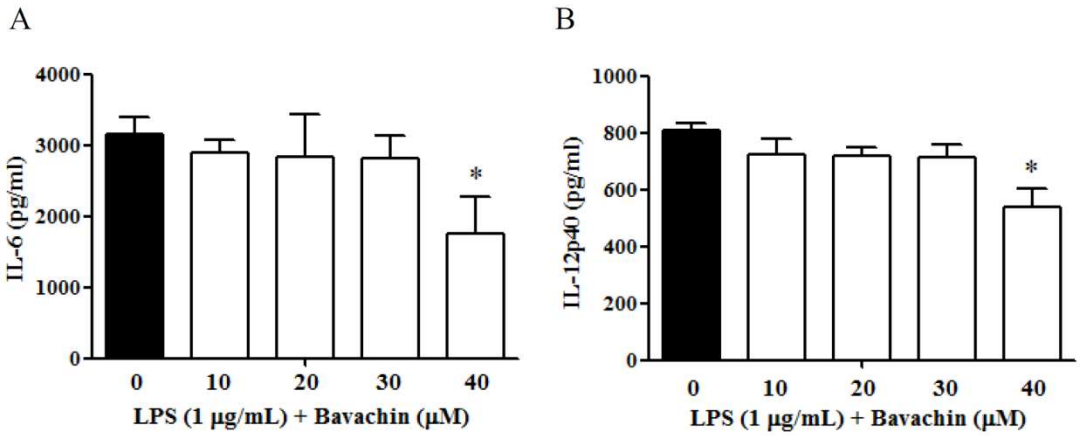

Fig. 8. The effects of bavachin on the production of IL-6 and IL-12p40 by LPS-stimulated J774A.1 cells. Cells were treated with bavachin (0~40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. The supernatant was collected. The concentrations of (A) IL-6 and (B) IL-12p40 in the supernatant. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ vs LPS alone.

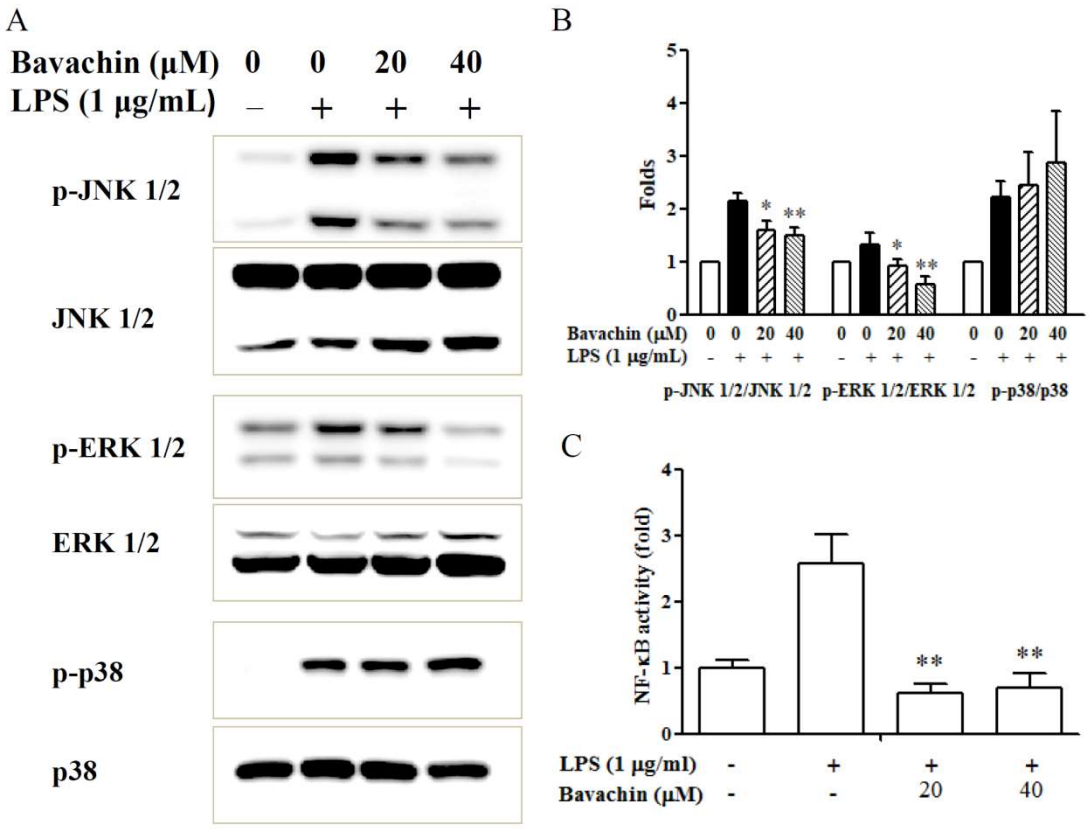

Fig. 9. The effects of bavachin on the activation of MAPKs by LPS-stimulated J774A.1 cells, and the activity of NF-κB in LPS-stimulated J-Blue cells. J774A.1 cells were treated with bavachin (0, 20 and 40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 1 h. (A) The expression levels of p-JNK 1/2, JNK 1/2, p-ERK 1/2, ERK 1/2, p-p38 MAPK and p38 MAPK. The representative of Western blotting results was shown. (B) The intensities of bands were quantified from three separate experiments and normalized to untreated samples. (C) J-Blue cells were treated with bavachin (0, 20 and 40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. The supernatant was collected for SEAP activity assay. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ and ** $p< 0.01$ vs LPS alone.

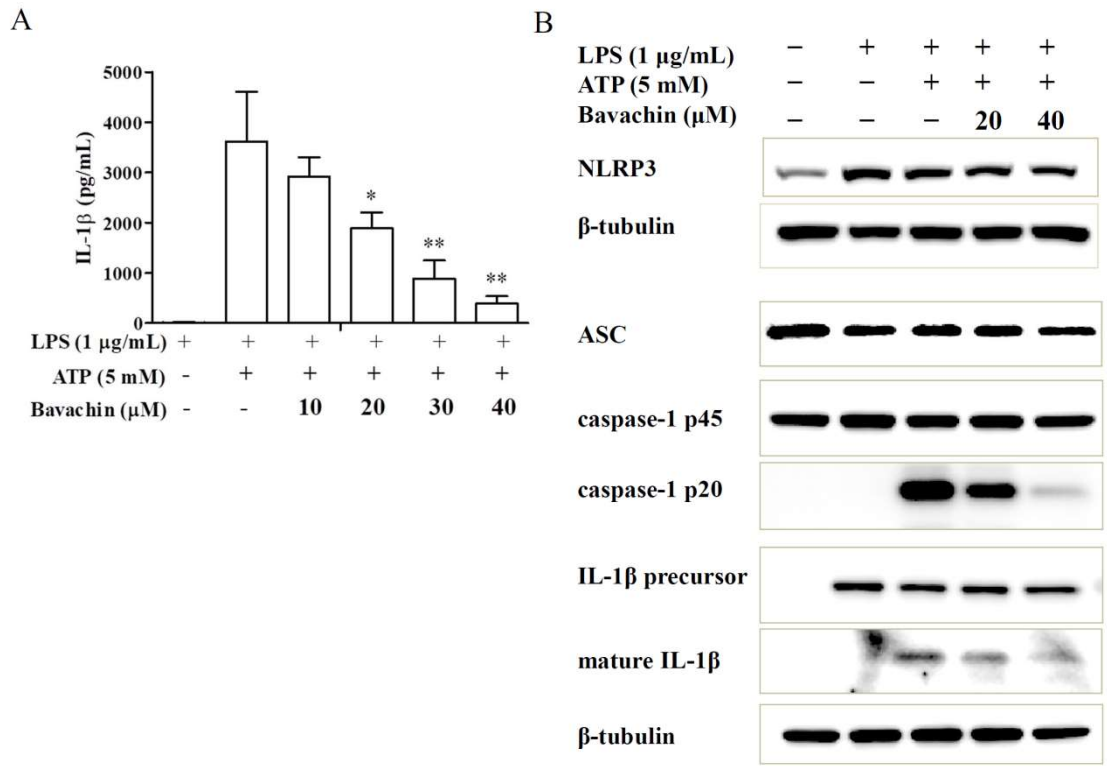

Fig. 10. The effects of bavachin on the production of IL-β and the expression of NLRP3 inflammasome complex. J774A.1 cells were treated with LPS (0 and 1 μg/mL) for 5 h, and then treated with bavachin (0~40 μM) for 1 h following treatment of ATP (5 mM) for 30 min. (A) The concentration of IL-1β in the supernatant. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ and ** $p< 0.01$ vs LPS plus ATP alone. (B) The expression levels of NLRP3, ASC, capase-1, IL-1β and β-tubulin were analyzed using Western blotting.

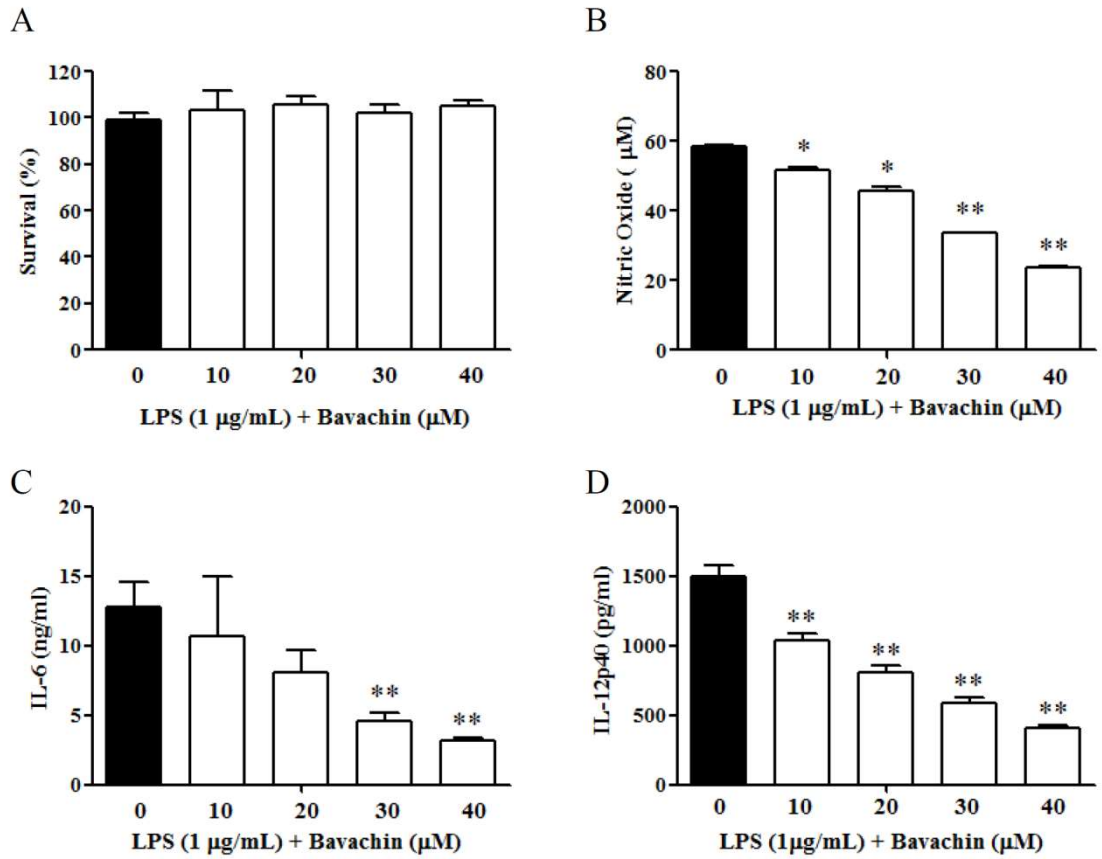

Fig. 11. The effects of bavachin on the production of NO, IL-6 and IL-12p40 by LPS-stimulated murine peritoneal macrophages. Cells were treated with bavachin (0~40 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. (A) The cell viability. (B) The concentration of NO, (C) IL-6 and (D) IL-12p40 in the supernatant. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ and ** $p< 0.01$ vs LPS alone.

3.4 Discussion

These experimental results demonstrated that bavachin which a flavonoid is derived from *Psoralea corylifolia* L. decreased LPS-stimulated production of PGE_2 and NO via the inhibitory effects on mPGES-1 and iNOS. In addition, bavachin suppressed LPS-induced IL-6 and IL-12p40 production, and inhibited MAPKs and NF-κB activation. On the other hand, bavachin suppressed NLRP3 inflammasome-primed IL-1β production and reduced caspase-1 activation and mature IL-1β protein expression level. Moreover, bavachin also reduced the production of NO, IL-6 and IL-12p40 in LPS-stimulated murine peritoneal macrophages.

iNOS catalyzes the reaction in which L-Arginine converts to NO, and NO is oxidized to cytotoxic $ONOO^-$ by O_2^-. Overproduction of NO leads to tissue destruction in several inflammatory diseases [78]. Bavachin not only decreased LPS-induced NO production by J774A.1 cells, but also exerted inhibitory effect on LPS-induced NO production by murine peritoneal macrophages. The experimental results suggested that bavachin suppressed LPS-induced production of NO through decreasing iNOS expression.

PGE_2 crucially contributes to edema, fever and pain, and increases vascular

permeability and immune cell infiltration. The process of PGE_2 synthesis is COX-2 transforms AA to PGH_2, and then mPGES-1 converts PGH_2 to PGE_2 [23]. These results indicated that bavachin inhibited LPS-induced PGE_2 production and mPGES-1 expression, but did not decrease COX-2 expression. Thus, it suggests that bavachin reduces the PGE_2 production through inhibitory effect on mPGES-1 (PGH_2 converts to PGE_2), not COX-2 (AA converts to PGH_2).

IL-6/IL-12 family includes several similar structure in cytokines which share the subunits to induce inflammation and regulate adaptive immune system [26]. IL-6 elicits activity of B and T cells [25], and IL-12 induces T-cell differentiation [79]. Our results demonstrated that bavachin not only suppressed LPS-induced IL-6 and IL-12p40 production by J774A.1 cells, but also exerted inhibitory effects on IL-6 and IL-12p40 production by LPS-activated murine peritoneal macrophages. Taken together, bavachin reduced pro-inflammatory cytokine IL-6 and IL-12p40 by LPS-activated macrophages.

IL-1β is considered that is a critical contributor to several immune-related diseases. NLRP3 inflammasome crucially contribute to prime mature IL-1β, and is associated with pathogenesis of several immune-related diseases [12]. Our results indicated that

bavachin inhibited NLRP3 inflammasome-primed caspase-1 activation and IL-1β production. It is suggested that bavachin is a potentially anti-inflammatory supplement candidates in the treatment of these immune-related diseases.

Chapter 4. The anti-inflammatory effects of corylin in LPS-induced inflammation

4.1 Introduction of corylin (*Psoralea corylifolia* L. (Fabaceae))

Corylin is another flavonoid which derived from *Psoralea corylifolia* L. [52, 55]. It has been reported that corylin exerts antioxidant activities [80] and increases osteoblastic proliferation in UMR106 cells [81]. In addition, corylin suppresses IL-6-triggered STAT3 activation in Hep3B cells [58]. Nevertheless, the effects of corylin on LPS-induced inflammation are unclear.

4.2 Methods

Ethics statement

The animal care and experimental protocols were approved by the same committee as Chapter 3 (Permit Number: 104092).

Reagents

Corylin was obtained from ChemFaces (Wuhan, Hubei, PRC), and endotoxin levels in corylin were confirmed fewer than 0.03 EU/mL. HMGB1 ELISA kit was obtained from Aviva Systems Biology (San Diego, USA). Nuclear protein isolation-translocation assay kit was obtained from Fivephoton Biochemicals (San Diego, USA). Mouse antibody

against mouse HMBG1 was obtained from OriGene Technologies, Inc. (Rockville, USA). Rabbit antibody against mouse Lamin A/C was obtained from GeneTex, Inc. (Irvine, USA). The all cell culture, NO determination, cell viability, cytokine measurement, NF-κB promoter reporter assay and Western blotting reagents were the same as the reagents in Chapter 3.

Cell culture

Murine macrophage cell line RAW264.7 cells were obtained from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan), and were cultured in RPMI-1640 medium with 10% FBS, penicillin (100 units/mL) and streptomycin (100 units/mL) in humidified incubator containing 5% CO_2 at 37 ˚C and maintained growth by being passaged every other day. In addition, the protocols of murine peritoneal macrophage isolation and culture were the same as the protocols in Chapter 3. The cells were seeded at a concentration of 1×10^6 cells/mL in 96-well plates for cell viability, NO determination, and cytokine measurement.

Experimental protocols

The experimental protocols of cell viability (MTT assay), NO determination (Griess

reagent), cytokine measurement (ELISA), NF-κB promoter reporter assay (J-Blue cells) and Western blotting were the same as the experimental protocols in Chapter 3.

LPS-induced systemic inflammation and sepsis mouse model

The experiments were carried out in the following groups: DMSO group (vehicle control), LPS group (LPS plus DMSO); LPS plus corylin (20 mg/kg body weight) group and LPS plus corylin (40 mg/kg body weight). Mice were intraperitoneally administered with DMSO or corylin. After 1 h, experimental systemic inflammation and sepsis were induced by intraperitoneally injecting LPS (50 mg/kg body weight). The mouse serum samples were harvested for 4 h after LPS administration for the measurement of NO and TNF-α. Survival was monitored for 120 h after LPS administration.

Histopathology and Biochemistry analysis

Mice were intraperitoneally injected with DMSO or corylin. After 1 h, experimental sepsis was induced by intraperitoneally injecting LPS (50 mg/kg body weight). After LPS administration for 20 h, tissues were harvested and fixed with 4% formaldehyde solution, and then embedded in paraffin and sectioned. The tissue sections were

counterstained with hematoxylin and eosin (H&E). The mouse blood samples were harvested and quantified the activities of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), creatinine (CRE) and blood urea nitrogen (BUN).

Statistical analysis

All data are expressed as means ± standard deviation (SD). Each value is the mean of three independent experiments. GraphPad Prism 5 (San Diego, CA, USA) was used to determine the *p* values using one-way ANOVA followed by Tukey post-hoc test, and survival analysis was performed using Log-Rank test. The significant difference was set at $*p < 0.05$; $**p < 0.01$.

4.3 Results, figures and tables

Corylin inhibited LPS-induced PGE_2 and NO production and COX-2 and iNOS expression by RAW264.7 cells

To elude the cytotoxicity of corylin, RAW264.7 cells were treated with corylin (0, 2.5, 5, 10 and 20 μM) for 1 h prior to LPS (1 μg/ml) treatment for 24 h. The results indicated that all concentration of corylin treatment did not affect cell viability (Fig. 12A). To examine the effect of corylin on LPS-induced production of NO and PGE_2, cells were

treated with corylin (0, 2.5, 5, 10 and 20 μM) for 1 h prior to LPS (1 μg/ml) treatment for 24 h. Corylin dramatically reduced LPS-induced production of PGE_2 and NO (Fig. 12B and C). Additionally, corylin reduced LPS-induced expression of COX-2 and iNOS (Fig. 12D).

Corylin suppressed LPS-induced production of TNF-α, IL-6 and HMGB1 in RAW264.7 cells

RAW264.7 cells were treated with corylin (0, 2.5, 5, 10 and 20 μM) for 1 h prior to LPS (1 μg/ml) treatment for 24 h. Corylin reduced LPS-stimulated production of TNF-α, IL-6 and HMGB1 concentration-dependently (Fig. 13A, B and C). In addition, LPS increased translocation of nucleus HMGB1 into cytoplasm. Corylin inhibited LPS-induced translocation of nucleus HMGB1 (Fig. 13D).

Corylin suppressed LPS-induced phosphorylation of MAPKs by RAW264.7 cells, and LPS-induced activation of NF-κB in J-Blue cells.

These experimental results indicated that LPS dramatically increased phosphorylation of MAPKs, and corylin significantly reduced LPS-stimulated phosphorylation of MAPKs (Fig. 14A and B). In addition, activation of NF-κB was markedly increased by

LPS, and corylin also significantly suppressed LPS-induced NF-κB activity by J-Blue cells (Fig. 14C).

Corylin exerted inhibitory effects on LPS-induced production of NO, TNF-α and IL-6 in murine peritoneal macrophages

To confirm the effects of corylin in LPS-stimulated murine peritoneal macrophages, cells were treated with corylin (0, 2.5, 5, 10 and 20 μM) for 1 h prior to LPS (1 μg/ml) treatment for 24 h. As shown in Fig. 15A, the results indicated that all concentrations of corylin treatment did not affect cell viability of murine peritoneal macrophages. Additionally, corylin dramatically reduced LPS-induced production of NO, TNF-α and IL-6 *ex vivo* (Fig. 15B, C and D).

Corylin repressed LPS-induced tissue damage in LPS-treated mice

Mice were intraperitoneally administered with DMSO or corylin. After 1 h, experimental systemic inflammation and sepsis were induced by intraperitoneally injecting LPS (50 mg/kg body weight). After LPS administration for 20 h, lung, liver and kidney were damaged by LPS-induced systemic inflammation. It was observed that the alveolar wall swelling was augmented, and pulmonary alveoli were decreased in

LPS-stimulated lung damage. The treatment of corylin improved the alveolar wall swelling, and increased the LPS-declined the pulmonary alveolus. On LPS-induced liver damage, the treatment of corylin repressed LPS-induced cavities of liver tissue and infiltration of inflammatory cells. On LPS-induced kidney damage, it decreased brush borders and renal epithelial cells, and exhibited that renal tubular epithelial cells were sloughed in LPS-challenged mice. The treatment of corylin inhibited LPS-induced decreases in epithelial cells and brush borders, and reduced the sloughing of tubular epithelial cells in kidney (Fig. 16). In addition, AST, ALT, BUN and CRE were dramatically elevated by LPS administration. The treatment of corylin significantly decreased LPS-induced increases in AST, ALT, BUN and CRE (Tab. 1).

Corylin reduced the production of NO and TNF-α in serum, and increased the survival rate of LPS-treated mice

Mice were intraperitoneally administered with DMSO or corylin. After 1 h, experimental systemic inflammation and sepsis were induced by intraperitoneally injecting LPS (50 mg/kg body weight). After LPS administration for 4 h, the blood samples were harvested. The survival was monitored for 120 h after LPS administration. The results suggested the treatment of corylin reduced the levels of NO and TNF-α in

serum after LPS administration (Fig. 17A and B). Furthermore, the treatment of corylin increased survival rate significantly (Fig. 17C).

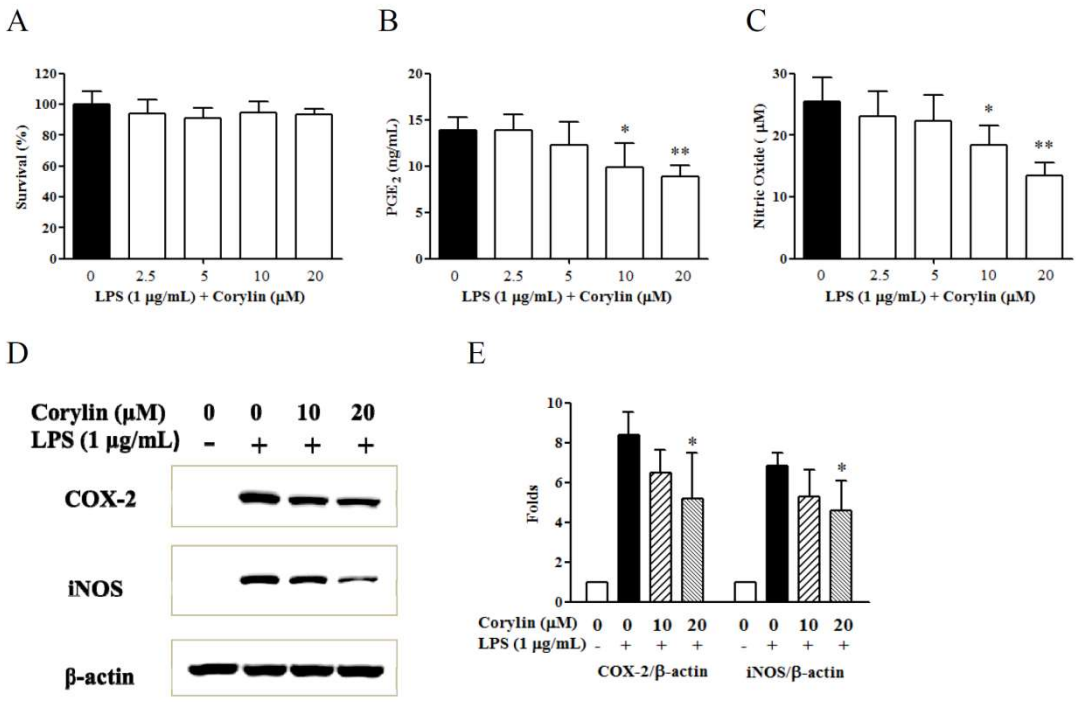

Fig. 12. The effects of corylin on LPS-induced PGE_2 and NO production, and COX-2 and iNOS expression in RAW 264.7 cells. Cells were treated with corylin (0~20 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. (A) The cell viability. (B) The concentration of PGE_2 and (C) NO in the supernatant. Cells were treated with corylin (0, 10 μM and 20 μM) for 1 h prior to LPS (1 μg/mL) treatment for 6 h. (D) The expression levels of COX-2 and iNOS. (E) The intensities of bands were quantified from three separate experiments and normalized to untreated samples. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ vs LPS alone.

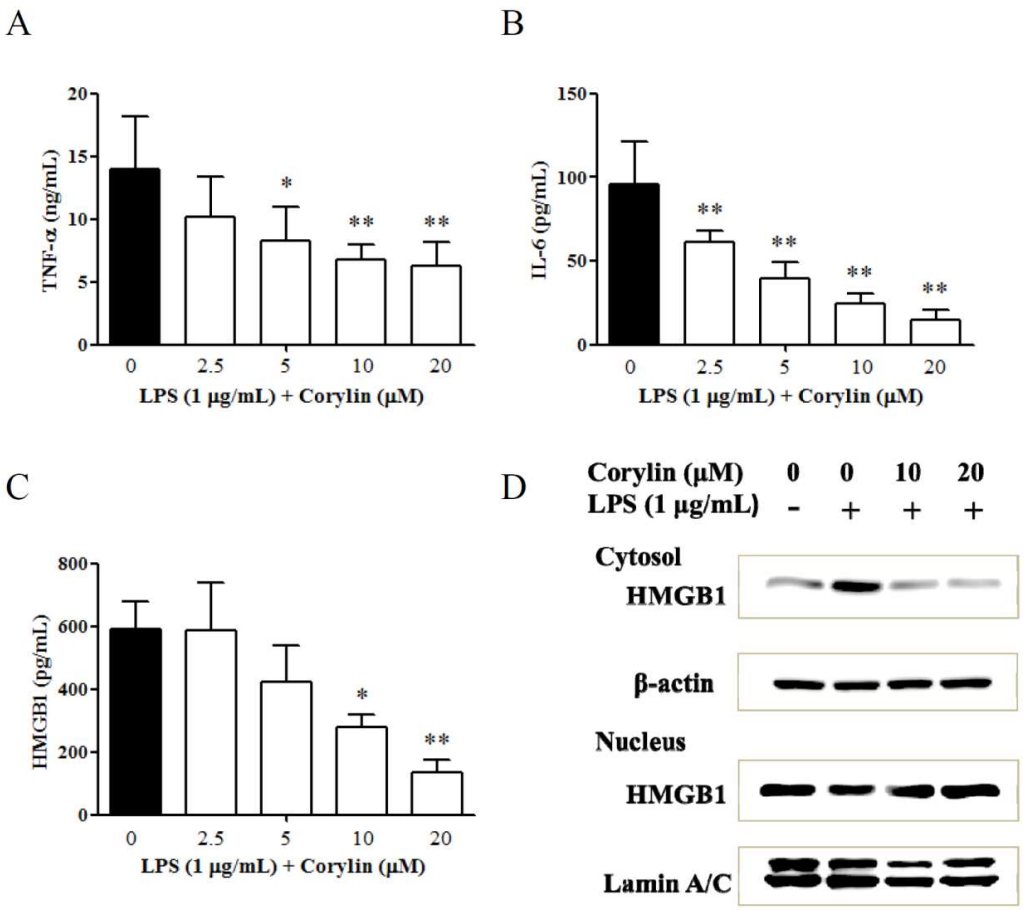

Fig. 13. The effects of corylin on LPS-induced TNF-α, IL-6 and HMGB1 production in RAW 264.7 cells. Cells were treated with corylin (0~20 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. The concentrations of (A) TNF-α, (B) IL-6 and (C) HMGB1 in the supernatant. Cells were treated with corylin (0, 10 μM and 20 μM) for 1 h prior to LPS (1 μg/mL) treatment for 6 h. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ vs LPS alone. (D) The expression of HMGB1 in nucleus and cytosol were determined by Western blotting.

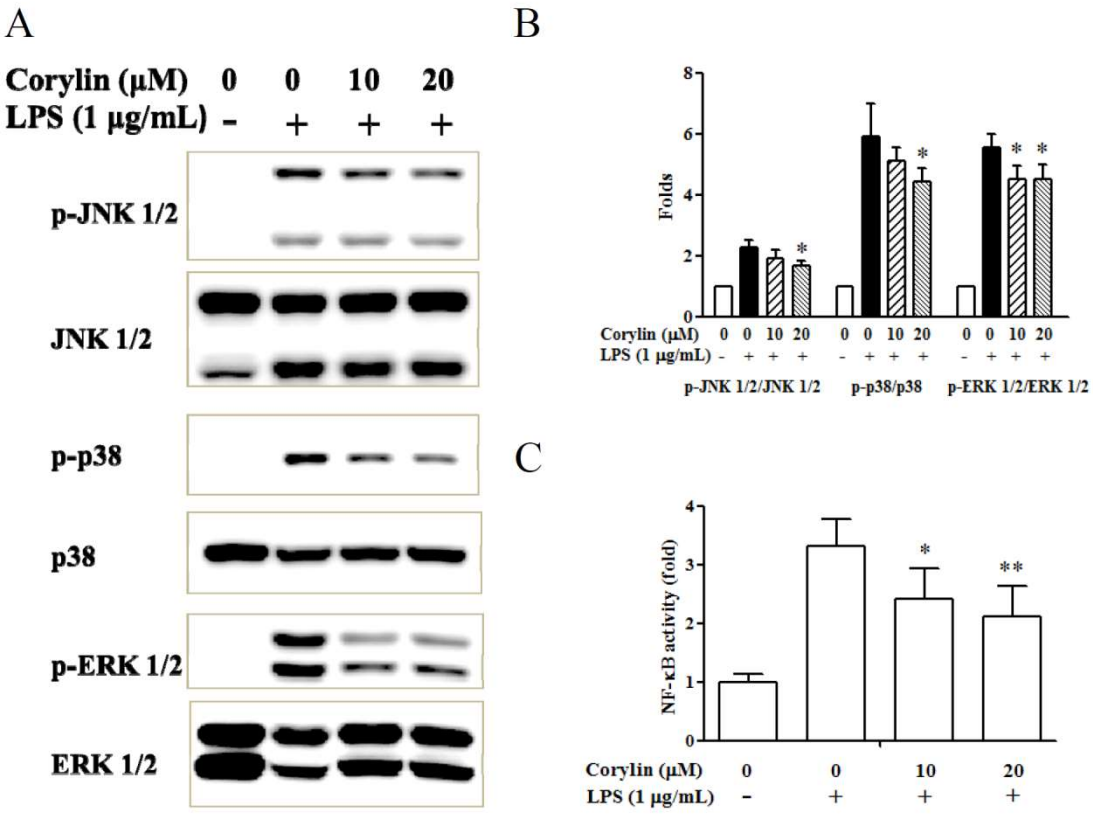

Fig. 14. The effects of corylin on LPS-induced phosphorylation of JNK 1/2, p38 MAPK and ERK 1/2 in RAW 264.7 cells and the LPS-induced activation of NF-κB in J-Blue cells. Cells were treated with corylin (0, 10 μM and 20 μM) for 1 h prior to LPS (1 μg/mL) treatment for 30 min. (A) The expression levels of p-JNK 1/2, JNK 1/2, p-ERK 1/2, ERK 1/2, p-p38 MAPK and p38 MAPK. The representative of Western blotting results was obtained in three separate experiments. (B) The intensities of bands were quantified from three separate experiments and normalized to untreated samples. (C) J-Blue cells were treated with corylin (0, 10 and 20 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. The supernatant was collected for SEAP activity assay. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ and ** $p< 0.01$ vs LPS alone.

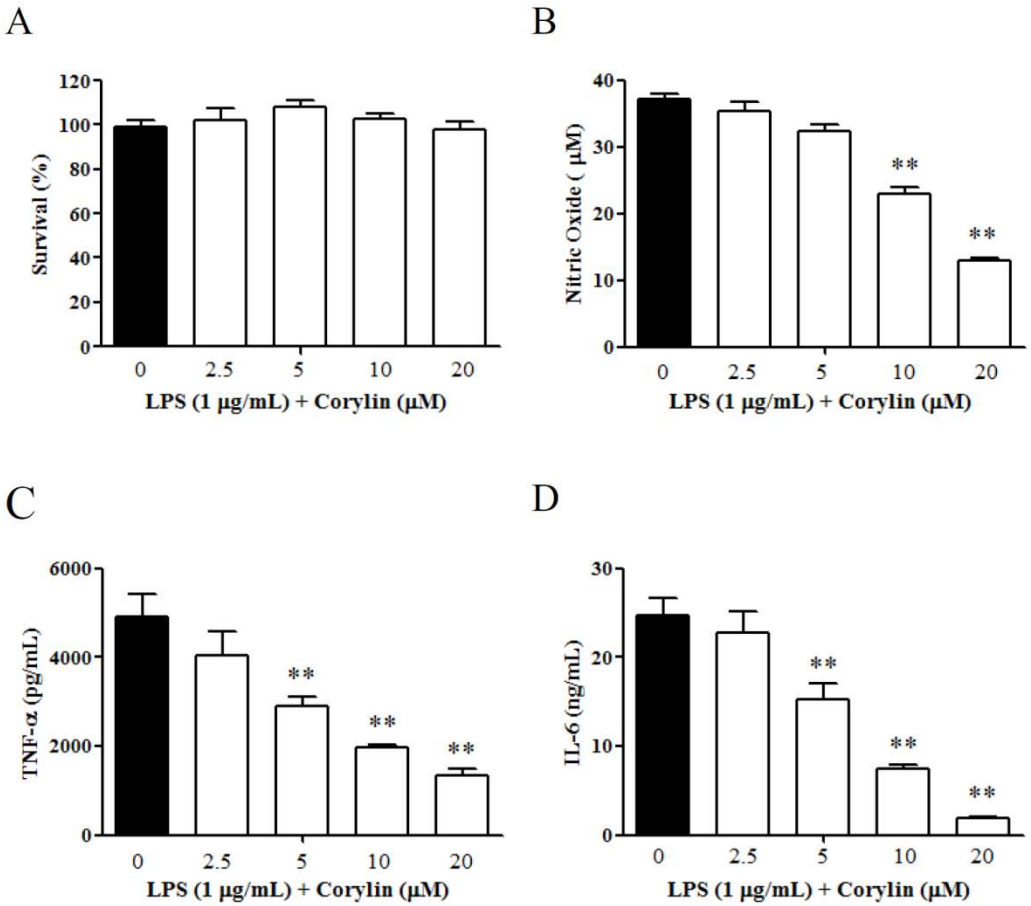

Fig. 15. The effects of corylin on LPS-induced NO, TNF-α and IL-6 production in murine peritoneal macrophages. Cells were treated with corylin (0~20 μM) for 1 h prior to LPS (1 μg/mL) treatment for 24 h. (A) The cell viability. (B) The concentrations of NO, (C) TNF-α and (D) IL-6 in the supernatant. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p< 0.05$ vs LPS alone.

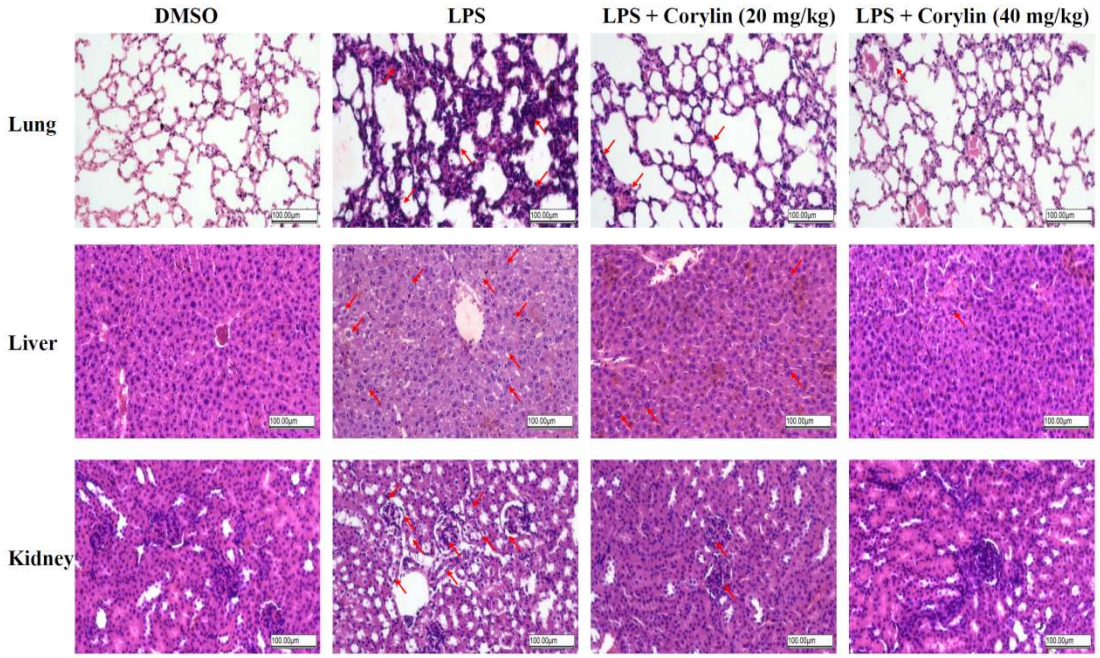

Fig. 16. The effects of corylin on LPS-induced tissue damage of lung, liver and kidney in mice. Female C57BL/6 mice were treated with DMSO or corylin (20 or 40 mg/kg, i.p.) for 1 h prior to LPS administration (50 mg/kg, i.p.). After LPS administration for 20 h, lung, liver and kidney tissues were harvested. The results show H&E-staining (× 200). The red arrows indicate the infiltration of inflammatory cells and damage sites.

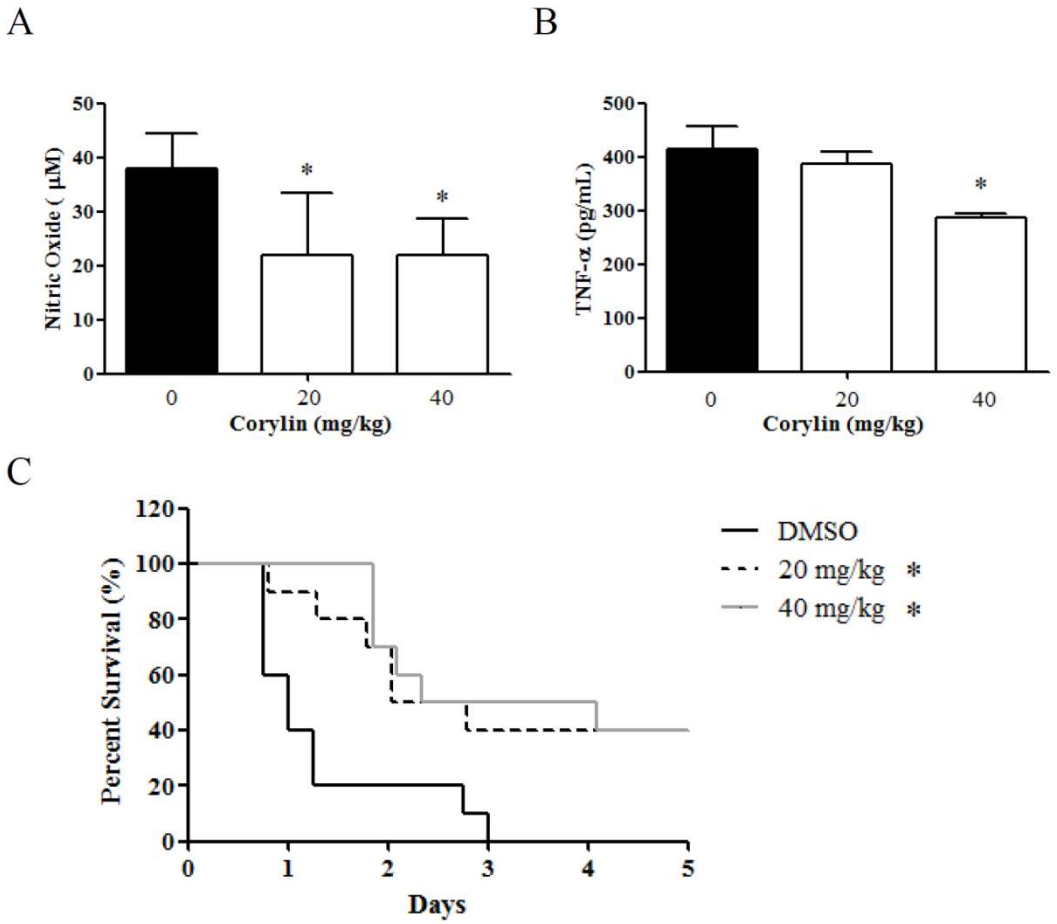

Fig. 17. The effects of corylin on NO and TNF-α concentrations in serum and survival rate by LPS-treated mice. Female C57BL/6 mice were treated with DMSO or corylin (20 or 40 mg/kg, i.p.) for 1 h prior to LPS administration (50 mg/kg, i.p.). After LPS administration for 4 h, the blood samples were collected. The concentration of (A) NO and (B) TNF-α in the mouse serum. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: * $p<$ 0.05 and ** $p<$ 0.01 vs LPS alone. (C) The survival was monitored for 120 h (n=10). Statistical significance was represented as follows: * $p<$ 0.05 vs DMSO.

Tab. 1. The effects of corylin on LPS-induced liver and kidney damage markers.

	DMSO	LPS	Corylin 20 mg/kg	Corylin 40 mg/kg
AST	200.00±3.00	619.75±97.39**	333.50±31.25+	289.00±34.53+
ALT	46.50±0.50	291.33±85.61*	72.40±5.61+	69.33±4.18+
BUN	28.40±1.20	150.50±11.93**	41.66±5.44++	40.3±9.90++
CRE	0.31±0.01	0.93±0.21**	0.26±0.04++	0.28±0.02++

Female C57BL/6 mice were treated with DMSO or corylin (20 or 40 mg/kg, i.p.) for 1 h prior to LPS administration (50 mg/kg, i.p.). After LPS administration for 20 h, the blood samples were harvested. The levels of ALT, AST, BUN and CRE were quantified (n=5). Statistical significance was represented as follows: *: $p<0.05$, **: $p<0.01$ vs DMSO; +: $p<0.05$, ++: $p<0.05$ vs LPS.

4.4 Discussion

The present results demonstrated that corylin suppressed LPS-stimulated TNF-α, IL-6, and NO production by RAW264.7 cells and murine peritoneal macrophages. Additionally, corylin decreased LPS-induced production of PGE_2 and HMBG1, and inhibited LPS-induced expression of iNOS and COX-2 and translocation of nucleus HMBG1 by RAW264.7 cells. It is suggested that corylin repressed LPS-induced inflammation through inhibitory effects on MAPKs and NF-κB. In LPS-stimulated systemic inflammation, the treatment of corylin repressed LPS-induced ALT, AST, BUN, CRE, NO and TNF-α, and improved LPS-induced tissue damage. Furthermore, the treatment of corylin decreased the mortality from LPS-induced systemic inflammation.

Bavachin and corylin are flavonoids that derived from *Psoralea corylifolia* L.. These experimental results suggested that corylin exerted stronger inhibitory effects on LPS-induced NO production by murine peritoneal macrophages than bavachin. The half maximal inhibitory concentration (IC_{50}) of corylin was approximate 20 μM, and bavachin was over 20 μM. Similar to bavachin, corylin also reduced LPS-induced production of NO and PGE_2, and inhibited LPS-induced expression of iNOS and

COX-2. It is suggested that bavachin reduced the PGE_2 production through inhibitory effect on mPGES-1 (PGH_2 converts to PGE_2), and corylin repressed the PGE_2 production through inhibitory effect on COX-2 (AA converts to PGH_2). In addition, corylin suppressed phosphorylation of JNK 1/2, p38 MAPK and ERK 1/2, but bavachin did not affect p38 MAPK. Furthermore, corylin inhibited the production and translocation of HMBG1. Thus, corylin was further confirmed the anti-inflammatory effects on LPS-stimulated systemic inflammation by LPS-treated mice (*in vivo*).

These experimental results indicated that mice exhibited inflammatory cascades (NO and TNF-α), multiple organ damage (lung, liver and kidney), sepsis and septic shock by LPS administration. The treatment of corylin improved LPS-induced tissue damage, and suppressed LPS-increased levels of ALT, AST, BUN and CRE. Additionally, corylin reduced LPS-induced production of NO and TNF-α, and increased the survival rate of LPS-triggered sepsis.

Chapter 5. Conclusions and suggestions for future research

The LPS-induced inflammation is broadly involved with development of many inflammatory diseases. Notably, TLR4-mediated signaling transduction pathway plays an essential role in LPS-induced inflammation. TLR4-mediated signaling such as MAPKs and NF-κB contribute to produce pro-inflammatory mediators and cytokines by LPS-stimulated macrophages. Particularly, the acute inflammatory cascades lead to systemic inflammatory response syndrome, sepsis, multiple organ failure, and even death. Unfortunately, there are no specific therapeutic agents or supplementation on treatment of exercise-induced systemic inflammation currently. Therefore, the anti-inflammatory effects of polyphenols which are isolated from *Camellia sinensis* (L.) O. Kuntze and *Psoralea corylifolia* L. on LPS-induced inflammation were investigated in this thesis.

These experimental results in Chapter 2 demonstrated EGCG exerted the strongest inhibitory effects on LPS-induced TNF-α and IL-6 production by RAW264 cells among polyphenols in green tea (*Camellia sinensis*). Additionally, pyrogallol-type structure might contributes to inhibit LPS-induced TNF-α and IL-6 production crucially, and flavan-3-ol structure regulates p38 MAPK. The green tea extracts (contain 41% EGCG)

exhibits anti-fatigue effect on swimming time [82], and enhance muscle strength recovery after downhill running [83]. Especially, green tea extract administration represses muscle inflammation induced by downhill running [83]. Thus, it suggests that EGCG is a potentially anti-inflammatory supplementation for treatment of exercise-induced inflammation.

The experimental results in Chapters 3 and 4 suggested that bavachin and corylin, the polyphenols which are derived from *Psoralea corylifolia* L., repressed production of pro-inflammatory mediators and cytokines by LPS-activated macrophages. In addition, bavachin and corylin inhibited LPS-triggered MAPK and NF-κB activity. Moreover, bavachin repressed NLRP3 inflammasome-derived IL-1β production and caspase-1 activation. Notably, corylin exhibited anti-inflammatory effect on LPS-induced systemic inflammation in mice (*in vivo*). Taken together, bavachin and corylin are potentially anti-inflammatory natural product candidates in the treatment of LPS-induced systemic inflammation (sepsis and exercise-induced endotoxemia).

In this thesis, the anti-inflammatory effects of polyphenols which are derived from plants were confirmed using cell culture system, and anti-inflammatory natural product

candidates were selected. The candidates were confirmed by the anti-inflammatory effects in animal study. These experimental protocols and results provide the investigation of new anti-inflammatory natural products for future research. To examine the effects of oral corylin administration in animal study and to investigate the anti-inflammatory effects of corylin in human study are the suggestions for future corylin research.

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References

[1] R. Medzhitov, Origin and physiological roles of inflammation, Nature 454(7203) (2008) 428-35.
[2] A. Iwasaki, R. Medzhitov, Regulation of adaptive immunity by the innate immune system, Science 327(5963) (2010) 291-5.
[3] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124(4) (2006) 783-801.
[4] S.L. Raymond, D.C. Holden, J.C. Mira, J.A. Stortz, T.J. Loftus, A.M. Mohr, L.L. Moldawer, F.A. Moore, S.D. Larson, P.A. Efron, Microbial recognition and danger signals in sepsis and trauma, Biochim Biophys Acta (2017) (in press).
[5] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140(6) (2010) 805-20.
[6] Z.A. Ibrahim, C.L. Armour, S. Phipps, M.B. Sukkar, RAGE and TLRs: relatives, friends or neighbours?, Mol Immunol 56(4) (2013) 739-44.
[7] T. Kawai, S. Akira, Toll-like receptors and their crosstalk with other innate receptors in infection and immunity, Immunity 34(5) (2011) 637-50.
[8] S. Akira, K. Takeda, Toll-like receptor signalling, Nat Rev Immunol 4(7) (2004) 499-511.
[9] S.K. Drexler, B.M. Foxwell, The role of toll-like receptors in chronic inflammation, Int J Biochem Cell Biol. 42(4) (2010) 506-18.
[10] Y. Zhong, A. Kinio, M. Saleh, Functions of NOD-Like Receptors in Human Diseases, Front Immunol 4 (2013) 333.
[11] Y.K. Kim, J.S. Shin, M.H. Nahm, NOD-Like Receptors in Infection, Immunity, and Diseases, Yonsei Med J 57(1) (2016) 5-14.
[12] T. Strowig, J. Henao-Mejia, E. Elinav, R. Flavell, Inflammasomes in health and disease, Nature 481(7381) (2012) 278-86.
[13] S. Kesavardhana, T.D. Kanneganti, Mechanisms governing inflammasome activation, assembly and pyroptosis induction, Int Immunol (2017) (in press).
[14] S.E. Hardison, G.D. Brown, C-type lectin receptors orchestrate antifungal immunity, Nat Immunol 13(9) (2012) 817-22.
[15] F. Osorio, C. Reis e Sousa, Myeloid C-type lectin receptors in pathogen recognition and host defense, Immunity 34(5) (2011) 651-64.
[16] K.M. Quicke, M.S. Diamond, M.S. Suthar, Negative regulators of the RIG-I-like receptor signaling pathway, Eur J Immunol 47(4) (2017) 615-28.
[17] L.A. O'Neill, A.G. Bowie, The family of five: TIR-domain-containing adaptors in

Toll-like receptor signalling, Nat Rev Immunol 7(5) (2007) 353-64.
[18] E.F. Kenny, L.A. O'Neill, Signalling adaptors used by Toll-like receptors: an update, Cytokine 43(3) (2008) 342-9.
[19] K.A. Fitzgerald, D.C. Rowe, B.J. Barnes, D.R. Caffrey, A. Visintin, E. Latz, B. Monks, P.M. Pitha, D.T. Golenbock, LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF, J Exp Med 198(7) (2003) 1043-55.
[20] C. Bogdan, Nitric oxide synthase in innate and adaptive immunity: an update, Trends Immunol 36(3) (2015) 161-78.
[21] C. Szabo, K. Modis, Pathophysiological roles of peroxynitrite in circulatory shock, Shock 34 Suppl 1 (2010) 4-14.
[22] M. Lind, A. Hayes, M. Caprnda, D. Petrovic, L. Rodrigo, P. Kruzliak, A. Zulli, Inducible nitric oxide synthase: Good or bad?, Biomed Pharmacother 93 (2017) 370-5.
[23] K. Kawahara, H. Hohjoh, T. Inazumi, S. Tsuchiya, Y. Sugimoto, Prostaglandin E2-induced inflammation: Relevance of prostaglandin E receptors, Biochim Biophys Acta 1851(4) (2015) 414-21.
[24] R. Zamora, Y. Vodovotz, T.R. Billiar, Inducible nitric oxide synthase and inflammatory diseases, Mol Med 6(5) (2000) 347-73.
[25] W. Schulte, J. Bernhagen, R. Bucala, Cytokines in sepsis: potent immunoregulators and potential therapeutic targets--an updated view, Mediators Inflamm 2013 (2013) 165974.
[26] H. Hasegawa, I. Mizoguchi, Y. Chiba, M. Ohashi, M. Xu, T. Yoshimoto, Expanding Diversity in Molecular Structures and Functions of the IL-6/IL-12 Heterodimeric Cytokine Family, Front Immunol 7 (2016) 479.
[27] K. Sugama, K. Suzuki, K. Yoshitani, K. Shiraishi, T. Kometani, IL-17, neutrophil activation and muscle damage following endurance exercise, Exerc Immunol Rev 18 (2012) 116-27.
[28] J. Cohen, The immunopathogenesis of sepsis, Nature 420(6917) (2002) 885-91.
[29] B.G. Chousterman, F.K. Swirski, G.F. Weber, Cytokine storm and sepsis disease pathogenesis, Semin Immunopathol 39(5) (2017) 517-28.
[30] H. Wang, O. Bloom, M. Zhang, J.M. Vishnubhakat, M. Ombrellino, J. Che, A. Frazier, H. Yang, S. Ivanova, L. Borovikova, K.R. Manogue, E. Faist, E. Abraham, J. Andersson, U. Andersson, P.E. Molina, N.N. Abumrad, A. Sama, K.J. Tracey, HMG-1 as a late mediator of endotoxin lethality in mice, Science 285(5425) (1999) 248-51.
[31] K. van Wijck, K. Lenaerts, J. Grootjans, K.A. Wijnands, M. Poeze, L.J. van Loon, C.H. Dejong, W.A. Buurman, Physiology and pathophysiology of splanchnic hypoperfusion and intestinal injury during exercise: strategies for evaluation and

prevention, Am J Physiol Gastrointest Liver Physiol 303(2) (2012) G155-68.
[32] W. Pires, C.E. Veneroso, S.P. Wanner, D.A.S. Pacheco, G.C. Vaz, F.T. Amorim, C. Tonoli, D.D. Soares, C.C. Coimbra, Association Between Exercise-Induced Hyperthermia and Intestinal Permeability: A Systematic Review, Sports Med 47(7) (2017) 1389-403.
[33] C.L. Lim, G. Wilson, L. Brown, J.S. Coombes, L.T. Mackinnon, Pre-existing inflammatory state compromises heat tolerance in rats exposed to heat stress, Am J Physiol Regul Integr Comp Physiol 292(1) (2007) R186-94.
[34] C.L. Lim and K. Suzuki, Systemic inflammation mediates the effects of endotoxemia in the mechanisms of heat stroke, Biol Med (Aligarh) 9(1) (2016) 1000376.
[35] G.P. Lambert, Intestinal barrier dysfunction, endotoxemia, and gastrointestinal symptoms: the 'canary in the coal mine' during exercise-heat stress?, Med Sport Sci 53 (2008) 61-73.
[36] M. Lamprecht, S. Bogner, G. Schippinger, K. Steinbauer, F. Fankhauser, S. Hallstroem, B. Schuetz, J.F. Greilberger, Probiotic supplementation affects markers of intestinal barrier, oxidation, and inflammation in trained men; a randomized, double-blinded, placebo-controlled trial, J Int Soc Sports Nutr 9(1) (2012) 45.
[37] C.M. Shing, J.M. Peake, C.L. Lim, D. Briskey, N.P. Walsh, M.B. Fortes, K.D. Ahuja, L. Vitetta, Effects of probiotics supplementation on gastrointestinal permeability, inflammation and exercise performance in the heat, Eur J Appl Physiol 114(1) (2014) 93-103.
[38] T. Ashton, I.S. Young, G.W. Davison, C.C. Rowlands, J. McEneny, C. Van Blerk, E. Jones, J.R. Peters, S.K. Jackson, Exercise-induced endotoxemia: the effect of ascorbic acid supplementation, Free Radic Biol Med 35(3) (2003) 284-91.
[39] B.J. Fisher, D. Kraskauskas, E.J. Martin, D. Farkas, P. Puri, H.D. Massey, M.O. Idowu, D.F. Brophy, N.F. Voelkel, A.A. Fowler, 3rd, R. Natarajan, Attenuation of sepsis-induced organ injury in mice by vitamin C, JPEN J Parenter Enteral Nutr 38(7) (2014) 825-39.
[40] Y.C. Chang, M.H. Tsai, W.H. Sheu, S.C. Hsieh, A.N. Chiang, The therapeutic potential and mechanisms of action of quercetin in relation to lipopolysaccharide-induced sepsis in vitro and in vivo, PLoS One 8(11) (2013) e80744.
[41] L.W. Soromou, N. Chen, L. Jiang, M. Huo, M. Wei, X. Chu, F.M. Millimouno, H. Feng, Y. Sidime, X. Deng, Astragalin attenuates lipopolysaccharide-induced inflammatory responses by down-regulating NF-kappaB signaling pathway, Biochem Biophys Res Commun 419(2) (2012) 256-61.

[42] L. Arab, F. Khan, H. Lam, Tea consumption and cardiovascular disease risk, Am J Clin Nutr 98(6 Suppl) (2013) 1651S-9S.
[43] M.H. Pan, Y.C. Tung, G. Yang, S. Li, C.T. Ho, Molecular mechanisms of the anti-obesity effect of bioactive compounds in tea and coffee, Food Funct 7(11) (2016) 4481-91.
[44] N. Khan, H. Mukhtar, Tea polyphenols for health promotion, Life sciences 81(7) (2007) 519-33.
[45] J.H. Park, J.H. Bae, S.S. Im, D.K. Song, Green tea and type 2 diabetes, Integr Med Res 3(1) (2014) 4-10.
[46] Y.S. Lin, Y.J. Tsai, J.S. Tsay, J.K. Lin, Factors affecting the levels of tea polyphenols and caffeine in tea leaves, J Agric Food Chem 51(7) (2003) 1864-73.
[47] C. Braicu, M.R. Ladomery, V.S. Chedea, A. Irimie, I. Berindan-Neagoe, The relationship between the structure and biological actions of green tea catechins, Food Chem 141(3) (2013) 3282-9.
[48] H.S. Oz, Chronic Inflammatory Diseases and Green Tea Polyphenols, Nutrients 9(6) (2017) (in press).
[49] E.G. de Mejia, M.V. Ramirez-Mares, S. Puangpraphant, Bioactive components of tea: cancer, inflammation and behavior, Brain Behav Immun 23(6) (2009) 721-31.
[50] E. Hong Byun, Y. Fujimura, K. Yamada, H. Tachibana, TLR4 signaling inhibitory pathway induced by green tea polyphenol epigallocatechin-3-gallate through 67-kDa laminin receptor, J Immunol 185(1) (2010) 33-45.
[51] D.S. Wheeler, P.M. Lahni, P.W. Hake, A.G. Denenberg, H.R. Wong, C. Snead, J.D. Catravas, B. Zingarelli, The green tea polyphenol epigallocatechin-3-gallate improves systemic hemodynamics and survival in rodent models of polymicrobial sepsis, Shock 28(3) (2007) 353-9.
[52] B. Chopra, A.K. Dhingra, K.L. Dhar, Psoralea corylifolia L. (Buguchi) - folklore to modern evidence: review, Fitoterapia 90 (2013) 44-56.
[53] E. Seo, Y.S. Oh, H.S. Jun, Psoralea corylifolia L. Seed Extract Attenuates Nonalcoholic Fatty Liver Disease in High-Fat Diet-Induced Obese Mice, Nutrients 8(2) (2016) 83.
[54] P.G. Latha, D.A. Evans, K.R. Panikkar, K.K. Jayavardhanan, Immunomodulatory and antitumour properties of Psoralea corylifolia seeds, Fitoterapia 71(3) (2000) 223-31.
[55] X. Zhang, W. Zhao, Y. Wang, J. Lu, X. Chen, The Chemical Constituents and Bioactivities of Psoralea corylifolia Linn.: A Review, Am J Chin Med 44(1) (2016) 35-60.
[56] C.C. Cheng, Y.H. Chen, W.L. Chang, S.P. Yang, D.M. Chang, J.H. Lai, L.J. Ho,

Phytoestrogen bavachin mediates anti-inflammation targeting Ikappa B kinase-I kappaB alpha-NF-kappaB signaling pathway in chondrocytes in vitro, Eur J Pharmacol 636(1-3) (2010) 181-8.
[57] E. Szliszka, D. Skaba, Z.P. Czuba, W. Krol, Inhibition of inflammatory mediators by neobavaisoflavone in activated RAW264.7 macrophages, Molecules 16(5) (2011) 3701-12.
[58] S.W. Lee, B.R. Yun, M.H. Kim, C.S. Park, W.S. Lee, H.M. Oh, M.C. Rho, Phenolic compounds isolated from Psoralea corylifolia inhibit IL-6-induced STAT3 activation, Planta Med 78(9) (2012) 903-6.
[59] N. Sueoka, M. Suganuma, E. Sueoka, S. Okabe, S. Matsuyama, K. Imai, K. Nakachi, H. Fujiki, A new function of green tea: prevention of lifestyle-related diseases, Ann N Y Acad Sci 928 (2001) 274-80.
[60] M. Maeda-Yamamoto, Human clinical studies of tea polyphenols in allergy or life style-related diseases, Curr Pharm Des 19(34) (2013) 6148-55.
[61] R. Johnson, S. Bryant, A.L. Huntley, Green tea and green tea catechin extracts: an overview of the clinical evidence, Maturitas 73(4) (2012) 280-7.
[62] M. Monobe, K. Ema, Y. Tokuda, M. Maeda-Yamamoto, Enhancement of phagocytic activity of macrophage-like cells by pyrogallol-type green tea polyphenols through caspase signaling pathways, Cytotechnology 62(3) (2010) 201-3.
[63] Y. Fujimura, D. Umeda, K. Yamada, H. Tachibana, The impact of the 67kDa laminin receptor on both cell-surface binding and anti-allergic action of tea catechins, Arch Biochem Biophys 476(2) (2008) 133-8.
[64] H. Tachibana, K. Koga, Y. Fujimura, K. Yamada, A receptor for green tea polyphenol EGCG, Nat Struct Mol Biol 11(4) (2004) 380-1.
[65] S.Y. Joo, Y.A. Song, Y.L. Park, E. Myung, C.Y. Chung, K.J. Park, S.B. Cho, W.S. Lee, H.S. Kim, J.S. Rew, N.S. Kim, Y.E. Joo, Epigallocatechin-3-gallate Inhibits LPS-Induced NF-kappaB and MAPK Signaling Pathways in Bone Marrow-Derived Macrophages, Gut and liver 6(2) (2012) 188-96.
[66] U. Singh, J. Tabibian, S.K. Venugopal, S. Devaraj, I. Jialal, Development of an in vitro screening assay to test the antiinflammatory properties of dietary supplements and pharmacologic agents, Clin Chem 51(12) (2005) 2252-6.
[67] H. Huang, A. Fletcher, Y. Niu, T.T. Wang, L. Yu, Characterization of lipopolysaccharide-stimulated cytokine expression in macrophages and monocytes, Inflamm Res 61(12) (2012) 1329-38.
[68] K.V. Ramana, A.B. Reddy, R. Tammali, S.K. Srivastava, Aldose reductase mediates endotoxin-induced production of nitric oxide and cytotoxicity in murine macrophages,

Free Radic Biol Med 42(8) (2007) 1290-302.
[69] R. Bingisser, C. Stey, M. Weller, P. Groscurth, E. Russi, K. Frei, Apoptosis in human alveolar macrophages is induced by endotoxin and is modulated by cytokines, Am J Respir Cell Mol Biol 15(1) (1996) 64-70.
[70] J. Xaus, M. Comalada, A.F. Valledor, J. Lloberas, F. Lopez-Soriano, J.M. Argiles, C. Bogdan, A. Celada, LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-alpha, Blood 95(12) (2000) 3823-31.
[71] C.C. Chen, J.K. Wang, p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages, Mol Pharmacol 55(3) (1999) 481-8.
[72] H. Karahashi, F. Amano, Endotoxin-tolerance to the cytotoxicity toward a macrophage-like cell line, J774.1, induced by lipopolysaccharide and cycloheximide: role of p38 MAPK in induction of the cytotoxicity, Biol Pharm Bull 26(9) (2003) 1249-59.
[73] W.D. Li, C.P. Yan, Y. Wu, Z.B. Weng, F.Z. Yin, G.M. Yang, B.C. Cai, Z.P. Chen, Osteoblasts proliferation and differentiation stimulating activities of the main components of Fructus Psoraleae corylifoliae, Phytomedicine 21(4) (2014) 400-5.
[74] D. Xin, H. Wang, J. Yang, Y.F. Su, G.W. Fan, Y.F. Wang, Y. Zhu, X.M. Gao, Phytoestrogens from Psoralea corylifolia reveal estrogen receptor-subtype selectivity, Phytomedicine 17(2) (2010) 126-31.
[75] Z.B. Weng, Q.Q. Gao, F. Wang, G.H. Zhao, F.Z. Yin, B.C. Cai, Z.P. Chen, W.D. Li, Positive skeletal effect of two ingredients of Psoralea corylifolia L. on estrogen deficiency-induced osteoporosis and the possible mechanisms of action, Mol Cell Endocrinol 417 (2015) 103-13.
[76] H. Lee, H. Li, M. Noh, J.H. Ryu, Bavachin from Psoralea corylifolia Improves Insulin-Dependent Glucose Uptake through Insulin Signaling and AMPK Activation in 3T3-L1 Adipocytes, Int J Mol Sci 17(4) (2016) 527.
[77] K.F. Hua, F.L. Yang, H.W. Chiu, J.C. Chou, W.C. Dong, C.N. Lin, C.Y. Lin, J.T. Wang, L.H. Li, H.W. Chiu, Y.C. Chiu, S.H. Wu, Capsular Polysaccharide Is Involved in NLRP3 Inflammasome Activation by Klebsiella pneumoniae Serotype K1, Infect Immun 83(9) (2015) 3396-409.
[78] N. Olson, A. van der Vliet, Interactions between nitric oxide and hypoxia-inducible factor signaling pathways in inflammatory disease, Nitric Oxide 25(2) (2011) 125-37.
[79] S. Zundler, M.F. Neurath, Interleukin-12: Functional activities and implications for disease, Cytokine Growth Factor Rev 26(5) (2015) 559-68.
[80] J.M. Guo, X.C. Weng, H. Wu, Q.H. Li, K.S. Bi, Antioxidants from a Chinese

medicinal herb - Psoralea corylifolia L., Food Chemistry 91(2) (2005) 287-292.
[81] D. Wang, F. Li, Z. Jiang, Osteoblastic proliferation stimulating activity of Psoralea corylifolia extracts and two of its flavonoids, Planta Med 67(8) (2001) 748-9.
[82] T. Murase, S. Haramizu, A. Shimotoyodome, A. Nagasawa, I. Tokimitsu, Green tea extract improves endurance capacity and increases muscle lipid oxidation in mice, Am J Physiol Regul Integr Comp Physiol 288(3) (2005) R708-15.
[83] S. Haramizu, N. Ota, T. Hase, T. Murase, Catechins suppress muscle inflammation and hasten performance recovery after exercise, Med Sci Sports Exerc 45(9) (2013) 1694-702.