Inhibition of lipopolysaccharide-induced inflammatory responses by 1'-acetoxychavicol acetate

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Graduate School of Biological Sciences Doctoral Thesis Abstract

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Abstract

In the presence of pathogen components or endogenous danger signals, inflammatory response is often the first immune response which will be activated. Upon challenged by gram-negative bacteria, the lipopolysaccharide (LPS) released by these bacteria can be recognized and detected by Toll-like receptor 4 (TLR4). The activation of TLR4 results in a series of downstream signaling pathways, leading to the production of various inflammatory mediators such as tumor necrosis factor (TNF) and interleukin (IL)-6 via NF-κB transcription factor. However, dysregulation in this delicate and tightly controlled response contributes to excessive inflammatory responses which cause unnecessary damage to the host homeostasis. Hence, inhibitors are often used to circumvent such dysregulations. Acetoxychavicol acetate (ACA) is a bioactive compound extracted from the rhizome of tropical ginger and has demonstrated anti-inflammatory and apoptosis-inducing properties. In this study, the effect of ACA on LPS-induced inflammatory responses was elucidated. ACA was found to inhibit the gene expression and cytokine production of inflammatory cytokines, namely TNF-α and IL-6, in mouse bone marrow-derived macrophages and PMA-differentiated human macrophages THP-1. Further investigation revealed that ACA inhibited the phosphorylation of p65 by stabilizing IκBα complex in the NF-κB pathway. In addition, ACA also decreased the phosphorylation of p38, JNK and ERK, which are crucial signaling proteins in the MAPK pathway that also contributes to the expression of TNF-α and IL-6. Using overexpression system in HEK293T cells, ACA attenuated the non-degradative K63-linked polyubiquitination on TRAF6, an E3 ubiquitin ligase playing a central role in TLR4-mediated signaling pathway. The current results suggest that ACA may be a potent anti-inflammatory agent for therapeutic purposes.

1.0 Introduction

1.1 Innate immunity

The innate immunity is an important component of the mammalian immune system against various internal and external threats. It is often the first response activated to protect the host from pathogen infections. This is due to its ability to recognize pathogen-associated molecular patterns (PAMPs) derived from the pathogens or danger-associated molecular patterns (DAMPs) released when cells are damaged, using the germline encoded pattern-recognition receptors (PRRs). The presence of a wide variety of PRRs enabled the recognition of various types of PAMPs. Upon detection of PAMPs, the PRRs then activate a series of downstream innate immune pathways, leading to the production of pro-inflammatory cytokines and other mediators. The innate immunity is also crucial in triggering the antigen-specific adaptive immunity. Working together, innate and adaptive immunity are essential in the elimination of invading pathogens.

1.1.1 PRRs

The concept of PRRs was first proposed by Charles Janeway [1] and Toll-like receptors (TLRs) were the first to be discovered. Soon after the discovery of TLRs, several other distinct classes, including RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), and intracellular DNA sensors such as cGAS [2, 3] were also discovered (Figure 1.1). Among these, TLRs remain the best characterized PRRs to date.

To date, a total of 10 TLRs were discovered in human while 12 in mice [4]. They can be classified into two large subfamilies based on their localization, i.e. cell surface and intracellular TLRs. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are categorized as the cell surface TLRs; while TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13 are grouped into the intracellular TLRs [5]. These TLRs are responsible to recognize their respective PAMPs or DAMPs, either as a homo- or heterodimer along with a co-receptor or accessory molecule [6]. The first TLR-ligand being discovered is TLR4 which recognizes lipopolysaccharide (LPS), a component of gram-negative bacteria cell wall [7-9]. TLR2, which heterodimerizes with TLR1 or TLR6, can recognize diacylated or triacylated lipopeptides of bacterial origins respectively [10-13]. TLR5 was shown to sense bacterial flagellin [14], while TLR11 can detect a component of uropathogenic bacteria [15], and when dimerized with TLR12, is able to bind to *Toxoplasma gondii* profilin protein [16, 17]. Other than that, TLR13 was found to recognize bacterial ribosomal RNA [18]. Several other

TLRs are involved in antiviral responses, namely TLR3 (double-stranded RNA (dsRNA)) [19], TLR7 and TLR8 (viral single-stranded RNA (ssRNA))[20-22]; and lastly, TLR9 (CpG-rich hypomethylated DNA motifs)[23]. Subsequently, it was found that TLR7, TLR8 and TLR9 can also recognize host nucleic acids in response to danger signals originated from infection or tissue damage[24-26]. Upon recognition of their respective PAMPs or DAMPs, TIR domain-containing adaptor proteins such as myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) are recruited by TLRs. All TLRs recruits MyD88 as adaptor molecules, except for TLR3 which utilizes TRAM and TRIF, whereas TLR4 can recruit both MyD88 and TRIF. Then, this initiates the downstream signal transduction pathways which often lead to the activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB). The activation of NF-κB is represented by the phosphorylation of NF-κB p65 subunit that is then translocated across the nuclear membrane to initiate the transcription of pro-inflammatory cytokines. Additionally, interferon regulatory factors (IRFs) or mitogen-activated protein kinases (MAPKs) are often activated along NF-κB. Next, the expression of related cytokines is regulated, triggering in the subsequent immune responses, with inflammation as the one of the most common responses being induced.

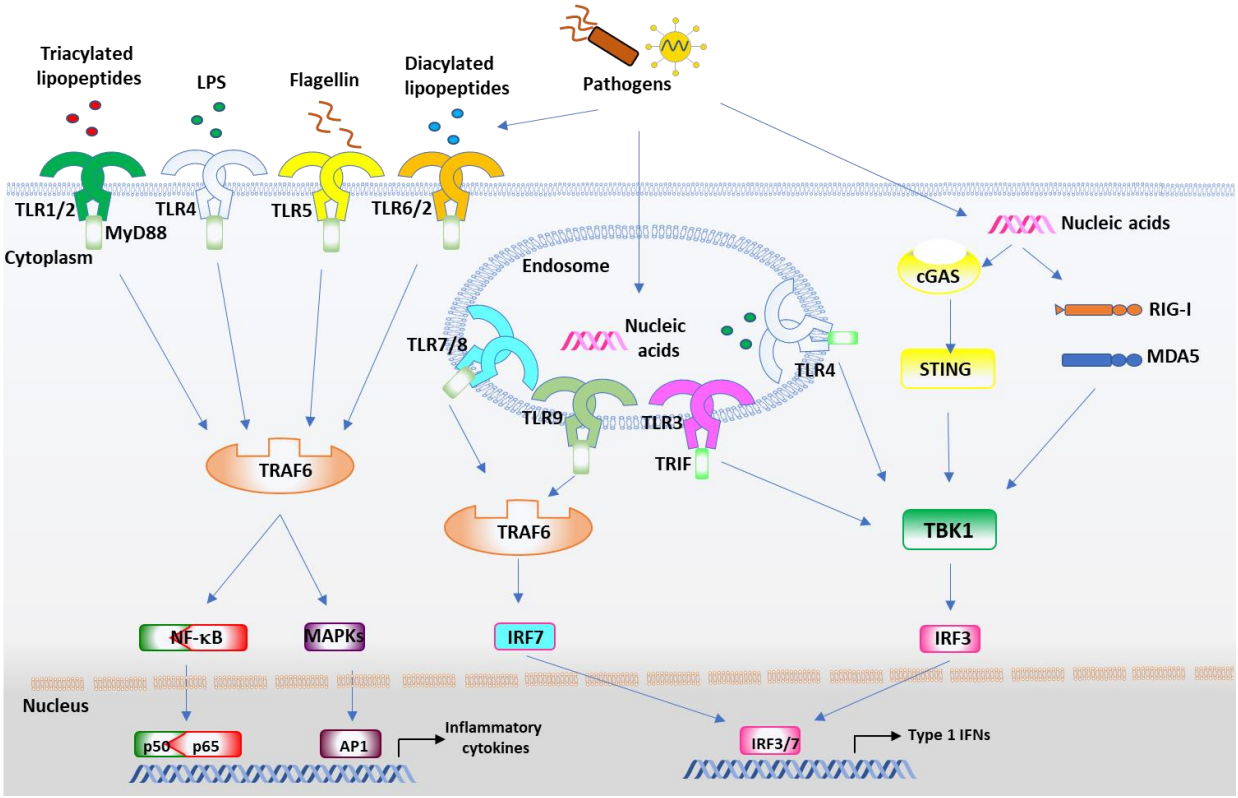

Figure 1.1. PRRs of innate immunity. Each PRRs is responsible to detect specific components released by pathogens like bacteria or viruses. All TLRs recruits MyD88, except for TLR3 which recruits TRIF. TLR4 can recruit both adaptors depending on its localization. (PRR: pattern recognition receptors; TLR: Toll-like receptors; MyD88: myeloid differentiation primary response 88; TRIF: TIR-domain-containing adapter-inducing interferon-β; TRAF6: TNF receptor associated factor 6; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; MAPK: mitogen-activated protein kinase; IRF: interferon regulatory factor)

1.1.2 TLR4 signaling pathway

As mentioned above, TLR4 is activated by LPS released in the presence of gram-negative bacteria. The binding of LPS to TLR4 enables the formation of an activated TLR4 homodimer which then activate the intracellular signaling [27]. Upon dimerization, TIR domain-containing adaptor proteins (TIRAP)/MyD88 and TRIF-related adaptor molecule (TRAM)/TRIF will be recruited to the Toll/interleukin-1 receptor (TIR) domain of TLR4. Interleukin-1 receptor associated kinases (IRAK)-4 and IRAK1/2 kinases are then recruited to MyD88 to form a complex known as myddosome [28, 29]. Next, myddosome can trigger the signaling cascade leading to the activation of NF-κB and MAPKs. This is known as the MyD88-dependent pathway (Figure 1.2). Alternatively, when TRIF is recruited to TLR4, TRIF-dependent pathway is activated. Other than NF-κB and MAP kinases, IRF3 is activated to induce type I IFNs and several other inflammatory cytokines in this pathway [5]. MyD88-dependent pathway is initiated from the LPS/MD-2/TLR4 complex formation located on the plasma membrane while TRIF-dependent pathway is started when the complex is internalized into the endosomes [30].

In the MyD88-dependent pathway, several other downstream molecules are involved. After the dimerization of TLR4 and recruitment of IRAK4, IRAK4 activates IRAK1 to autophosphorylate itself and being released from MyD88 [31, 32]. The released IRAK1 then recruits TNF receptor associated factor 6 (TRAF6) which is a RING-domain E3 ubiquitin ligase. Next, TRAF6 generates K63-linked polyubiquitination chains, together with ubiquitin-conjugating enzyme UBC13 and UEV1A to autoubiquitinate itself. These chains then recruit and activate several other preassembled protein complexes such as IκB kinase (IKK) regulatory subunit and TGFβ-activated kinase 1 (TAK1) [33, 34]. These complexes are responsible for the regulation of IKK and MAPK signaling to control the transcription factors NF-κB and AP-1, respectively.

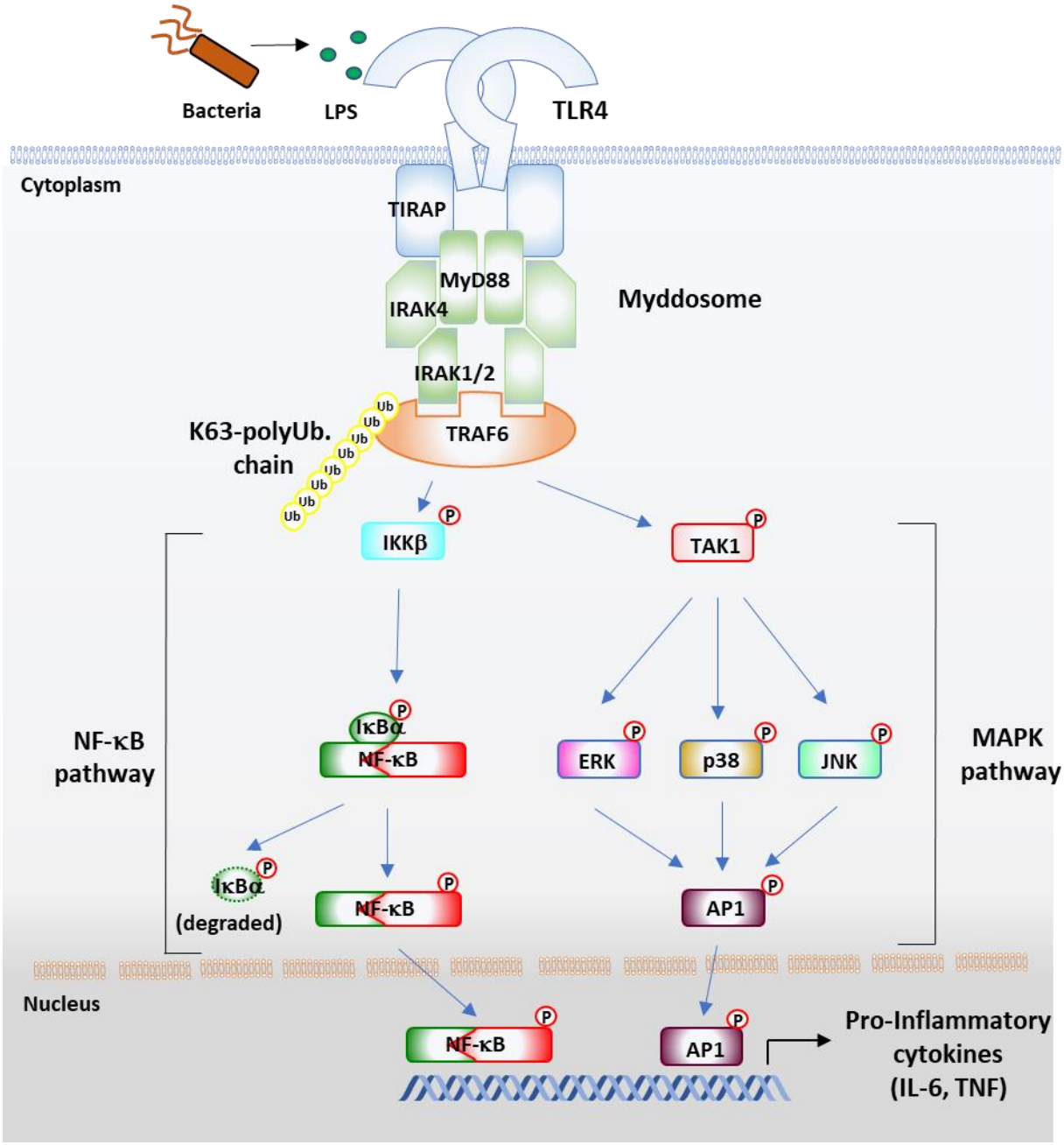

Figure 1.2. TLR4 signaling pathway. Upon recognition of LPS, TLR4 recruits TIRAP/MyD88 adaptor proteins, triggering the MyD88-dependent pathway; or when endocytosed, recruits TRAM/TRIF adaptor proteins to activate the TRIF-dependent pathway. (TLR: Toll-like receptors; TIRAP: TIR domain-containing adaptor proteins; MyD88: myeloid differentiation primary response 88; IRAK: Interleukin-1 receptor associated kinase; TRAF6: TNF receptor associated factor 6; TAK1: TGFβ-activated kinase 1; IKK: IκB kinase; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; ERK: extracellular signal-regulated kinase; JNK: Jun N-terminal kinase; MAPK: mitogen-activated protein kinase)

1.1.3 NF-κB and MAPK pathways

NF-κB family consists of inducible transcription factors that are involved in many crucial biological processes, such as immunity and the subsequent inflammatory responses, cell growth, proliferation and survival, and oncogenesis [35, 36]. The transcription factors are able to bind to the conserved κB DNA element as homo- or hetero-dimers in the promotor or enhancer region of the target genes [36]. In unstimulated condition, the precursor NF-κBs are sequestered in the cytoplasm as inactive complex. Other than the precursor NF-κBs, the inactive complex also includes inhibitory proteins such as IκBα, IκBβ and several other proteins with similar structure, all contains an ankyrin-repeat domain mediating binding and inhibition of NF-κB, which serves as a inhibitory subunit of the complex [36].

Following the recruitment of the IKK complex and TAK1 kinases to the activated TRAF6, the IKKs activate the inhibitory subunit complex, particularly the IκBα is signaled for degradation by the IKKs [37, 38] (Figure 1.3). The degradation allows the precursor NF-κB, p105 to be processed by proteosome into the mature NF-κB p50, thus triggering the nuclear translocation of p50-containing NF-κB complexes, particularly the p50/RelA (p65) heterodimer [39]. This in turn initiates the transcription of pro-inflammatory cytokines. This pathway is activated rapidly in response to a wide range of inflammatory stimuli, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), or exposure to LPS [36].

MAPK pathways comprised of a core tier of three kinases: a MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK. The upstream kinases MAP3K, phosphorylates MAP2K, which in turn responsible to activate the MAPK via dual phosphorylation of the THR-X-Tyr motif, in which X can be any amino acid. [40]. Then, the phosphorylated MAPKs activate the AP-1 subunits such as c-fos and c-jun, leading to their nuclear translocation to initiate the transcription of pro-inflammatory cytokines [41]. To date, extracellular signal-regulated kinase (ERK), p38 MAPK, Jun N-terminal kinase (JNK) are the most extensively studied MAPKs for their relation with innate immunity.

Similar to NF-κB pathway, the activation of MAPK pathway requires the formation of TRAF6 complex. Then, different MAP3Ks are activated. Following the formation of TRAF6, TAK1, which is the MAP3K of JNK and p38, is recruited to the polyubiquitinated TRAF6 [42, 43]. Next,

the MAP2Ks for p38 (MKK3 and MKK6) and JNK (MKK4 and MKK7) are phosphorylated and then activate p38 and JNK respectively [42].

Meanwhile, ERK1/2, often referred to as the classical MAPKs, requires the activation of tumor progression locus 2 (TPL2, the MAP3K for ERK1/2), which phosphorylates MKK1 and MKK2 (MAP2K for ERK1/2) that regulate ERK1/2 [44]. It is interesting to note that TAK1 not only directly regulates the p38 and JNK pathways, it is also indirectly related to the activation of TPL2. Under physiological condition, TPL2 forms a stable but inactive complex with the NF-κB precursor, p105 [44]. The complex inhibits the processing of p105 and prevented the release of TPL2 to phosphorylate MKK1 and MKK2 [45, 46]. However, after the formation of TRAF6 polyubiquitination complex, TAK1 activates the IKK complex, whereby the IKK phosphorylates p105 to induce the hydrolysis of p105. This allows TPL2 to be released from the complex to phosphorylate MKK1/2, subsequently activating ERK1/2 [44, 47]. Therefore, TRAF6 and TAK1 function as a master kinase mediating the activation of both NF-κB and MAPK signaling pathways, creating an interesting crosstalk between both pathways.

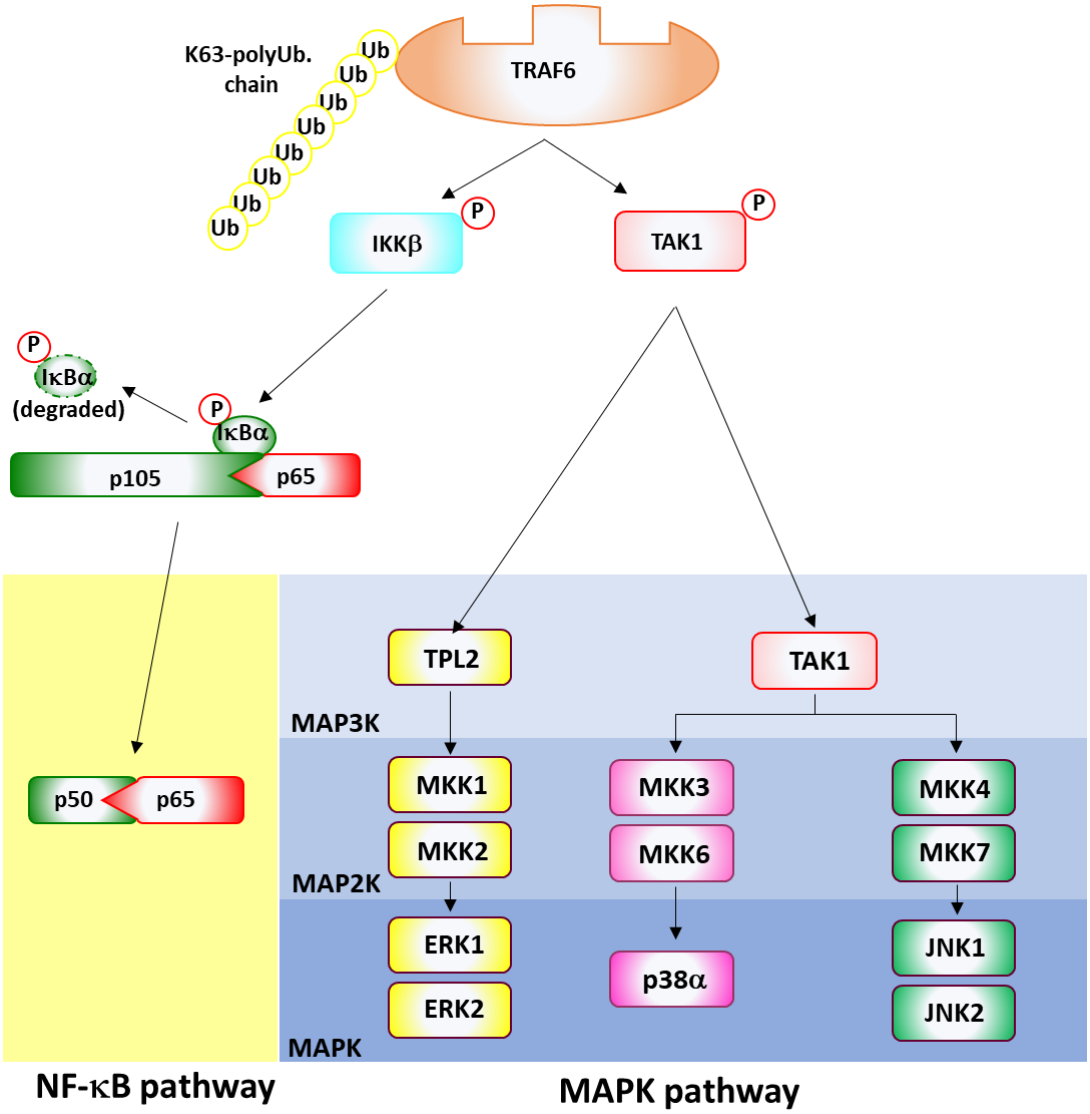

Figure 1.3. The hierarchical activation of NF-κB and MAPK pathway. In NF-κB pathway, p105 is constitutively inactivated by the inhibitory protein, IκBα. Upon activation of the upstream receptors, IKKβ signals the degradation of IκBα, releasing p105 to be processed by proteosome into the active p50 form. Together with p65, the heterodimer p50/p65 is translocated across the nuclear membrane to initiate transcription of NF-κB related genes. In MAPK pathway, each MAPKs are activated by the specific MAP2Ks, which are also activated by their own MAP3Ks. Several other MAP3Ks exist, though this figure only listed the MAP3Ks which are activated via LPS stimulation. TRAF6 and TAK1 are involved in the activation of both NF-κB and MAPK pathways, where TAK1 acts as the MAP3K of both p38 and JNK1/2 while indirectly involved in the release of TPL2, the MAP3K of ERK1/2. (TRAF6: TNF receptor associated factor 6; TAK1: TGFβ-activated kinase 1; IKK: IκB kinase; TPL2: tumor progression locus 2; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; MAPK: mitogen-activated protein kinase; MAP2K: MAPK kinase; MAP3K: MAPK kinase kinase; ERK: extracellular signal-regulated kinase; JNK: Jun N-terminal kinase)

1.1.4 TRAF6 and mROS

The activation of TLR4 also leads to increase in mitochondrial reactive oxygen species (mROS) production. mROS generation has been demonstrated to be contributing to the antibacterial activity in macrophages [48]. It was found that the generation of mROS is related to TRAF6, and another component known as evolutionarily conserved signaling intermediate in Toll pathways (ECSIT). ECSIT is a cytosolic protein previously found to be related to the NF-κB signaling and also plays a role in bone morphogenetic protein signaling pathway [49]. Previously it was demonstrated that ECSIT localizes in mitochondria and aids in the formation of mitochondrial respiratory complex I, which is part of the electron transport chain and the main ROS generation site of mitochondria [50]. The localization of ESCIT at mitochondria also mediates the activation of RLRs during antiviral responses [51]. The activation of ECSIT requires interaction with TRAF6 and the ubiquitin ligase function of TRAF6 is crucial for its activation [48] (Figure 1.4). Meanwhile, ECSIT acts as a mediator for TRAF6-TAK1 complex interaction during TLR4 activation, whereby the knockdown of ECSIT impairs NF-κB activation [52]. Furthermore, TRAF6 also localizes to mitochondria during RLR activation (for example, RIG-I or MDA5 activation) by interacting with IPS-1 [49], hinting that TRAF6 regulation may be crucial in mROS generation for antibacterial and antiviral activity via interaction with ECSIT.

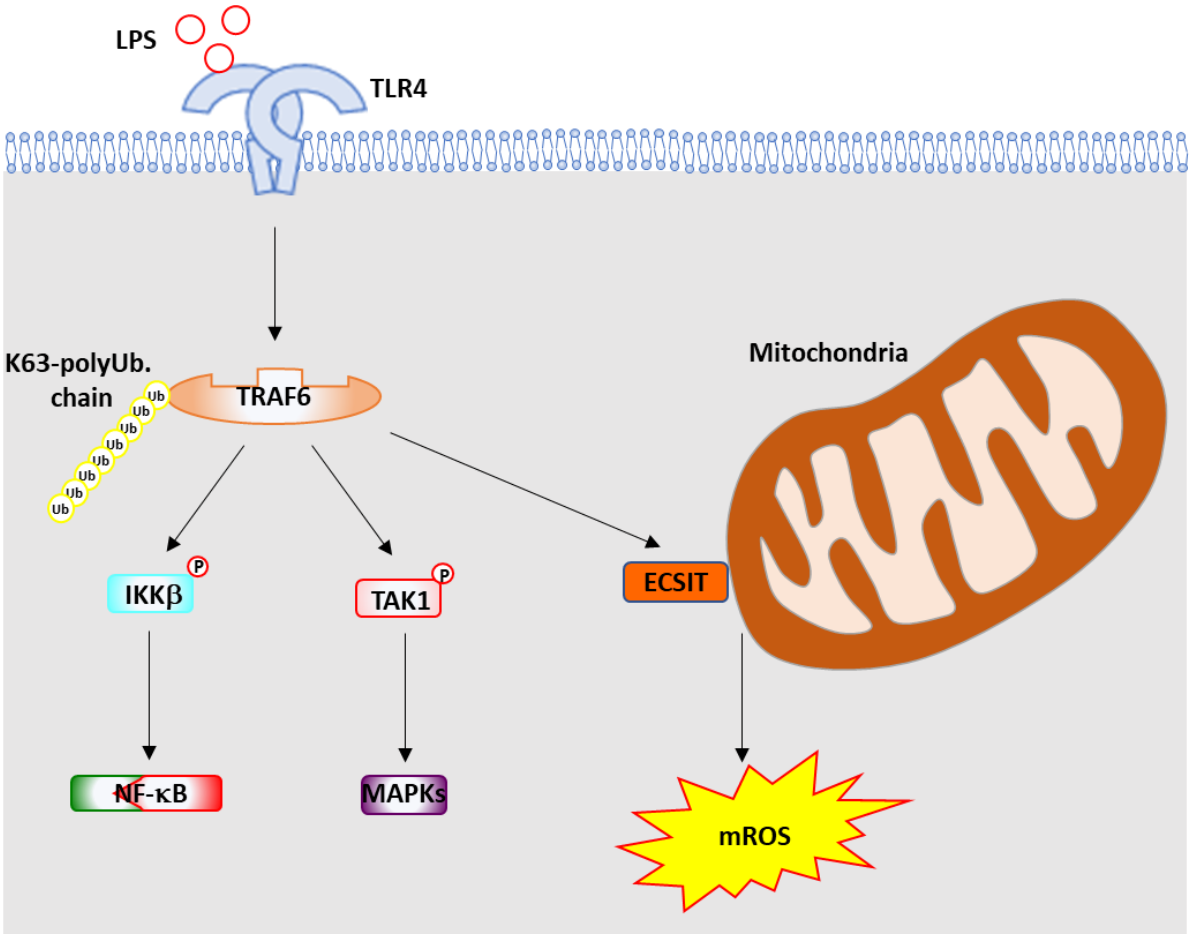

Figure 1.4. Interaction of TRAF6 and mitochondria. TRAF6 localizes to mitochondria, interacting and activating TAK1, IKKβ and ECSIT complex upon TLR4 activation, leading to NF-κB and MAPK signaling pathway activation. The interaction of TRAF6 with ECSIT also leads to mROS generation. (TLR: Toll-like receptors; TRAF6: TNF receptor associated factor 6; TAK1: TGFβ-activated kinase 1; ECSIT: evolutionarily conserved signaling intermediate in Toll pathways; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; MAPK: mitogen-activated protein kinase; mROS: mitochondrial reactive oxygen species)

1.1.5 Inflammation diseases related to TLR4 activation

Normally, the occurrence of inflammation benefits the host by assisting the clearance of invading pathogen. However, in certain situation, unnecessary host response to PAMPs or DAMPs leading to excessive or chronic inflammation can bring harm to the host. One good example is the life-threatening sepsis. Sepsis is defined as a dysregulation or over activation of host response to infection that can result in multi organ failure [53]. The origin of sepsis can be anywhere in the host system, with the presence of an invading pathogen with sufficient load and virulence within the host [54]. It was reported that gram-negative bacteria are responsible for 62% of the reported studies, with *Escherichia coli*, *Klebsiella* sp. and *Pseudomonas aeruginosa* as the leading cause of sepsis [55]. The expected mortality rate for sepsis is estimated from 20-50%, depending on countries or areas [56].

In addition to bacterial infections, it was also found that TLR4 can be activated by various DAMPs from sterile inflammation. The examples of such DAMPs are fibronectins, hyaluronan, saturated fatty acids from cellular damage, and high mobility group box 1 (HMGB1) [57, 58]. These dysregulation of TLR4 signaling responses have been linked with autoimmune, cardiovascular and neurological inflammation diseases [59-61]. Furthermore, recent studies have discovered that TLR4 antagonist treatment can protect mice from lethal influenza infection, in which the oxidized phospholipid released during viral infection is able to stimulate TLR4-dependent immune response to cause acute lung injury. The application of TLR4 antagonists in the viral infected mice reduced their mortality [62, 63]. These observations have prompted researchers in search for potential TLR4 antagonist for therapeutic purposes.

1.2 1'-acetoxychavicol acetate (ACA)

In recent years, natural compounds with therapeutic effect from plant origin have attracted the interest of many researchers. Usually, herbs or spices which have been used as traditional medicine are the target for such purposes. ACA is one of such active compounds extracted from the rhizomes or seeds of *Alpinia galanga* and *Alpinia conchigera* of the ginger family (Figure 1.5).

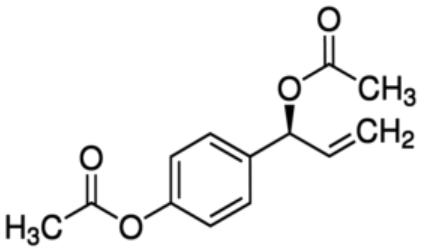

Figure 1.5. The chemical structure of 1'-acetoxychavicol acetate.

To date, various studies have been conducted on ACA and it was found that ACA can regulate a diversity of biological processes, such as anti-cancer properties, gastroprotective effects, anti-allergic activity, antimicrobial activity, anti-dementia effect and also anti-inflammation properties [64]. One of the pharmacological effects of ACA which attracted much attention is the anti-cancer aspect, in which it is reported to be effective against colorectal adenocarcinoma cells [65, 66], cervical cancer cells [67], squamous carcinoma cells [68], prostate cancer cells [69], breast cancer cells [70, 71], myeloma cells [72] and Ehrlich ascites tumor cells [73, 74]. The anti-cancer property of ACA is mediated through inducing apoptosis, suppression of angiogenesis, cell proliferation, and more recently, suppression of microRNA expression [64].

It is worth to note that the mechanism in which ACA suppresses proliferation and induces apoptosis of cancer cells via inhibiting the nuclei translocation of NF-κB [70, 72]. Further investigation has shown that ACA actually acted upstream of the NF-κB pathway, i.e., inhibiting the phosphorylation and degradation of IκBα, thus prevented the release of p105, the precursor of NF-κB to be processed into p50 to activate the NF-κB pathway [70, 75]. This has also contributed to the anti-inflammation activity of ACA. Despite with the knowledge of ACA is capable of inhibiting the degradation of IκBα, the actual target of ACA remained elusive.

Additionally, our lab recently also demonstrated that ACA is able to attenuate the activation of nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome by inhibiting the production of mROS [76]. NLRP3 inflammasome is an intracellular multimeric protein complex involved in the regulation of maturation and secretion of the pro-inflammatory cytokines, IL-1β and IL18, at the same time induces pyroptosis, an immunogenic cell death [77]. The activation of NLRP3 inflammasome is tightly regulated and requires two signals. The first signal is known as priming step, in which the presence of PAMPs or DAMPs is recognized by TLRs, activating NF-κB pathway to mediate the transcription of the inflammasome-related complex such as NRLP3, apoptosis-associated speck-like protein containing a CARD (ASC) and immature pro-IL-1β [78]. A second signal is required for the assembly of inflammasome complex that processes pro-IL-1β into the mature and functional IL-1β. A variety of PAMPs and DAMPs can serve as the second signal for NLRP3 activation, for example adenosine triphosphate (ATP), uric acid crystals, silica and bacterial toxin [79]. It is suggested that NLRP3 does not recognizes PAMPs and DAMPs directly. Instead, their presence causes physiological changes in the cell, mainly by affecting the mitochondria to generate mROS, thus activating NLRP3 inflammasome [80]. Our lab demonstrated that ACA inhibited the production of mROS, subsequently attenuating the activation of NLRP3 inflammasome [76] (Figure 1.6). The inhibition is independent of the ability of ACA to affect NF-κB pathway. This observation suggested that ACA may function by affecting mitochondria directly.

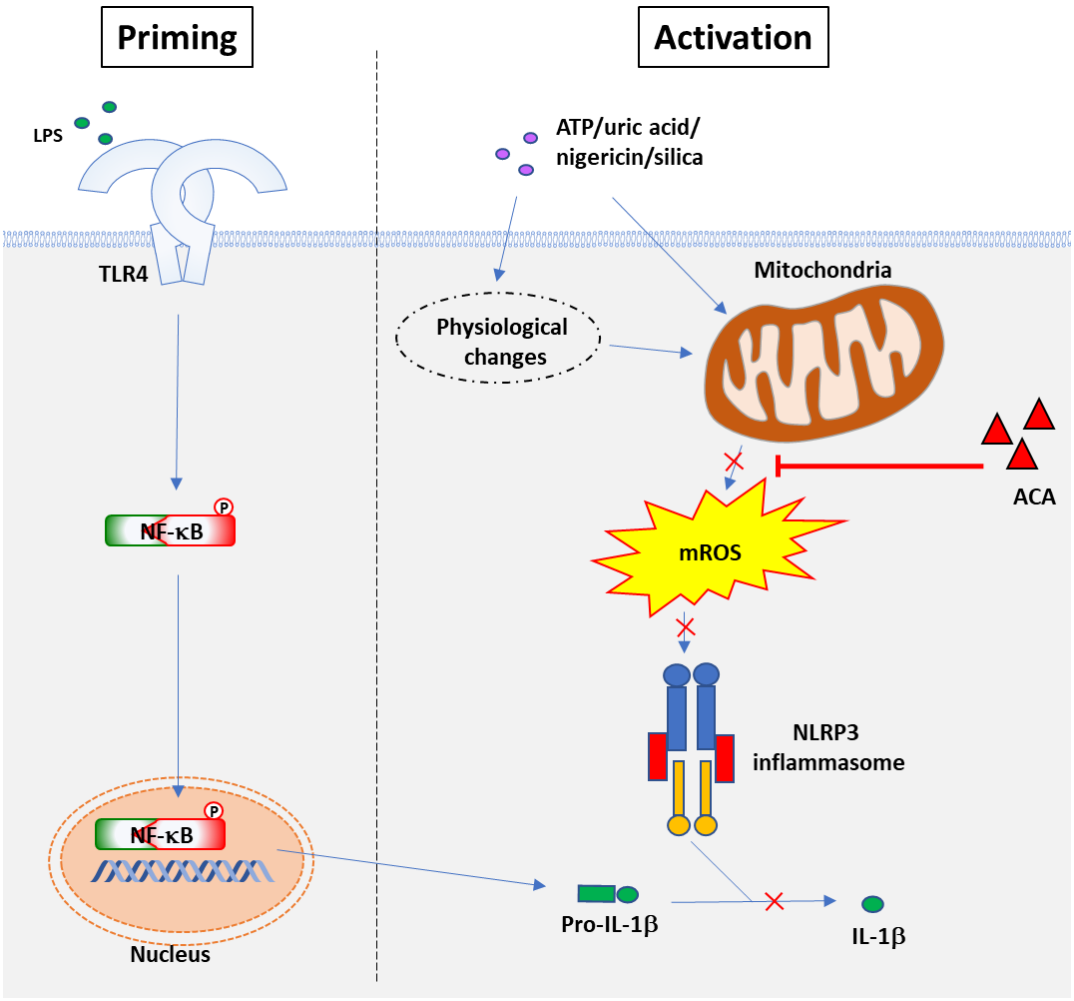

Figure 1.6. Proposed model for inhibition of NLRP3 inflammasome activation by ACA. mROS generation by mitochondria acts as the activation signal for NLRP3 inflammasome activation. ACA inhibited the mROS generation thus inhibiting the activation of NLRP3 inflammasome, attenuating the production of IL-1β. (TLR: Toll-like receptors; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; mROS: mitochondrial reactive oxygen species)

Lately, the world is plagued by coronavirus disease 2019 (COVID-19) caused by the virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In the severe cases of the infectious disease, patient often suffers from acute respiratory distress syndrome (ARDS) or multiple organ failure, suspected to be the consequences of cytokine storm [81]. The virus infection causes a dysregulated high expression of pro-inflammatory cytokines, namely IL6 and TNF-α, through the activation of NF-κB pathway [82]. Multiple other factors including vascular endothelial growth factor, monocyte chemoattractant protein 1 and IL-8 are also recruited, thus resulting in severe

inflammation. ACA can be a potential anti-inflammatory agent in COVID-19 as it inhibits the activity of NF-κB pathway. Further study is required to harness the potential of ACA in this direction.

1.3 Research objectives

Multiple studies have demonstrated that ACA exhibited strong NF-κB inhibition property and is able to modulate various signaling pathways. Nevertheless, the exact target molecule of ACA has yet to be identified. The understanding of ACA's mechanism of action in anti-inflammation may contribute to the use of ACA in therapeutic purpose for inflammation-related diseases. The main objectives of this study are as stated below:

i. To determine the effect of ACA on LPS-induced inflammatory responses in macrophages.
ii. To elucidate the molecular mechanism of ACA in the modulation of LPS-induced inflammatory responses.
iii. To investigate the effect of ACA on in vivo septic shock model.

2.0 Materials and Methods

2.1 Mice

Wild type C57BL/6 (8-12 weeks old) mice were obtained from CLEA Japan. Animal maintenance and experimental procedures were in accordance with the guidelines of the Committee on Animal Research of Nara Institute of Science and Technology (NAIST).

2.2 Cells

Murine bone marrow derived macrophages (BMMs) were differentiated from mouse bone marrow cells in RPMI 1640 (Nacalai Tesque) containing 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific), antibiotic-antimycotic mixed solution (stabilized) (Nacalai Tesque), 100 μM of β-mercaptoethanol (Nacalai Tesque) and 20 ng/ml of murine macrophage colony-stimulating factor (M-CSF) (Biolegend) in 5% CO_2-humidified incubator. BMMs were harvested at day 7 using D-phosphate buffer saline (D-PBS) (Nacalai Tesque) with 10 mM EDTA and subjected to subsequent experiments. Human monocytic cell line THP-1 was cultured and maintained in RPMI 1640 containing 10% heat-inactivated FBS. THP-1 was differentiated into attaching macrophage by stimulation with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 2 days. The cells were allowed to rest for another 2 days in fresh culture medium before experiment. HEK293T cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated FBS.

2.3 Reagents

LPS, high molecular weight Polyinosinic-polycytidylic acid (HMW poly(I:C)), poly(deoxyadenylic-deoxythymidylic) (poly(dA:dT)) were purchased from InvivoGen. Poly(I:C) and poly(dA:dT) were mixed with Lipofectamine 2000 (Life Technologies) in the ratio 1:2 (μg:μl) in Opti-MEM (Life Technologies) for intracellular stimulation.

Table 2.1 List of ligands.

PRR	Ligand	Concentration
TLR4	LPS	100 ng/ml
MDA5	Poly(I:C)	1 μg /ml
cGAS	Poly(dA:dT)	1 μg /ml

2.4 Antibodies

Table 2.2 List of antibodies used.

Antibody	Company	Conc.
Mouse anti-actin monoclonal	Santa Cruz	1:1000
Rabbit anti-IRF3 monoclonal (D83B9)	Cell signaling	1:1000
Rabbit anti-phospho-IRF3 monoclonal (4D4G)	Cell signaling	1:1000
Rabbit anti-ECSIT polyclonal (HPA042979)	Sigma Aldrich	1:1000
Mouse anti-TRAF6 monoclonal (D10)	Santa Cruz	1:500
Mouse anti-IκB-α monoclonal (L35A5)	Cell signaling	1:1000
Rabbit anti-phospho-IκB-α monoclonal (Ser32)	Cell signaling	1:1000
Mouse anti-ERK1/2 monoclonal (C-9)	Santa Cruz	1:1000
Rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	Cell signaling	1:1000
Rabbit anti-JNK2 monoclonal (56G8)	Cell signaling	1:1000
Rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185)	Cell signaling	1:1000
Rabbit anti-p38 MAPK antibody	Cell signaling	1:1000
Rabbit anti-phospho-p38 MAPK monoclonal (Thr180/Tyr182)	Cell signaling	1:1000
Rabbit anti-NF-κB p65 monoclonal (D14E12)	Cell signaling	1:1000
Rabbit anti-phospho-NF-κB p65 monoclonal (Ser536)	Cell signaling	1:1000
Mouse anti-FLAG monoclonal (M2)	Sigma Aldrich	1:1000
Rabbit anti-HA polyclonal (H6908)	Sigma Aldrich	1:1000
Mouse anti-TOM20 monoclonal (F10)	Santa Cruz	1:1000
Goat anti-Lamin B polyclonal (M-20) (sc-6217)	Santa Cruz	1:1000
HRP-conjugated anti-mouse IgG monoclonal	Sigma	1:10000
HRP-conjugated anti-rabbit IgG monoclonal	Sigma	1:10000
HRP-conjugated anti-goat IgG monoclonal	Sigma	1:10000
Alexa Fluoro488 Goat anti-rabbit IgG (H+L)	Invitrogen	1:10000
Alexa Fluoro568 Goat anti-rabbit IgG (H+L)	Invitrogen	1:10000

2.5 RT-qPCR

Cells were seeded in 24 well plates (Corning) at 3 x 10^5 cells/well and allowed for overnight attachment. Cells were then stimulated with 100 ng/ml of LPS, 1 mg/ml of poly(I:C) or 1 mg/ml poly(dA:dT) for 1, 3, 6 and 8 hours. Total RNA was collected using Tri reagent (MRC).

2.5.1 RNA extraction and cDNA synthesis

Total RNA was extracted using Tri reagent according to the manufacturer's protocol. In brief, cell lysate collected using Tri reagent was mixed with chloroform and centrifuged at 13,000 rpm for 15 min at 4 °C. Solution in the upper phase were transferred to a new tube and equal volume of isopropanol was added to precipitate the RNA. Mixture was mixed by inverting for several times and then centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was discarded and pellets were washed with 75% ethanol. RNA pellets were then air dried and dissolved in deionized water (Nacalai Tesque). The concentration of total RNA was measured using Nanodrop (Thermo Scientific). ReverTra Ace synthesis kit (Toyobo) was used to synthesize of cDNA as follow:

Reagent	**Volume (µl)**
Total RNA (500 ng)	Up to 6 µl
Random primer	0.5
dNTPs	1
ReverTraAce (Toyobo)	0.5
ReverTraAce 5x buffer	2
Nuclease free water	Top up to 10 µl
Total volume	10

The condition for cDNA synthesis is as follow:

Temperature	**Time (min)**
30 °C	10
42 °C	60
99 °C	5

2.5.2 Real-time quantitative PCR

Following cDNA synthesis, the samples were subjected to real-time quantitative PCR (RT-qPCR) using Power SYBR Green PCR Master Mix (Applied Biosystems). The reaction solution was prepared as below:

Reagent	**Final concentration**	**Volume (μl)**
cDNA template	20 ng	2
Primers (forward + reverse)	10 mM	0.5
2x Power SYBR Green PCR Master Mix		5
Nuclease free water		2.5
Total volume		10

The list of RT-qPCR primers used was as below:

Table 2.3 List of primers used and the primer sequence for RT-qPCR.

Target	**Forward sequence (5'-3')**	**Reverse sequence (5'-3')**
Ifnb1	atggtggtccgagcagagat	ccaccactcattctgaggca
Cxcl10	cctgcaggatgatggtcaag	gaattcttgttcggcagtt
Il6	gtagctatagtactccagaagac	acgatgatgcacttgcagaa
Tnf	cacagaaagcatgatccgcgacgt	cggcagagaggaggttgactttct
Il12p40	agaccctgcccattgaactg	gaagctggtgctgtagttctcatatt
18s rRNA	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg

The reactions were performed using LightCycler 96 (Roche Diagnostics) with the condition as below:

	Temperature	**Time**	**No. of cycle**
Initial denaturation	95 °C	10 min	1
Denaturation	95 °C	10 s	50
Annealing and extension	60 °C	1 min	

2.6 Immunofluorescence microscopy

Coverslips were coated with poly-L-lysine (Sigma) in 24 wells plate for 30 min at room temperature and then washed with PBS. 3 x 10^5 BMMs were seeded on the coverslips and let attached overnight. Cells were stimulated with LSP for 20 minutes, with or without ACA treatment. Cells were then fixed on the coverslips with 4% paraformaldehyde (Nacalai) for 30 min and washed with PBS for three times. Next, the cells were permeabilized and blocked with PBS containing 100 mM glycine, 0.02% TritonX-100 and 1 % BSA for 30 min at room temperature. The coverslips were then incubated overnight with primary antibody diluted in 0.02% TritonX-100 in PBS at 4 °C overnight. After washing, the coverslips were incubated with Alexa Fluoro secondary antibody and Hoechst 33342 for nuclei staining. Coverslips were then mounted with Fluoro-KEEPER Anti-fade Reagent (Nacalai Tesque). The coverslips were observed with LSM 700 (Carl Zeiss) and analysis was performed with ImageJ.

2.7 Western Blot

For general protein detection, cells were seeded in assay plates and allowed for overnight attachment. Cells were then pretreated with ACA before stimulation with 100 ng/ml of LPS, and then lysed with cell lysis buffer. Next, the suspension was centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatant was collected and stored at -20 °C until further experiments.

2.7.1 Immunoprecipication

Cells were lysed with NP-40 lysis buffer (20 mM TRIS-HCl (pH8.0), 130 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, protease inhibitor cocktail), detached using cell scraper and sonicated two times for 10s in sonicator bath. For endogenous ubiquitination detection, 100 mM N-Ethylmaleimide was supplemented to the lysis buffer. Cell lysates were centrifuged at 15,000 rpm for 5 min at 4 °C. One part of the supernatant was kept as input and the remaining supernatant was incubated with the respective primary antibody for immunoprecipication in a new tube. The lysates were mixed for 3 hours at 4 °C before the addition of nProtein A Sepharose (Cytiva). The mixture was then allowed to mix for another 1 hour at 4 °C. The beads were pellet at 15,000 rpm for 1 min and washed three times with lysis buffer. Proteins were detached from the beads by adding 2X SDS sample buffer and heating for 10 min at 95°C. The suspension was centrifuged at 15,000 rpm for 1 min at 4 °C. The supernatant was collected and stored at -20 °C until further experiments.

2.7.2 SDS-PAGE

Acrylamide gel was cast using TGX FastCast premixed acrylamide solutions (12%) (Bio-Rad). Protein samples were mixed with SDS sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.01% BPB and 0.2 M DTT) and heated at 95 °C for 5m before being loaded into the acrylamide gel. The samples were run with 20 mA in SDS-PAGE running buffer (0.25 M Tris, 1% SDS, and 1.92 M glycine).

2.7.3 Western blot analysis

Proteins separated in SDS-PAGE were transferred to Immobilon-P transfer membrane (Merck Millipore) at 200 mA in Transfer Buffer (25 mM Tris, 20% Methanol, 1.92 mM glycine). Membrane was blocked by incubating with 5% skim milk (Nacalai) or 5% BSA for one hour with shaking at room temperature. The, membrane was incubated with primary antibody diluted in TBST buffer (0.5 M tris, 1.38 M NaCl, 0.027 M KCl, 0.05% Tween-20) with 5% BSA at 4 °C overnight. After three times washing with TBST buffer, membrane was incubated with secondary antibody diluted in 2% skim milk with shaking for 30 min at room temperature. After washing, membrane was incubated using Immobilon Forte Western HRP substrate (Merck Millipore) and visualized with Image Quant LAS-4000 (GE Healthcare).

2.7.4 TRAF6 ubiquitin-ligase activity assay

HEK293T cells were seeded and allowed to attached overnight in assay plate. The cells were transiently transfected with 1 μg of pCMV2-FLAG-TRAF6 and 2.5 μg of HA-tagged ubiquitin using polyethylenimine (PEI) mixed in OptiMEM media (Table 2.4). Cell culture media was replaced with fresh DMEM supplemented with 10% FBS, with or without ACA. The cells were incubated for 24 hours before lysis with NP-40 lysis buffer and detached using cell scraper. Cell lysates were centrifuged at 15,000 rpm for 5 min at 4 °C and transferred to a new tube. FLAG-TRAF6 was immunoprecipitated using anti-FLAG antibody and nProtein A Sepharose. Sepharose beads were then washed three times with the lysis buffer and detached from the beads by adding 2X SDS sample buffer and heating for 3 min at 95°C. The suspension was centrifuged at 15,000 rpm for 1 min at 4 °C. The supernatant was collected and blotted against anti-HA antibody to visualize the TRAF6 ubiquitin-ligase activity.

Table 2.4 Plasmid used in TRAF6 ubiquitin-ligase activity assay

Plasmid	Protein tag	Protein description
pCMV2-FLAG-TRAF6	FLAG	Human TRAF6
pcDNA3-HA-Ub	HA	Wild type ubiquitin
pcDNA3-HA-K48	HA	K48-specific ubiquitin (all lysine mutated to arginine except for lysine at position-48)
pcDNA3-HA-K63	HA	K63-specific ubiquitin (all lysine mutated to arginine except for lysine at position-63)

2.8 Luciferase reporter assay

HEK293 cells were seeded and allowed to attached overnight in assay plate. The cells were transiently transfected with 50 ng of pEF-Bos empty vector, pEF-Bos-MyD88, pCMV2-FLAG-TRAF6 or pEF-Bos-IKKβ along pGL3-NF-κB-Luc and 10 ng pTK-RL using Lipofectamine 2000 mixed in OptiMEM media. The cells were incubated for 6 hours before treatment with 10 μM of ACA for 24 hours. Then, the luciferase activity was measured using Dual-Luciferase Reporter Assay kit (Promega). Briefly, the cells were lysed using Passive Lysis Buffer for 15 minutes. Luciferase Assay Reagent II and Stop & Glo reagent were added to the cell lysis using TriStar^2 LB942 Multidetection Microplate Reader (Berthold). Firefly and Renilla luciferase activities were then measured by the microplate reader. Promoter activity of NF-kB from Firefly were normalized by Renilla luciferase as internal control and then promoter activity induced by each plasmid were normalized using empty vector as control.

2.9 Mitochondria ROS detection and measurement

Cells were seeded and allowed to attached overnight in 24 well assay plate. The cells were preteated with ACA for 30 min before exposure to LPS for 30 and 60 min. Next, cells were washed with PBS once and incubated with 2.5 μM of MitoSox Red reagent (Invitrogen) in cell culture medium for 20 min in incubator, protected from light. Cells were washed with ice cold PBS and detached by gently pipetting with PBS supplemented with 10 mM EDTA. Detached cells were collected in a new tube and spin down at 3000 rpm for 5 min. Cell pellets were washed with ice cold PBS and resuspended in FACS buffer. Then, the mROS generation were measured by flow cytometry.

3.0 Results

3.1 ACA inhibits mRNA expression of pro-inflammatory cytokines

Previous studies have reported that ACA treatment is able to inhibit the downstream effectors of inflammation pathway such as NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2. Here, I have investigated the effect of ACA treatment on the mRNA expression of pro-inflammatory cytokines upon LPS stimulation. Murine bone marrow-derived macrophages (BMMs) were pretreated with various doses of ACA (0.5, 1, 2.5, 5 μM) prior to exposure to LPS stimulation, poly(I:C) or poly(dA:dT) transfection for 3 hours. The total mRNA of the cells was collected, reverse transcribed to cDNA and rt-qPCR was performed to examine the cytokines mRNA expression. In untreated condition, LPS stimulation increased the mRNA expression of *Il6, TNF, Il12b, and Ip10*, which are regulated by NF-κB and MAPK. When treated with 2.5 and 5 μM, the expression of these cytokines was significantly reduced in BMM cells (Figure 3.1A). As a conformation, the expression of these cytokines was also reduced when BMMs were exposed to transfection of poly(I:C) and poly(dA:dT) (Figure 3.1B and 3.1C). The results therefore suggest that ACA indeed inhibits the expression of NF-κB- or MAPK-dependent pro-inflammatory genes.

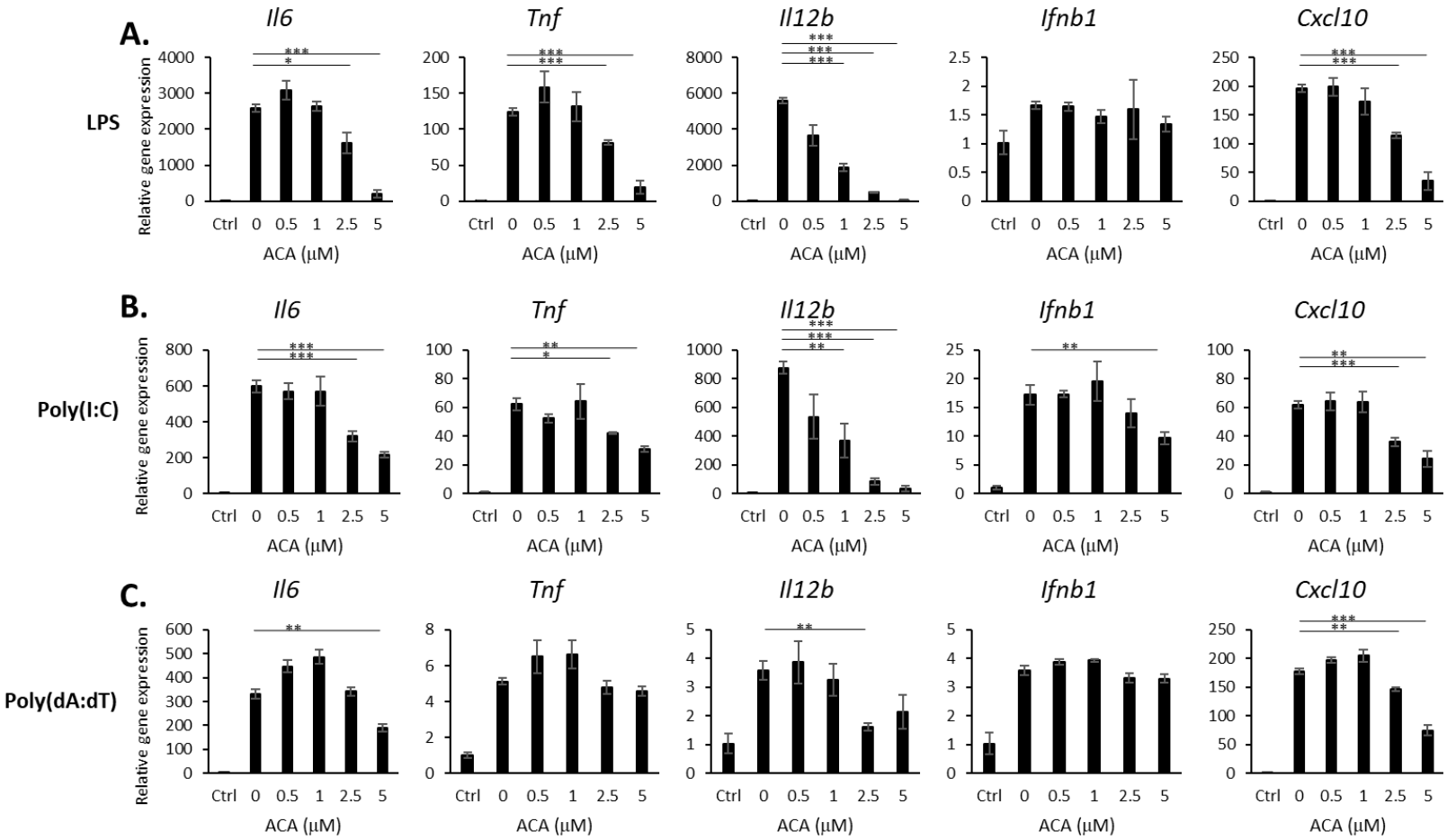

Figure 3.1. ACA treatment inhibited the mRNA expression of *Il6, Tnf, Il12b, and Cxcl10*. BMMs were treated with ACA with concentration from 0.5 – 5 μM stimulated with (A) LPS; or transfected with (B) poly (I:C) or (C) poly(dA:dT). 18s rRNA was used as the internal control. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n=3).

3.2 ACA inhibits IL-6 and TNF-α production

Following the transcription of mRNA, the mRNAs are translated into protein, in this case, the pro-inflammatory cytokines. In order to measure the production of cytokines, BMMs were treated with various concentration of ACA (0.625 – 10 μM) and stimulated with 100 ng/ml of LPS for 3, 6 and 9 hours. The production of IL-6 and TNF-α in the culture supernatant was collected and measured using ELISA.

The findings revealed that ACA treatment significantly reduced the production of IL-6 and TNF-α in a dose-dependent manner. ACA effectively inhibited the production of IL-6 in BMM from the concentration of 1.25 μM in 3, 6, 9 hours post-stimulation (Figure 3.2A); whereas the concentration of 2.5 μM inhibited the production of TNF-α in all 3, 6, 9 hours post-stimulation (Figure 3.2B). The results complimented the observation from rt-qPCR, in which ACA treatment is able to inhibit the cytokines production under the NF-κB or MAPK regulation.

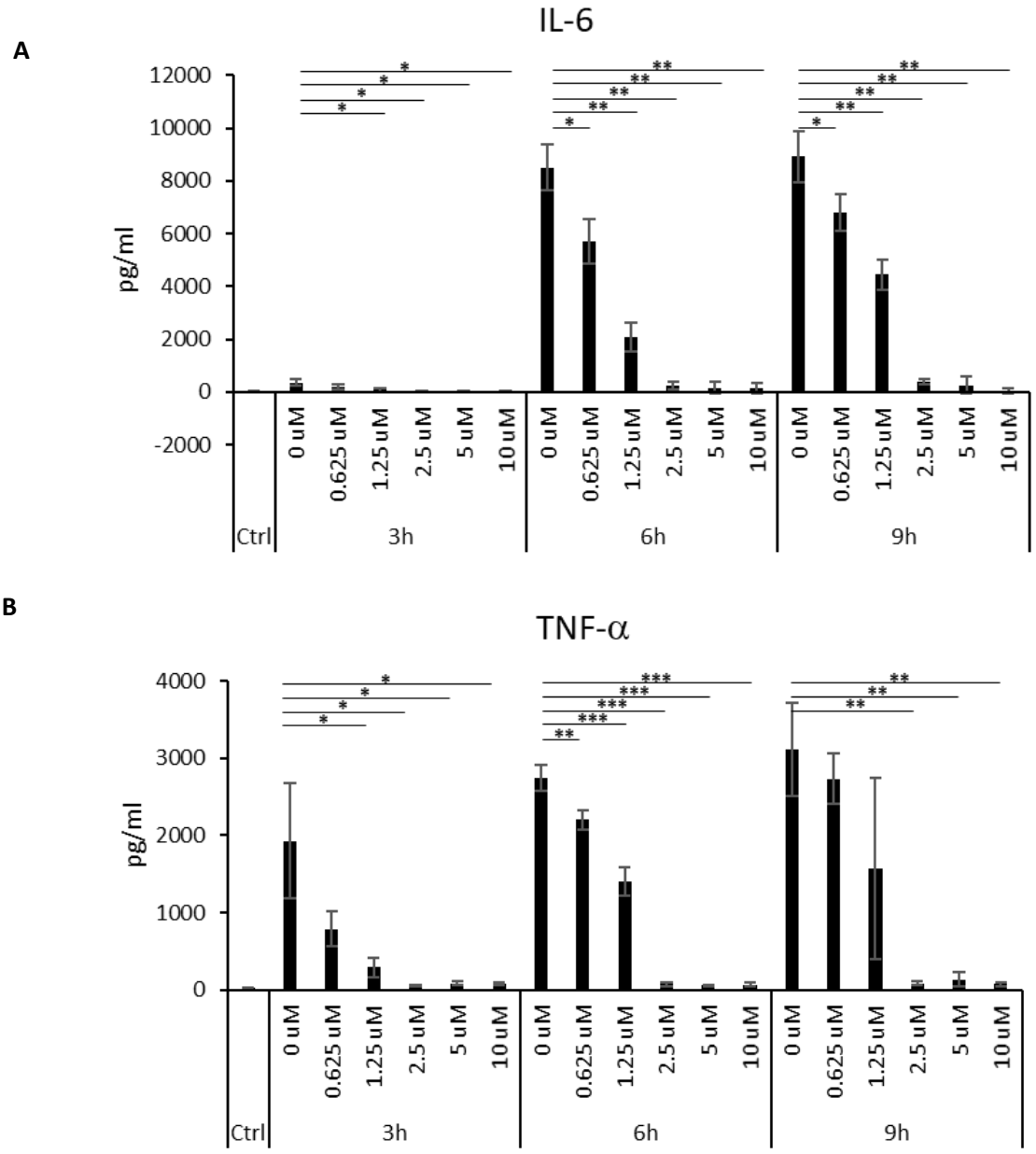

Figure 3.2. ACA treatment inhibited the production of IL-6 and TNF-α in a dose-dependent manner. BMMs were treated with ACA with concentration from 0.625 – 10 μM prior to stimulation with LPS. The concentration of (A) IL-6 and (B) TNF-α were measured by ELISA. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n=3).

3.3 ACA attenuates p65 nuclear translocation

Prior to the transcription of pro-inflammatory cytokines, the transcription factor p65 needs to be transported across the nucleus membrane to initiate the NF-κB response. The nuclear translocation of p65 can be visualized using immunofluorescence microscopy. BMMs were treated with 2.5 μM of ACA and stimulated with LPS for 20 minutes before the cells were fixed and stained with anti-p65 antibody for immunofluorescence microscopy. In unstimulated cells, no nuclear translocation was observed (Figure 3.3A). In comparison, p65 nuclear translocation was detected in nearly all LPS-stimulated cells. Whereas treated with ACA, only a few cells displayed nuclear localization. The number of cells with p65 nuclear localization were counted. Nuclear localization was observed in 96.5% of the treated cells as compared to 7.9% in the ACA-treated cells (Figure 3.3B). Thus, the results suggested that ACA inhibits the nuclear localization of p65 to suppress the production of IL-6 and TNF-α.

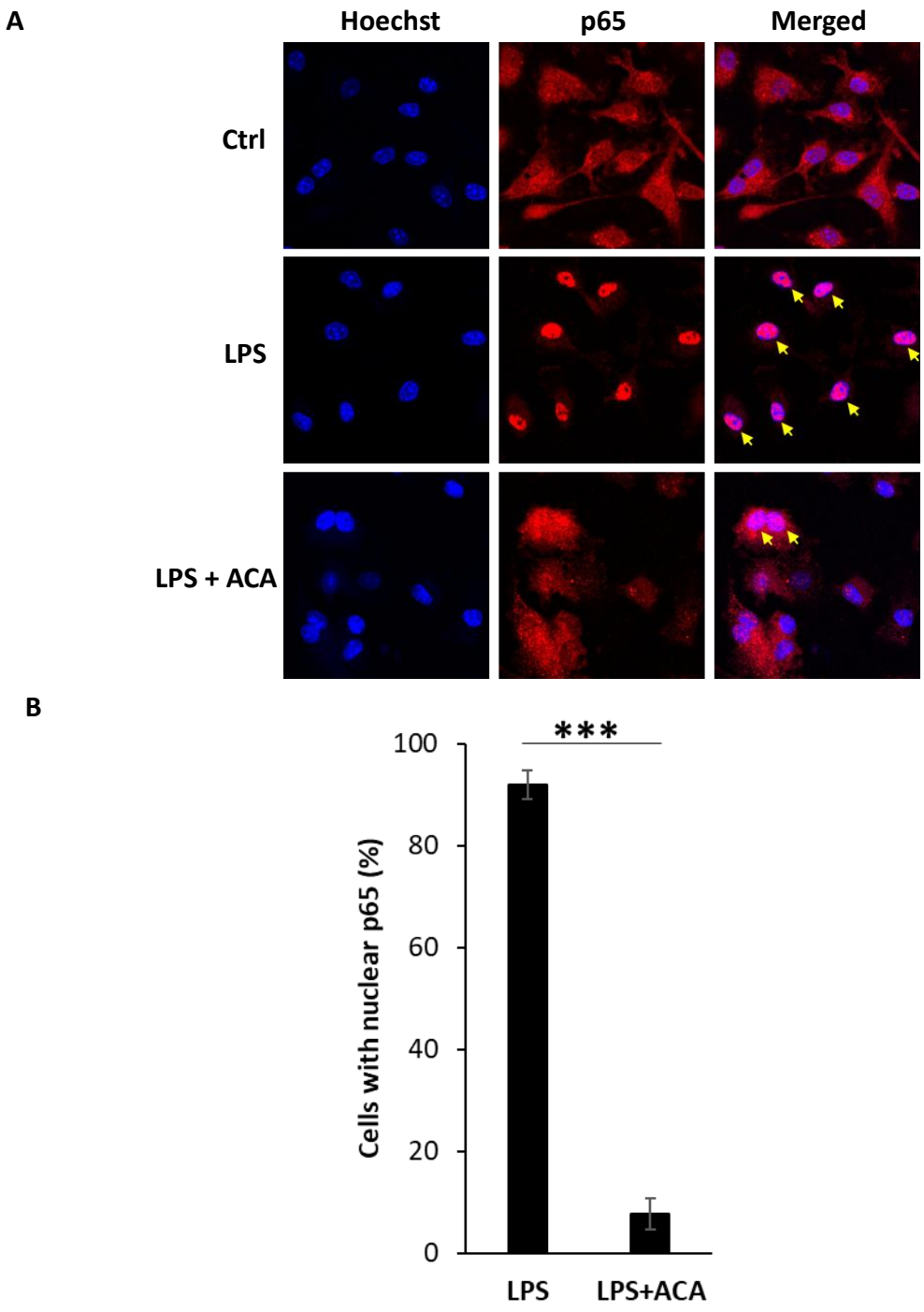

Figure 3.3. ACA inhibited the nuclear localization of p65. (A) BMMs were stimulated with LPS and treated by ACA, then subjected to immunostaining and observed under immunofluorescence microscope. Cells with nuclear p65 were indicated by yellow arrow. (B) More than 500 cells were counted and the percentage of cells with nuclear p65 in BMMs were tabulated. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n=10)

3.4 ACA suppresses the NF-κB pathway via inhibiting the phosphorylation and degradation of IκBα

In order to identify the target of ACA, BMMs were treated with ACA, stimulated with LPS for 10-60 minutes. The cell lysate was then collected for western blot analysis. In unstimulated condition, the NF-κB p65 remained unphosphorylated and stabilized by the inhibitory protein IκBα. Upon stimulation by LPS, IκBα is phosphorylated and then degraded. This allows p65 to be phosphorylated and translocated into the nucleus to initiate the downstream process. Based on the results, ACA inhibited the phosphorylation of p65 (Figure 3.4). This is consistent with the observation in which ACA treatment also inhibited the phosphorylation of IκBα in all time point. Furthermore, protein expression of total IκBα also demonstrated that ACA treatment inhibited the degradation of IκBα upon exposure to LPS, whereas in the untreated samples, IκBα was degraded rapidly upon exposure to LPS. In short, ACA suppresses the NF-κB pathway by inhibiting the phosphorylation and degradation of IκBα, thus preventing the phosphorylation and nuclear translocation of p65.

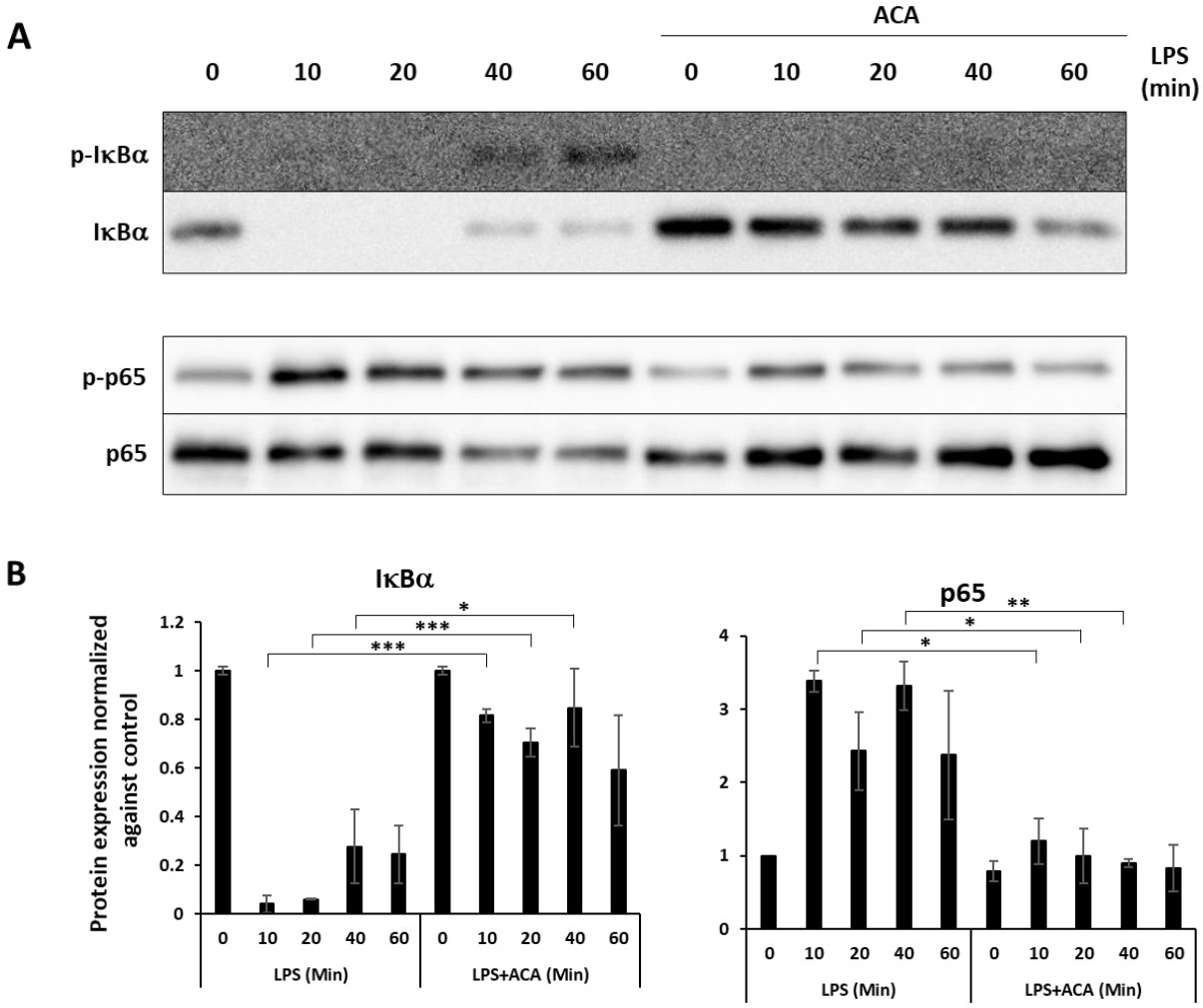

Figure 3.4. ACA inhibited the phosphorylation of p65, IκBα and prevented the degradation of IκBα. (A) Immunoblot analysis of cell lysates from BMMs treated with ACA and exposed to LPS for 10, 20, 40 and 60 minutes. (B) The relative band intensity was measured and normalized against unstimulated control. Data is representative of three independent experiments. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n=3)

3.5 ACA attenuates the phosphorylation of ERK, p38 and JNK in the MAPK pathway

Other than the NF-κB pathway, IL-6 and TNF-α production is also regulated by the MAPK pathway. The MAPKs need to be phosphorylated to activate the downstream pathway. In order to investigate whether ACA can also suppress the MAPK pathway, BMMs were treated with ACA, stimulated with LPS and the cell lysates were collected for western blot analysis. Based on the immunoblot observations, ACA treatment strongly suppressed the phosphorylation of all three MAPKs blotted, namely ERK, p38 and JNK (Figure 3.5). The results suggested that ACA simultaneously suppresses NF-κB and MAPK pathway, thus strongly inhibiting the production of pro-inflammatory cytokines.

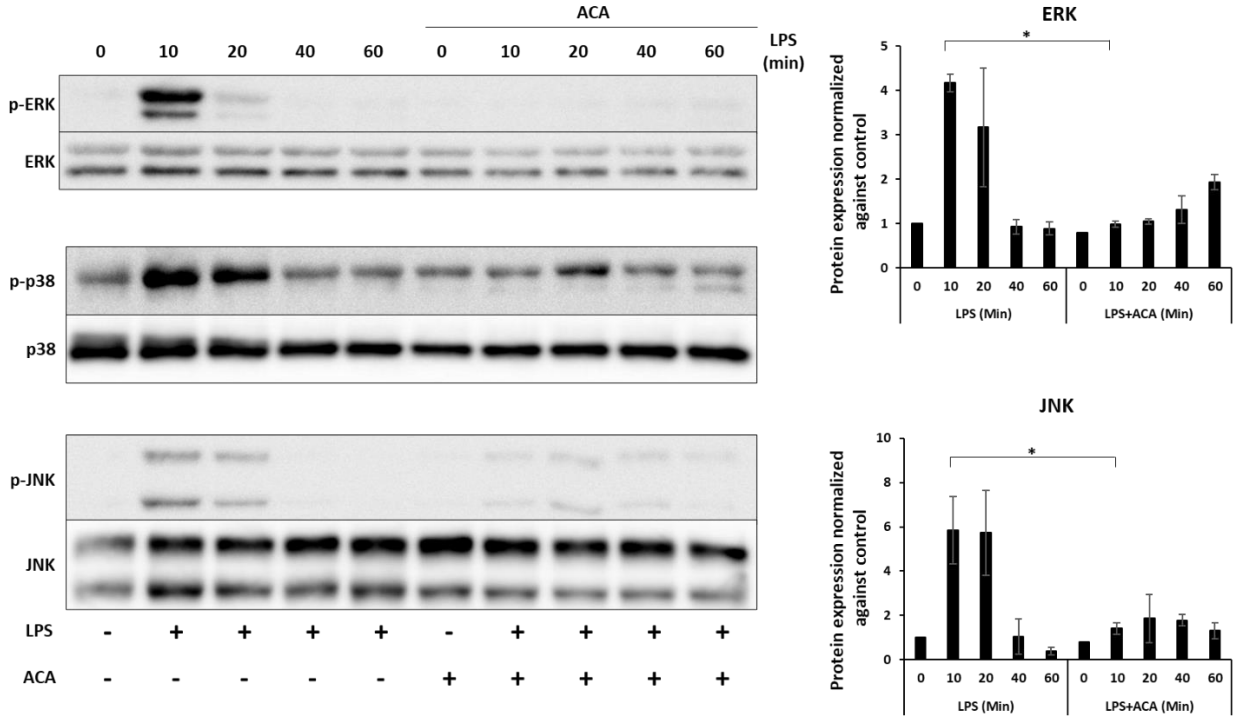

Fig 3.5. ACA treatment attenuated the phosphorylation of ERK, p38 and JNK. Immunoblot analysis of cell lysates from BMMs treated with ACA, exposed to LPS for 10, 20, 40 and 60 minutes; then blotted against the indicated antibody. The relative band intensity was measured and normalized against unstimulated control. Data is representative of at least two independent experiments. (*$p < 0.05$; n=3)

3.6 ACA suppresses phosphorylation of IRF3 induced by LPS stimulation

Alternatively, LPS stimulation can also activates TRIF-dependent pathway when TLR4 is activated. In this alternative pathway, the transcription factor IRF3 is phosphorylated and then translocated into nucleus to initiate the transcription of type 1 IFNs. As demonstrated in Figure 3.6, IRF3 was phosphorylated upon stimulation with LPS. When treated with ACA, the phosphorylation of ACA was suppressed. The suppression was also shown to be dosage dependent. The results complemented the observation in Section 3.1, whereby the gene expression of *ip10* (cytokine regulated by IRF3) was significantly reduced upon ACA treatment, suggested that ACA treatment is able to suppress the TRIF-dependent pathway in TLR4 activation.

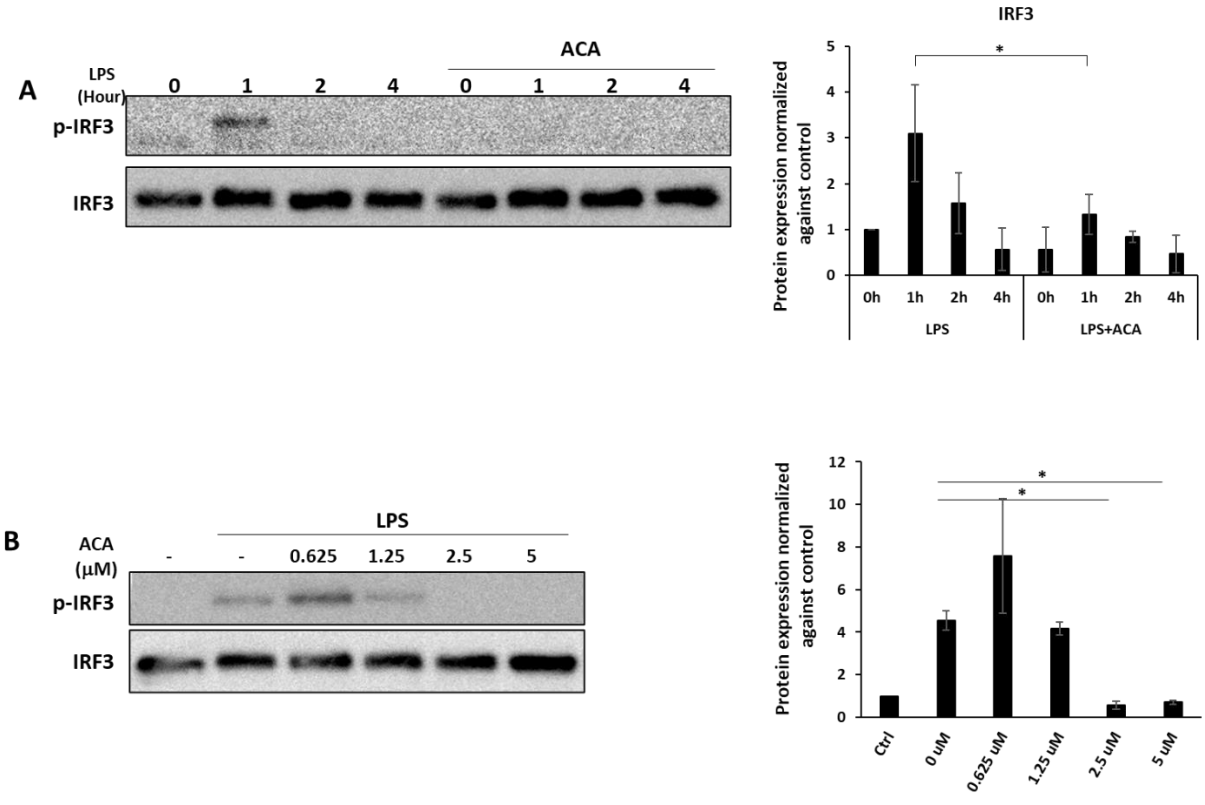

Fig 3.6. ACA treatment suppressed the phosphorylation of IRF3. Immunoblot analysis of cell lysates from BMMs treated with ACA, exposed to LPS for 1, 2 and 4 hours (A); or treated with various concentration of ACA before exposure to LPS for 1 hour (B). The cell lysate was blotted against the indicated antibody. The relative band intensity was measured and normalized against unstimulated control. Data is representative of at least two independent experiments. (*$p < 0.05$; n=2)

3.7 ACA attenuates MyD88- and TRAF6-induced NF-κB reporter gene expression

In order to further understand the effect of ACA in NF-κB innate immune pathway, I have transiently transfected HEK293T cells with plasmids expressing MyD88, TRAF6 and IKKβ respectively, along with luciferase reporter plasmid driven by NF-κB promoter. Then, the transfected cells were treated with ACA for 24 hours before the luciferase activity was measured. In untreated cells, the overexpression of MyD88, TRAF6 and IKKβ enhanced the NF-κB promoter activity (Fig 3.7). When the transfected cells were treated with ACA, the treatment significantly attenuated the NF-κB promoter activity driven by MyD88 and TRAF6 overexpression, but not IKKβ. The results suggested that ACA may act a step upstream on IKKβ, with most probable on TRAF6.

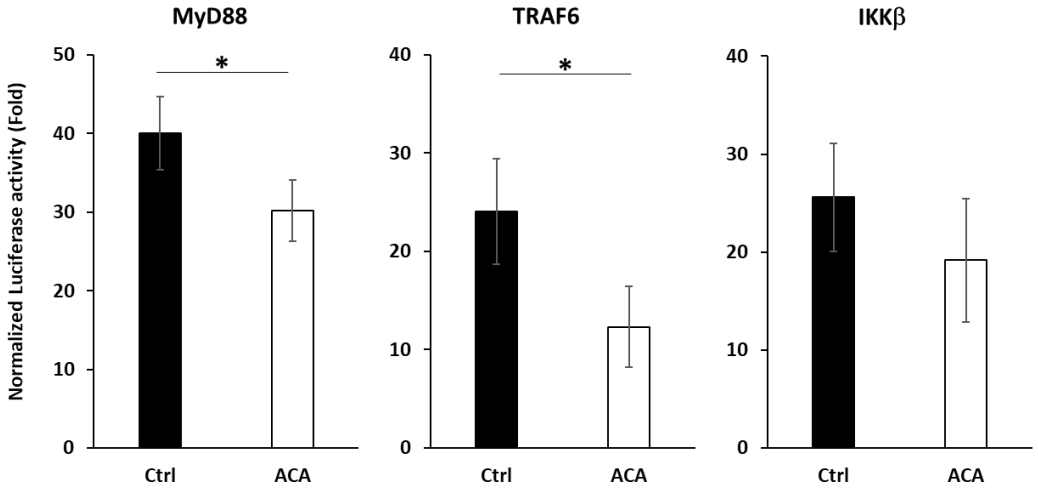

Figure 3.7. ACA treatment attenuated the NF-kB promoter activity induced by MyD88 and TRAF6. HEK293T cells were transiently transfected with empty vector, MyD88, TRAF6 and IKKβ along luciferase reporter plasmid driven by NF-κB promoter before treatment with 10 μM ACA. Luciferase activity was measured after 24 hours and normalized against cells transfected with empty vector. (*$p < 0.05$; n=3)

3.8 ACA inhibits NF-κB and MAPK pathway in THP-1

The previous sections have demonstrated that ACA inhibited both NF-κB and MAPK pathway induced by LPS stimulation in BMDM, a murine-originate primary cell. I have then proceeded to examine the effect of ACA in THP-1, a human monocytic cell line which displays macrophage properties when differentiated by PMA.

When treated with ACA, the expression of pro-inflammatory cytokines *Il6* and *Tnf* in THP-1 exposed to LPS stimulation was significantly reduced. The expression of *Tnf* and *Il6* was lowest when treated with ACA from 5 μM onwards (Figure 3.8A). Western blot analyses were performed to investigate the effect of ACA on the phosphorylation of signaling proteins in NF-κB and MAPK pathway. Similar to the observation in BMDM, ACA treatment suppressed the phosphorylation of p65 which is the transcription factor of NF-κB pathway (Figure 3.8B). ACA treatment also inhibited the phosphorylation of ERK and JNK, which are crucial signaling proteins in the MAPK pathway. The inhibition of the phosphorylation of these proteins are dosage-dependent. The results suggested that ACA can inhibit NF-kB and MAPK pathway in both BMDM from mice and THP-1 from human.

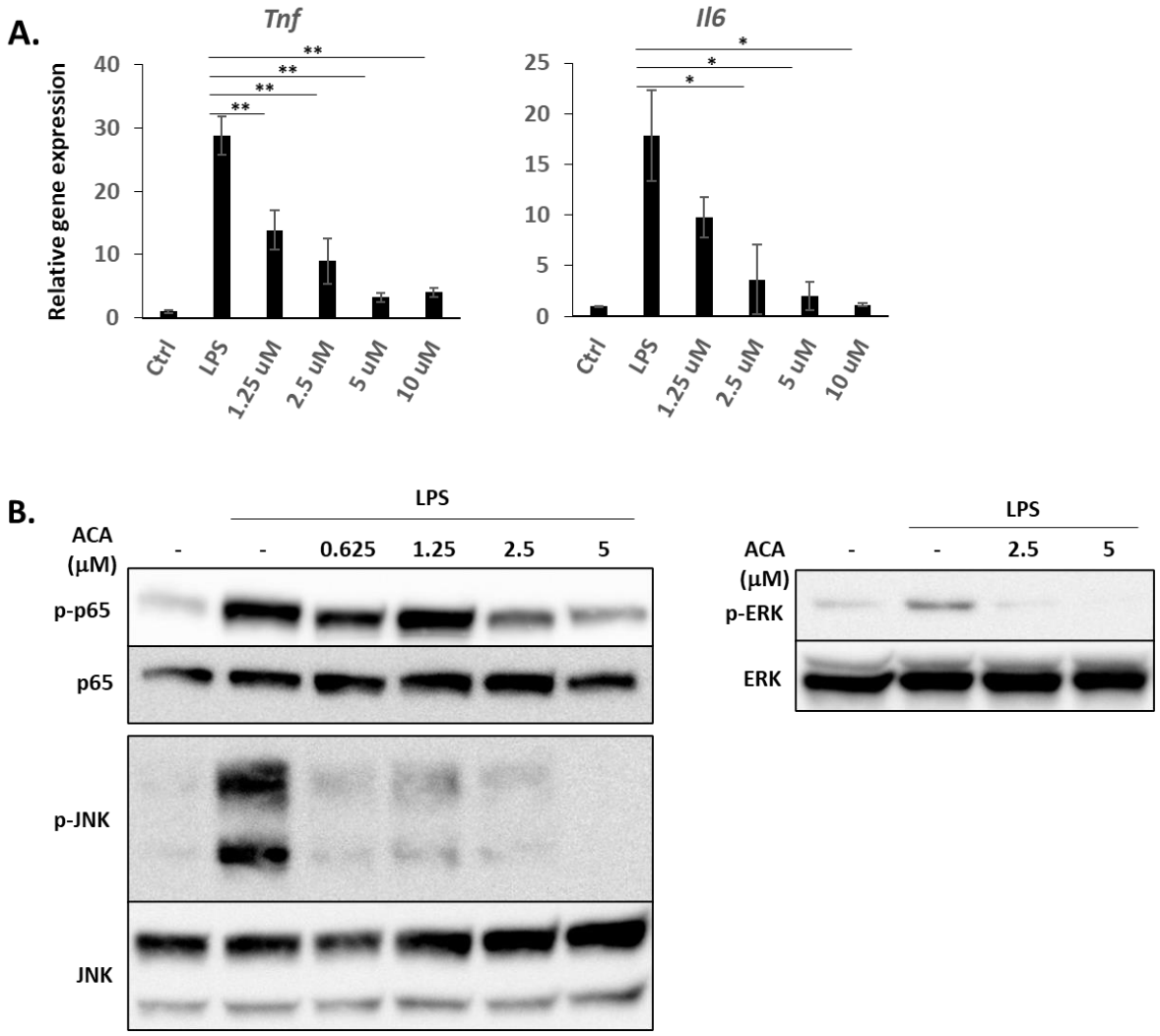

Figure 3.8. ACA inhibited NF-κB and MAPK pathway in THP-1. (A) THP-1 cells were treated with various concentration of ACA and exposed to LPS for 3 hours. The relative gene expression of *Tnf* and *Il6* were examined using qPCR analysis. (B) THP-1 cells were treated with ACA and exposed to LPS for 30 minutes. The cell lysates were collected and blotted to examine the phosphorylation of signaling proteins in NF-κB and MAPK pathway. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n=3).

3.9 ACA inhibits TRAF6 ubiquitin-ligase activity

MAPK and NF-κB pathway converged in the their upstream signaling process, in which TRAF6, an E3 ubiquitin ligase plays an important role. In order to investigate if ACA affects ubiquitination by TRAF6 in the signaling pathway, FLAG-TRAF6, together with different HA-tagged ubiquitin expression vectors (wild type ubiquitin, K48-specific ubiquitin, K63-specific ubiquitin) were transfected transiently into HEK293T cells and treated with ACA. IP was performed to precipitate FLAG-TRAF6 and blotted against anti-HA antibody.

Upon ACA treatment, the amount of wild type ubiquitin and K63-specific ubiquitin interacting with TRAF6 was reduced as compared to the untreated cells (Figure 3.9A). In comparison, K48-specific polyubiquitination was unaffected. This observation indicated that ACA treatment affected the formation of K63-specific polyubiquitination but not the formation of K48-specific polyubiquitination on TRAF6. As shown in Figure 3.8B, the reduction in K63-specific polyubiquitination was also dosage dependent. In addition, ACA treatment also reduced endogenous TRAF6 K63-linked polyubiquitination in BMDM and THP-1 (Figure 3.10). Altogether, these data suggested that ACA affected the ubiquitination function of TRAF6.

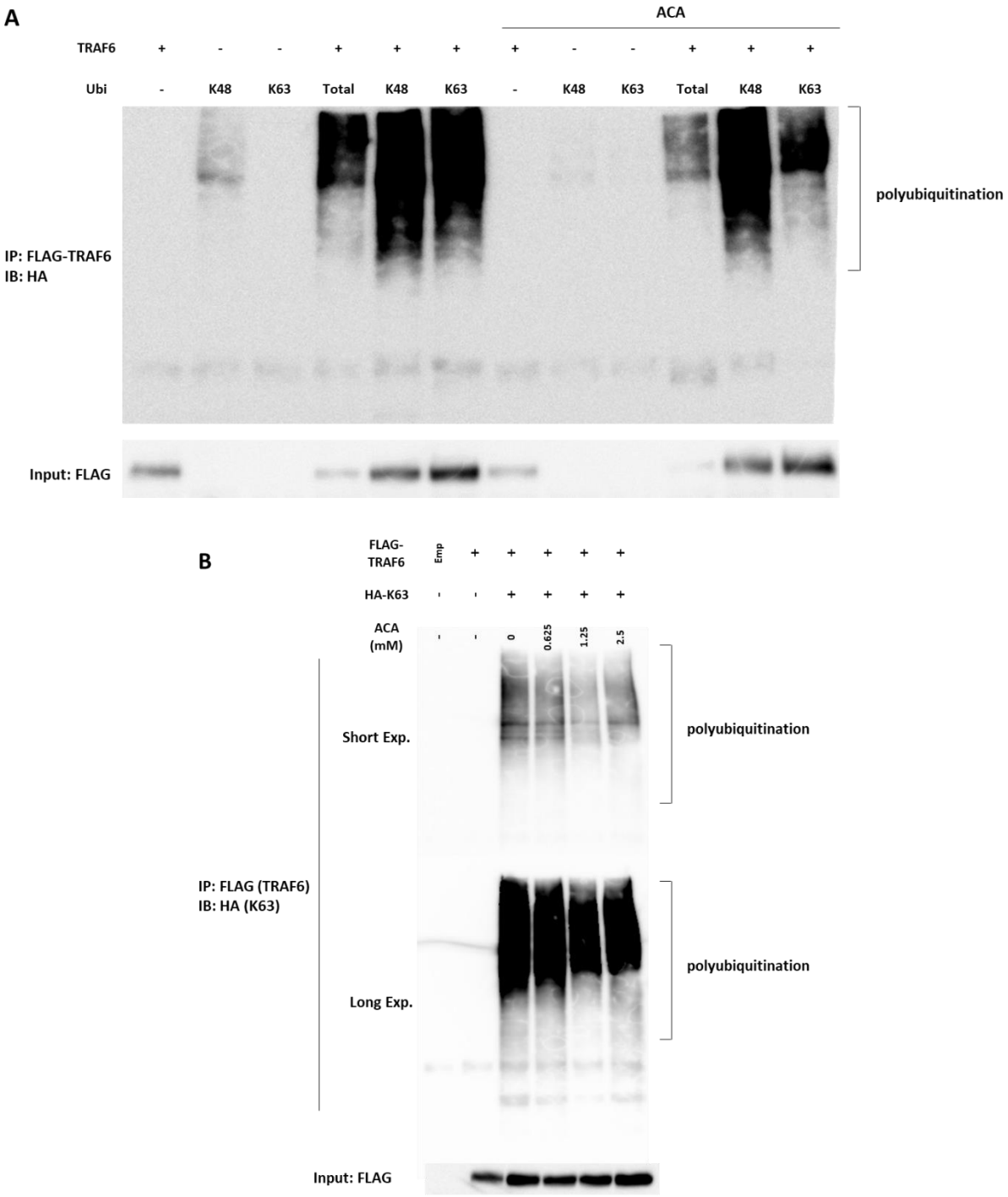

Figure 3.9. ACA inhibited TRAF6 ubiquitin-ligase activity in HEK293T overexpression system. (A) HEK293T cells were transfected with the FLAG-TRAF6, HA-Total ubiquitin, HA-K48-specific ubiquitin, HA-K63-specific ubiquitin and co-cultured overnight with 2.5 μM of ACA. (B) HEK293T cells co-transfected with FLAG-TRAF6 and HA-K63-specific ubiquitin, then co-cultured with various concentration of ACA as indicated. Cell lysates were immunoprecipitated with anti-FLAG antibody and blotted against anti-HA antibody.

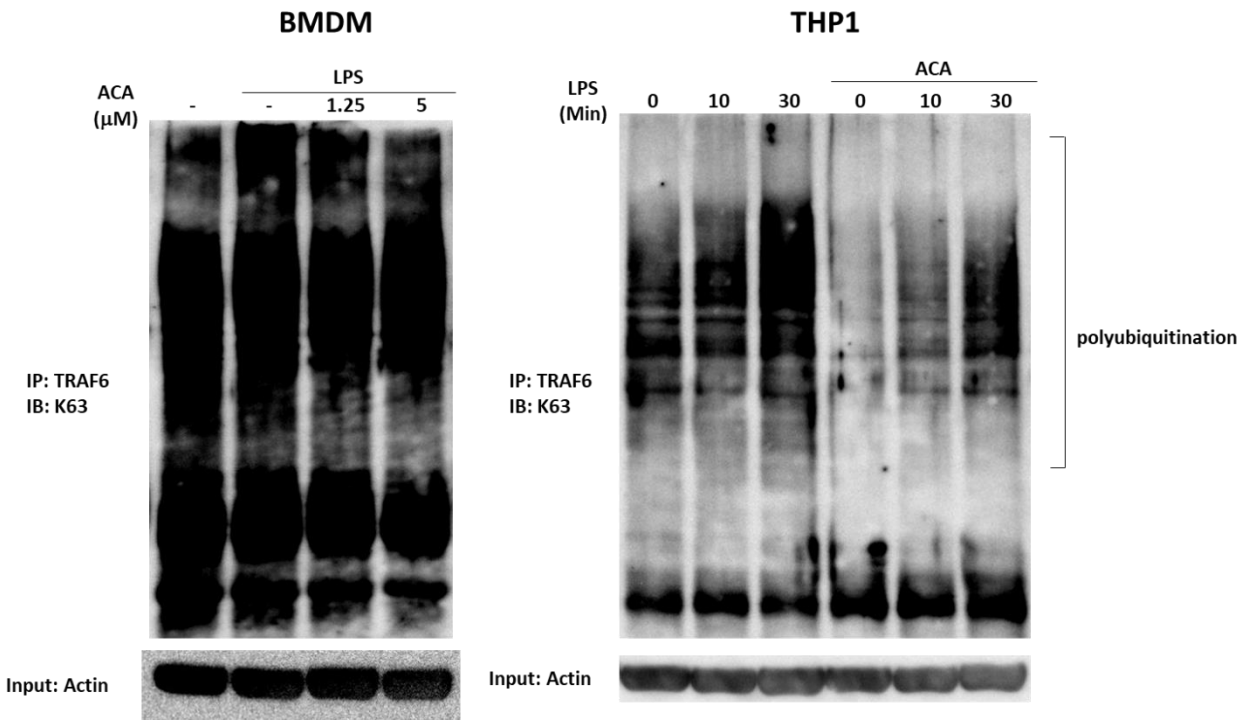

Figure 3.10. ACA inhibited endogenous TRAF6 ubiquitin ligase activity. BMDM and PMA-stimulated cells were treated with ACA before exposure to LPS. Cell lysates were immunoprecipitated with TRAF6 antibody and blotted against anti-K63-specific ubiquitin antibody.

3.10 ACA suppresses mtROS generation

In a recent study published by our lab, ACA was found to suppressed the mitochondrial ROS (mtROS) generation in NLRP3 inflammasome-activated cells. Several studies have demonstrated that TRAF6 ubiquitination is crucial to initiate the generation of mtROS in LPS-stimulated cells. Since ACA affects TRAF6 ubiquitin ligase activity, I then proceeded to examine the effect of ACA on the mtROS generation in LPS-stimulated cells. Cells were treated with ACA and exposed to LPS before stained by MitoSox Red reagent which targets superoxide in mitochondria. The generation of mtROS was then detected using flow cytometry. ACA treatment significantly reduced the mtROS generation induced by LPS stimulation in BMDM, represented by the decrease in relative mean fluorescence intensity (MFI) (Figure 3.11).

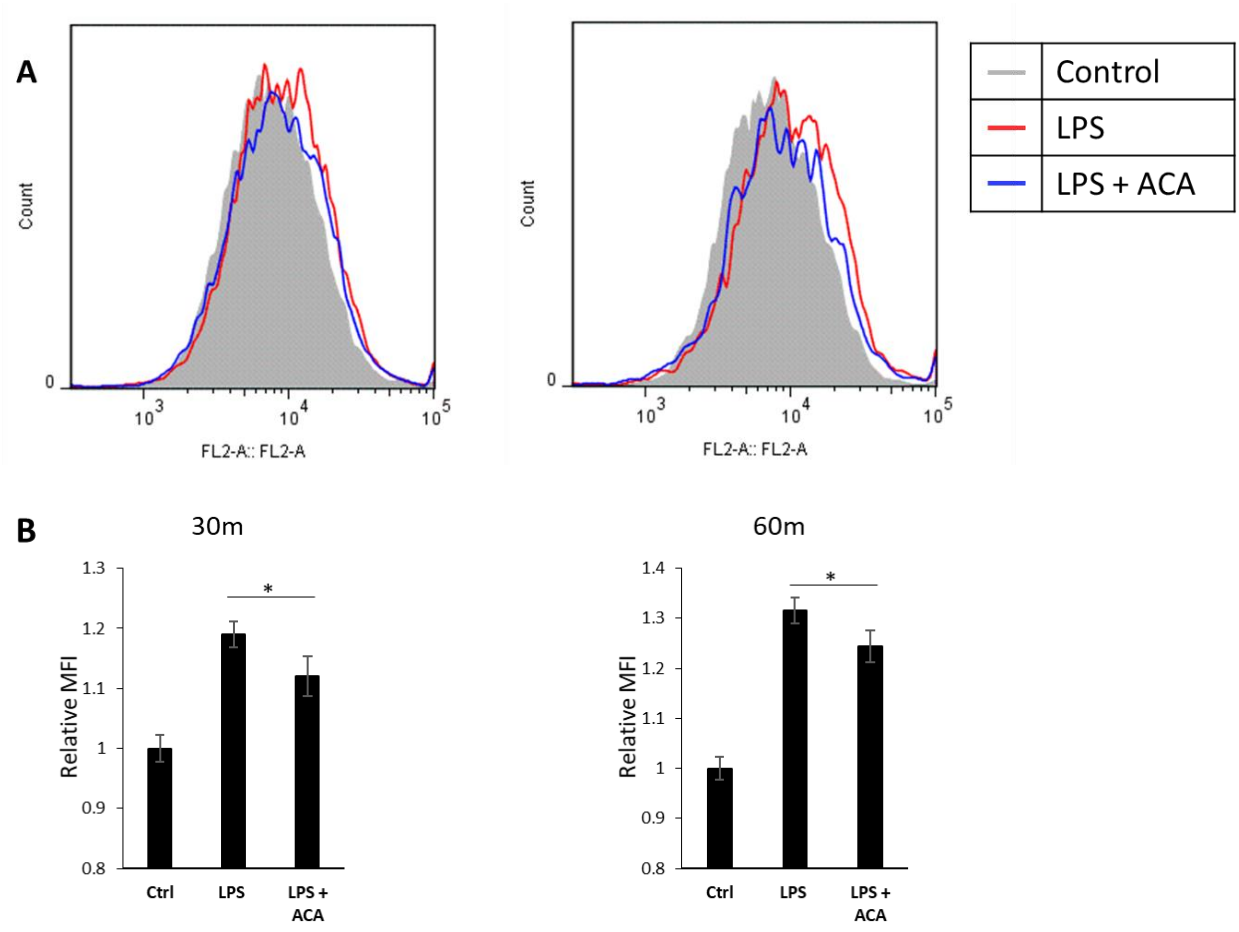

Figure 3.11. Effect of ACA treatment on mtROS generation. BMDMs were treated with ACA before exposure to LPS stimulation for 30 and 60 minutes. Cells were then stained with MitoSOX Red reagent and analyzed using flow cytometry. (A) Representative flow cytometry results of MitoSOX staining. (B) Relative MFI was calculated and tabulated. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n=3).

4.0 Discussion

The occurrence of inflammation is crucial in deterring the threats of danger signals originated from invading pathogens or damaged cells. However, excessive inflammatory responses are often detrimental to the host. Some of the examples of dysregulation of inflammation are septic shock, autoimmune and neurological inflammation. One of the solutions for excessive inflammation is the use of candidate compounds with immuno-modulating property for therapeutic purposes.

ACA is a compound extracted from the rhizome of tropical ginger. Various studies have showed that ACA has robust anti-cancer property which mainly executed through the induction of apoptosis. This is achieved by suppressing the NF-κB-regulated gene products [70, 72, 75]. These studies have also demonstrated that ACA regulates the NF-κB pathway by inhibiting the activation of IκBα.

In this study, ACA was evaluated as an anti-inflammatory compound and the mode of mechanism in which ACA exerts its anti-inflammatory property is elucidated. Firstly, ACA treatment significantly reduced the mRNA expression of *Il6*, *Tnf*, *Il12b* and *Ip10* regulated by NF-κB- and MAPK-dependent pathway. This is further strengthened by the fact that ACA treatment inhibited the IL-6 and TNF-α production in ELISA. These results complimented the previous studies in which ACA treatment is able to downregulate the NF-κB gene products.

In the activation of NF-κB pathway, p65 plays an important role by forming a heterodimer with p50. Under normal circumstances, the heterodimer is inactivated by the inhibitor protein complex, where the nuclear localization signal (NLS) was masked by IκBs [83]. The masking of NLS prevented the p50/p65 dimer to be translocated across the nuclear membrane to initiate the transcription of NF-κB gene products. Upon exposure to LPS, activation of TLR4 leads to the assembly of myddosome, signaling the activation of TRAF6 complex, phosphorylating TAK1 to activate the IKKs. The IKKs, particularly the IKKβ, is responsible to phosphorylate IkBα, allowing the degradation of p105 into p50 and exposing the NLS of the protein. This in turn permits the nuclear translocation of p50/p65 to carry out the transcription factor's function. In the current study, the LPS-induced p65 nuclear localization in ACA treated cells was almost completely obliterated. Furthermore, using western blot analysis, the phosphorylation of p65 in ACA treated cells was found to be suppressed. ACA treatment has also inhibited the phosphorylation and

degradation of IκBα which is responsible to inactivate the NF-κB complex. These observations validated the findings of previous studies in which ACA suppresses the NF-κB activation via inhibition of IκBα phosphorylation and degradation.

Other than the NF-κB pathway, MAPK pathway is another signaling pathway which regulates the expression of pro-inflammatory cytokines. Among the MAPKs, ERK, p38 and JNK are the most extensively studied MAPKs. Interestingly, the activation of these MAPKs is correlated with NF-κB. In LPS stimulation, upon activation of the TRAF6 ubiquitin complex, TAK1 is phosphorylated and act as the MAP3K for p38 and JNK [42]. At the same time, TAK1 also activates the IKK complex which simultaneously initiates the NF-kB pathway and ERK pathway [44, 47]. In this study, ACA treatment potently inhibited the phosphorylation of ERK, p38 and JNK. In other words, ACA treatment effectively inhibited the MAPK pathway activated by LPS stimulation.

Together with the inhibition on NF-κB pathway and MAPK pathway, ACA treatment effectively attenuated the production of pro-inflammatory cytokines. These observations gave hints to the further direction of this study. As mentioned above, the activation of NF-κB and MAPK pathway involves the common upstream signaling pathway; from the activation of TLR4, assembly of myddosome, and formation of TRAF6 ubiquitin complex. Previous studies discovered that ACA is able to inhibit the phosphorylation and degradation of IκBα. These results provided explanation for the inhibition of NF-kB signaling pathway. Nevertheless, this does not account for the inhibition of MAPK signaling pathway. It is highly possible that treatment with ACA affected further upstream of the signaling pathway, probably on TRAF6 ubiquitin complex. NF-kB luciferase reporter assay suggested that ACA affects the process above IKKβ and thus leading us to further investigate the effect of ACA on TRAF6 complex. It is noteworthy to point out that TRAF6 ubiquitin complex can control the TLR4-induced autophagy via K63-linked ubiquitination of beclin-1 (BECN1) [84]. On the other hand, a recent study has showed that ACA is able to induce autophagy in human non-small cell lung cancer cells [85]. Thus, it is likely that ACA exerts its anti-inflammatory effect via regulating TRAF6 complex.

In order to investigate the hypothesis, plasmids expressing tagged-TRAF6 and ubiquitin were transfected into HEK293T cell and treated with ACA to examine the effect of ACA on TRAF6 ubiquitination activity. Using this overexpression system, I have found that ACA treatment

reduced the total ubiquitination on TRAF6. There are two types of ubiquitination which are actively being studied, which are lysine 48 (K48)-linked polyubiquitination and K63-linked polyubiquitination. K48-linked polyubiquitination involves tagging the target protein for proteasome degradation, whereas K63-linked polyubiquitination mainly responsible for signal transduction [86]. ACA treatment decreased the K63-specific polyubiquitination but not K48-specific polyubiquitination on TRAF6. This observation suggested that ACA is capable of affecting the K63-linked ubiquitin ligase activity of TRAF6, thus disrupting the TLR4 downstream signaling pathway related to TRAF6 ubiquitination.

It is known that TLR4 activation by LPS stimulation results in mROS generation which contributes to antimicrobial activity in macrophages [48]. The generation of mROS is mainly contributed by the mitochondrial complex I [87]. Recent studies have identified ECSIT as a crucial member of the assembly and regulatory protein of mitochondrial complex I [50, 88]. The deletion of ECSIT in macrophage resulted in the failure of macrophages to produce mROS when exposed by LPS [88]. The ubiquitination of ECSIT by TRAF6 is found to be required for the assembly of mitochondrial complex I and subsequently mROS generation [48, 89]. Furthermore, another study has suggested that ECSIT interacts with TRAF6 and TAK1 to activate NF-κB [52]. In order to further validate the result in which ACA affects TRAF6 ubiquitination, I have investigated the effect of ACA on the production of mROS caused by LPS stimulation. As expected, ACA treatment resulted in reduction of mROS generation. This observation implied that ACA does in fact affects TRAF6 ubiquitination. The reduction in mROS generation is also compliments the findings by another member of our laboratory, in which ACA treatment inhibited mROS generation to attenuate NRLP3 inflammasome activation [76].

Collectively, ACA inhibited the production of pro-inflammatory cytokines from the activation of NF-κB and MAPK pathway. Further experiments revealed that ACA treatment attenuated the K63-linked ubiquitination ligase activity of TRAF6, resulting in the reduction in mROS generation, stabilizing IκBα complex, thus inhibiting the activation of NF-κB and MAPK pathway. However, the exact target of ACA remains elusive. Further in-depth investigation is required to reveal the underlying mechanism of various effects of ACA. Our laboratory's previous and current study found that ACA reduced mROS generation. It is possible that ACA imposes its affect directly on mitochondria. On the other hand, given that ACA affects K63-linked ubiquitination ligase activity

of TRAF6, it is possible that ACA exerts its effect by affecting K63-linked polyubiquitination, or even M1-linked polyubiquitination (also known as linear ubiquitination) which often forms a hybrid chain with K63-linked polyubiquitination in the regulation of innate immunity components [90]. TRAF6 is an E3 ubiquitin ligase, in which its activity requires interaction with ubiquitin-conjugating enzyme UBC13 and UEV1A. The effect of ACA on this interaction can be investigate by performing in vitro ubiquitination assay to narrow down the specific target of ACA. Then, pull-down assay can be performed with the target protein, followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis to identify the target molecule of ACA. To date, only a handful of inhibitors were discovered to inhibit this interaction and the inhibitor will be useful in limiting inflammation and in cancer therapy [91].

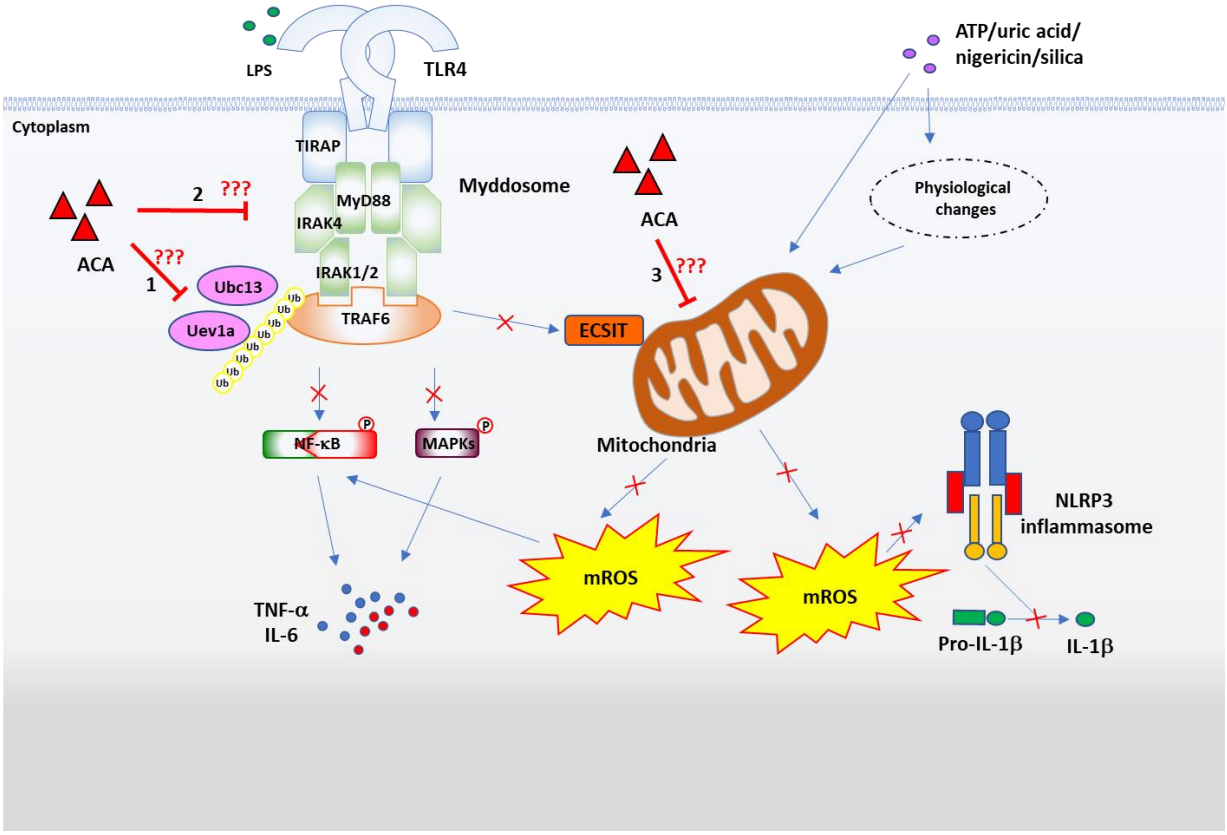

Figure 4. Proposed mechanism of ACA. Current finding suggests that ACA inhibits K63-linked polyubiquitination activity by TRAF6, thus attenuating the downstream NF-kB, MAPK and mROS generation. ACA possibly acts by: **(1)** affecting the interaction of E2 conjugating enzyme Ubc13 and Uev1a with TRAF6 ubiquitin ligase. **(2)** affecting the upstream signaling proteins in the myddosome formation. **(3)** acts directly on mitochondria thus affecting both NRLP3 inflammasome and TLR4 signaling pathway. (TLR: Toll-like receptors; TIRAP: TIR domain-containing adaptor proteins; MyD88: myeloid differentiation primary response 88; IRAK: Interleukin-1 receptor associated kinase; TRAF6: TNF receptor associated factor 6; TAK1: TGFβ-activated kinase 1; IKK: IκB kinase; ECSIT: evolutionarily conserved signaling intermediate in Toll pathways; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; MAPK: mitogen-activated protein kinase; mROS: mitochondrial reactive oxygen species)

5.0 Future perspective and directions

Up to now, my results suggested that ACA treatment affects both NF-κB and MAPK pathway, hinting that ACA regulates the upstream signaling molecules of TLR4. The suppression in these pathways is attributed to the inhibition of TRAF6 K63-linked polyubiquitination by ACA. Further study is required to identify the exact target of ACA. Hopefully the findings of this study can contribute to the development of ACA as a therapeutic drug against inflammation diseases such as septic shock, COVID-19 and autoimmune.

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List of abbreviations

PAMP	Pathogen-associated molecular pattern
DAMP	Danger-associated molecular pattern
PRR	Pattern-recognition receptor
TLR	Toll-like receptor
RLR	RIG-I-like receptor
NLR	Nod-like receptor
ALR	AIM2-like receptor
CLR	C-type lectin receptor
LPS	Lipopolysaccharide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IRF	Interferon regulatory factor
MAPK	Mitogen-activated protein kinase
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
MyD88	Myeloid differentiation primary response 88
TRIF	TIR-domain-containing adaptor-inducing interferon-β
TRAM	TRIF-related adaptor molecule
IRAK	Interleukin-1 receptor associated kinase
TRAF	TNF receptor associated factor
IKK	IκB kinase
IκB	Inhibitor of κB
TAK1	TGFβ-activated kinase 1
TNF	Tumor necrosis factor
IL	Interleukin
MAP3K	MAPK kinase kinase
MAP2K	MAPK kinase
ERK	Extracellular signal-regulated kinase

JNK	Jun N-terminal kinase
TPL2	Tumor progression locus 2
HMGB	High mobility group box
ECSIT	Evolutionarily conserved signaling intermediate in Toll pathways
ACA	1’-acetoxychavicol acetate

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