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CM1, a Chrysin Derivative, Protects Endotoxin-Induced Lethal Shock by Regulating the Excessive Activation of Inflammatory Responses

Running Title: Protective effects of CM1 on LPS-induced inflammation.

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Abstract: Human sepsis is one of the leading cause of death worldwide and is known to be a harmful damaging host inflammatory condition, primarily caused by endotoxin released by gram-negative bacteria. Despite antibiotics administration being widely used to treat disease, effective targeted therapeutic strategies for the sepsis are still lacking. Here, we demonstrate that CM1, a derivative of natural polyphenol chrysin, exerts anti-inflammatory activity by inducing the ubiquitin-editing protein TNFAIP3 and NAD-dependent deacetylase sirtuin 1 (SIRT1). We found that CM1 attenuated Toll-like receptor 4 (TLR4)-induced the generation of inflammatory cytokines by inhibiting the extracellular-signal-regulated kinase (ERK)/MAPK and nuclear factor kappa B (NF-κB) signaling pathway. In addition, the treatment of macrophages with CM1 induced the expression of TNFAIP3 and SIRT1 in TLR4-stimulated primary macrophages, however the anti-inflammatory properties of CM1 was disappeared by siRNA silencing of *TNFAPI3* or by the genetic and pharmacologic inhibition of SIRT1. Importantly, intravenously administration of CM1 resulted in the decreased susceptibility to endotoxin-induced sepsis, leading to inhibition of the generation of proinflammatory cytokines and neutrophils infiltration into lung than control mice. Collectively, these findings suggest that CM1 has the potential to be a treatment candidate for diverse inflammatory diseases including sepsis.

Keywords: sepsis; inflammation; toll-like receptor 4; CM1; TNFAIP3; sirtuin 1

1. Introduction

Sepsis is a life-threatening medical emergency with organ dysfunction that can lead to high mortality and morbidity, especially in intensive care units worldwide, and is characterized by an excessive systemic inflammation that occurs through uncontrolled and abnormal innate immune

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responses during overwhelming microbial infection [1,2]. Toll-like receptors (TLRs) are one of the best-characterized pattern recognition receptors (PRRs) and recognize pathogen-associated molecular patterns (PAMPs) that conserved structures of microbial species [3]. Among them, TLR4 is responsible for sensing lipopolysaccharide (LPS), a cell-wall component derived from outer membrane of gram-negative bacteria, which is known to be a main cause of sepsis. TLR4 stimulation initiates the transcriptional upregulation of various inflammatory mediators by the cooperation with TIR domain-containing adaptor molecules, such as myeloid differentiation primary-response protein 88 (MyD88) and TIR domain-containing adaptor inducing IFN-β (TRIF) [4].

TLR signaling pathway is tightly regulated by the distinct intracellular intermediators to prevent the development of autoimmune and inflammatory diseases. In TLR4 signaling cascades, E3 ubiquitin protein ligase TRAF6 play an essential role for the regulation of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling pathways through the catalysis of Lys63-linked polyubiqutination of TRAF6 itself with an E2 ubiquitin ligase complex of Ubc13 and Uev1A [5]. The tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20) is a TNF-induced zinc-finger protein and ubiquitin-editing enzyme that has a dual role with E3 ubiquitin ligase and a deubiquitinase, and has been shown to directly inhibit TRAF6 poly-ubiquitination as negative regulators of TLRs signaling [6]. Sirtuin1 (SIRT1) is a nicotinamide adenosine dinucleotide (NAD)-dependent protein deacetylase that modulate post-translational activity of histone and non-histone proteins and has been implicated in the multiple biological processes including inflammation, apoptosis, metabolism, and DNA repair [7]. In particular, SIRT1 is able to interacted with NF-κB complex and then catalyze the Lys310-linked deacetylation in the p65/RelA subunit, leading to the suppression of the transcriptional activation of the NF-κB-dependent genes [8]. Previous study has shown that the orphan nuclear receptor estrogen-related receptor α (NR3B1) functions as negative regulator in TLR-induced inflammation by controlling the transcriptional activation of *Tnfaip3* gene and SIRT1-mediated the suppression of p65 acetylation, leading to Increased susceptibility to endotoxin-induced septic shock [9].

Polyphenols are a class of natural compounds that found in various plant foods including fruits, vegetables, herbs, and wine and more than 8,000 types have been identified to date [10]. Flavonoids are most abundant type of polyphenols and have been investigated as an essential constituents of complementary therapeutic strategies in a variety of human health and diseases [11]. Flavonoid chrysin, also known as 5,7-dihydroxyflavone, has been found in a variety of plants, such as propolis, bitter melons and walnut pellicle and exerts various biological functions including antioxidant, anti-inflammatory, and anti-apoptotic activity, but has limitations in toxicity and safety [12,13]. We previously identified that CM1, a hydroxyethyl derivative of chrysin, is produced by the exposure of chrysin methanolic solution to ionizing radiation and exhibited anti-inflammatory effects in primary dendritic cells and dextran sodium salt (DSS)-induced colitis in mice [14]. In a follow-up study, we found that CM1 effectively attenuated TLR4-induced inflammation by upregulation of toll-interacting protein (Tollip) in RAW264.7 macrophage cell line [15]. In the present study, we investigated the anti-inflammatory activity of CM1 compared to chrysin and CM2 in TLR4-mediated inflammation using primary murine macrophages. Further, we identified that TNFAIP3 and SIRT1 play important roles in the anti-inflammatory function of CM1 during TLR4 stimulation. In particular, we found a novel role for CM1 as therapeutic candidate for the treatment of endotoxin-induced septic shock using the experimental *in vivo* mice models. These data demonstrate a previously uncharacterized functions and molecular mechanisms of CM1 in the regulation of excessive inflammation *in vitro* primary macrophages and *in vivo* mice model of sepsis.

2. Materials and methods

2.1. CM1 Preparation

CM1, a compound derived from chrysin, was obtained through a gamma irradiation process as outlined in a previously established protocol [16]. To commence the procedure, chrysin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in methanol to achieve a concentration of 1 mg/ml. This

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solution was then subjected to gamma irradiation using a 60Co source (AECL, IR-79, MDS Nordion International Co., Ltd., Ottawa, Canada) at a dose rate of 10kGy/h, accumulating to a total dose of 100 kGy. Dosimetry for this process was accurately calibrated with the use of 5 mm-diameter alanine dosimeters (Bruker Instruments, Rheinstette, Germany). Following irradiation, the chrysin solution was processed to remove the methanol, which was achieved using a rotary evaporator (RV10, IKA, Maribor, Germany).

2.2. Mice

C57BL/6 mice used in our experiments were purchased from Damool Science (Daejeon, Korea). Additionally, mSirt1−/− mice were generously provided by Prof. Byung-Hyun Park (Biochemistry Department at Chonbuk National University Medical School, Korea).

2.3. Preparation of Cell

Mice were utilized for isolating bone-marrow cells. The BMDMs underwent a differentiation protocol [9] for a period of 5 to 7 days, using a medium enriched with macrophage colony-stimulating factor (M-CSF). In a parallel study, HeLa cells (America Type Culture Collection, Manassas, Virginia, USA), a human cervical epithelial cancer cell line, were cultured following standard protocols. Both BMDMs and HeLa cells were propagated in Dulbecco's modified Eagle's medium (DMEM; Welgene, Gyeongsan, Korea) fortified with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Waltham, MA, USA) and 1% antibiotic-antimycotic (GibcoTM, Waltham, MA, USA).

2.4. Reagents and Antibodies

Chrysin (C80105), Sirtinol (S7942) and EX-527 (E7034) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide (LPS) from Escherichia coli 0111:B4 was procured from InvivoGen (San Diego, CA, USA). For immunoblotting, we used specific antibodies targeting phospho-ERK (4370), phospho-SAPK/JNK (4668), phospho-p38 (9211), IκB-α (9242), phospho-IKKα/β, A20/TNFAIP3 (5630), NF-κB p65 acetyl-K310 (3045), SIRT1 (8469), all sourced from Cell Signaling (Danvers, MA, USA).

2.5. Cell viability assays

To evaluate the cytotoxic effects of various chrysin derivatives on BMDMs, we employed the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). We first cultured BMDMs in 96-well plates, following the kit's guidelines. The cells were exposed to varying doses of chrysin derivatives and incubated for 18 hours. After this period, the medium was replaced with serum-free DMEM containing the CCK-8 reagent. We then measured cell viability by determining the absorbance at 450 nm using a SPECTROstar Nano microplate reader

2.6. RNA Extraction, RT-PCR and Real-Time Quantitative PCR

RNA extraction from the activated cells was performed utilizing Trizol reagent (Invitrogen, Waltham, MA, USA), adhering strictly to the guidelines specified by the manufacturer. This extracted RNA was subsequently reverse transcribed into cDNA using a Reverse Transcriptase Premix kit. For further analysis, the resultant cDNA underwent amplification using SolgTM 2X Taq PCR Pre-Mix (Solgent, Daejeon, Korea). The analysis of the amplified products was conducted in two ways: resolution via 1.5% agarose gel for RT-PCR and real-time quantitative PCR analysis using StepOneTM and StepOnePlusTM Software (Applied Biosystems, Waltham, MA, USA). Primer sequences utilized in these procedures were as follows: mouse TNFα (forward: 5′-AGCACAGAAAGCATGATCCG-3′; reverse: 5′-CTGATGAGAGGGAGGC CATT-3′), mouse IL-6 (forward: 5′-TACCACTTCACAAGTCGGAGGC-3′; reverse: 5′-CTGCAAGTG CATCATCGTTGTTC-3′), mouse β-actin (forward: 5′-TCATGAAGTGTGACGTTGACATCCGT-3′; reverse: 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′), human TNFAIP3 (forward: 5′-AGAGCAAC TGAGATCGAGCCA-3′; reverse: 5′-CTGGTTGGGATGCTGACACTC-3′), human TNFα (forward: 5′-

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TCCTCAGCCTCTTCTCCTTCCT-3′; reverse: 5′-ACTCCAAAGTGCAGCAGACAGA-3′), human IL-6 (forward: 5′-GTAGCCGCCCCACACAGA-3′; reverse: 5′-CATGTCTCCTTTCTCAGGGCTG-3′), human β-actin (forward: 5′-CGGAGTCAACGGATTTGGTCGTA-3′; reverse: 5′-AGCCTTCTCC ATGGTGGTGAAGAC-3′).

2.7. ELISA

Concentrations of TNFα and IL-6 in serum samples from mice and in supernatants from cultured cells were quantified using specific ELISA kits (R&D Systems, Minneapolis, MN, USA), strictly adhering to the protocols provided by the manufacturer. To determine the cytokine levels, standard curves were generated using recombinant TNFα and IL-6. These curves facilitated the accurate measurement of cytokine concentrations present in the samples

2.8. Western blot analysis

Protein isolation from cellular lysates was carried out using RIPA buffer supplemented with a freshly prepared protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Following the extraction, proteins were combined with SDS loading buffer and heated to promote denaturation. Subsequent separation of these proteins was achieved through SDS-PAGE, after which they were transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA). The membranes were then blocked in a 5% non-fat milk solution in TBST for 30 minutes at room temperature. Primary antibodies, diluted to 1:1000 in 5% BSA-TBST, were applied and incubated for 2 hours. After washing off the primary antibodies with TBST, the membranes were incubated with secondary antibodies, diluted at 1:10000 in milk-TBST, for an additional 2 hours. The protein bands were detected using the Immobilon Western HRP Substrate (Merck Millipore, Burlington, MA, USA) with a Fusion Solo System (Vilber Lourmat), and their intensities were quantified with Image J software

2.9. NF-κB p65 nuclear translocation

In this experiment, BMDMs were cultured on 22 mm diameter glass coverslips placed in 24-well plates. The cells underwent different treatments: they were either exposed to LPS at 100 ng/ml, LPS with chrysin (2.5 μg/ml), or LPS with CM1 (0.1, 0.5, or 2.5 μg/ml) for 30 min. After-treatment, cells were washed with PBS and then fixed using 4% paraformaldehyde for 1 hour at room temperature, followed by triple washing in PBS with 0.1% Triton X-100 (PBS-T) for 5 min each. Blocking was performed using 10% Normal Goat Serum (Abcam, Cambridge, MA, USA) in PBS for 30 minutes at ambient temperature. The cells were then incubated with the primary NF-κB p65 antibody (sc-8008) for 2 hours. Following this, they were washed and incubated with fluorescently labeled secondary antibodies (anti-mouse AlexaFluro 488) for another 2 hours at room temperature. Finally, DAPI staining (Sigma- Aldrich, St. Louis, MO, USA) was applied to the cell nuclei for 10 minutes. The stained cells were visualized and imaged using a LSM900 confocal microscope (Zeiss).

2.10. siRNA transfection

HeLa cells were subjected to transfection using 30 nM A20-specific siRNA (sc-37655) through the application of Lipofectamine RNAiMAX (Life Technologies Corporation, Carlsbad, CA, USA), following the guidelines provided by the manufacturer. The effec-tiveness and selectivity of the gene silencing were assessed using RT-PCR and western blot analyses. Comparative studies were conducted on cells transfected with either the negative control siRNA or siRNA-A20. Post-transfection, these cells exposed to (100 ng/ml) or LPS with CM1 (1 μg/ml) for 18 hours.

2.11. Mouse models of sepsis

Female mice aged between 6 and 8 weeks were selected for experimentation. Before initiating LPS-induced challenge, these mice were administered either sterile PBS or CM1 (at a dosage of 10 mg/kg) intravenously (i.v.) daily over a span of three days. Following this preparatory phase, they

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were subjected to an intraperitoneal (i.p.) injection of LPS from Escherichia coli O26:B6 (Sigma-Aldrich, St. Louis, MO, USA), at a concentration of 30 mg/kg.

2.12. Histology and immunohistochemistry

After euthanasia, spleens, livers, and lungs were swiftly removed from the mice, rinsed in PBS, and subsequently fixed in 10% formalin. Following fixation, these tissues were embedded in paraffin. For histological examination, thin sections (4 μm) were prepared from the paraffin blocks and stained using the hematoxylin and eosin (H&E) method. To specifically assess neutrophil infiltration, the sections underwent a detailed preparation process. Initially, they were deparaffinized and rehydrated via a series of immersions in xylene and graded ethanol solutions (100%, 95%, and 80%), followed by a final rinse in distilled water and PBS for 20 minutes. Post rehydration, these sections were immunohistochemically stained for neutrophils using the NIMP-R14 antibody (Abcam, Cambridge, MA, USA), to enable detailed analysis.

2.13. Statistical Analysis

In our research, we ensured that every experimental process was replicated a minimum of three times to confirm the consistency and accuracy of our findings. For the statistical evaluation, we employed a two-tailed paired Student's t-test to compare mean values, and survival data was analyzed using the log-rank test. We have represented all our results as mean values ± standard deviation (SD). The determination of statistical significance in the differences we observed was carried out using the GraphPad Prism software, version 5, with a p-value threshold of less than 0.05 indicating significant results.

3. Results

3.1. Cytotoxic effects of chrysin and its derivatives CM1 and CM2 in primary murine macrophages

To investigate the optimal concentration in term of toxicity and safety of chrysin and its derivatives CM1 and CM2, we first evaluated the cytotoxic effects of three candidates at different concentration by measuring the cell viability of BMDMs using CCK-8 assay. As shown in Figure 1A, the stimulation of BMDMs with chrysin had no significant effect on cell viability at various concentrations (0.1–20 μg/mL) after 18 hours or on solvent control-stimulated cells. However, cell viability was decreased by approximately 50% (for 5 μg/ml) and 85% (for 10 and 20 μg/ml) at 18 hours after treatment with CM1, respectively (Figure 1B). Additionally, CM2 did not showed the cytotoxicity up to a concentration of 50 μg/mL, but approximately 50% cells (Figure 1C). Based on these results, for subsequent experiments we used chrysin (0.1–20 μg/ml), CM1 (0.1–2.5 μg/ml), and CM2 (0.1–50 μg/ml) and evaluated the anti-inflammatory effects of these candidates in TLR4-mediated inflammation.

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Figure 1. Lee *et al*.

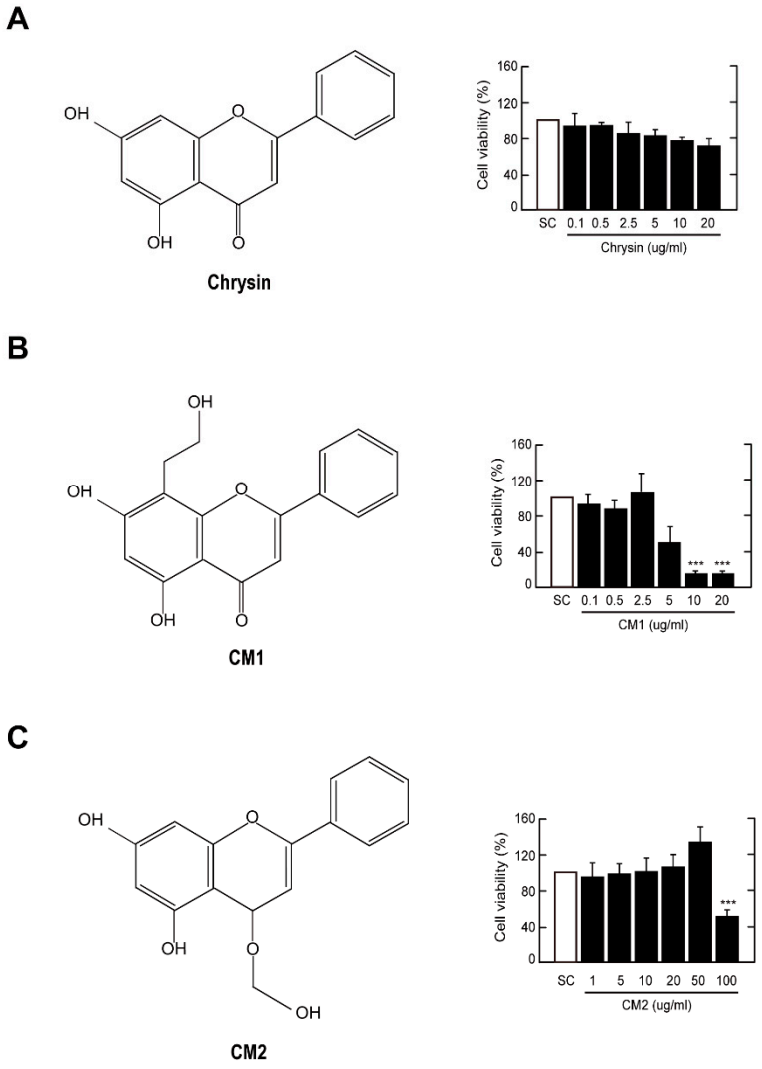

Figure 1. Chemical structures and cytotoxicity in chrysin and its derivatives. BMDMs were treated with different concentration of chrysin, CM1, or CM2 for 18 hours. Cell viability was measured using CCK-8 assay. Data are representative of three independent experiments and are presented as means ± SD. ***p < 0.001, compared with control cells (two-tailed Student's t-test). SC, solvent control (0.01% DMSO).

3.2. CM1, but not chrysin and CM2, negatively regulates TLR4-induced the generation of inflammatory cytokines in primary murine macrophages

Based on our previous results showing that CM1 effectively attenuated LPS-induced inflammatory responses in primary dendritic cells [14] and RAW264.7 macrophage cell line [15], we further examined the anti-inflammatory effects of CM1, including chrysin and CM2, on the LPS-stimulated primary macrophage BMDMs. As shown in Figure 2A–C, we stimulated BMDMs with

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LPS for various time periods and then assessed the mRNA (Figure 2A,B) and protein (Figure 2C) expressions of inflammatory cytokines, such as TNF-α and IL-6.

Figure 2. Lee *et al*.

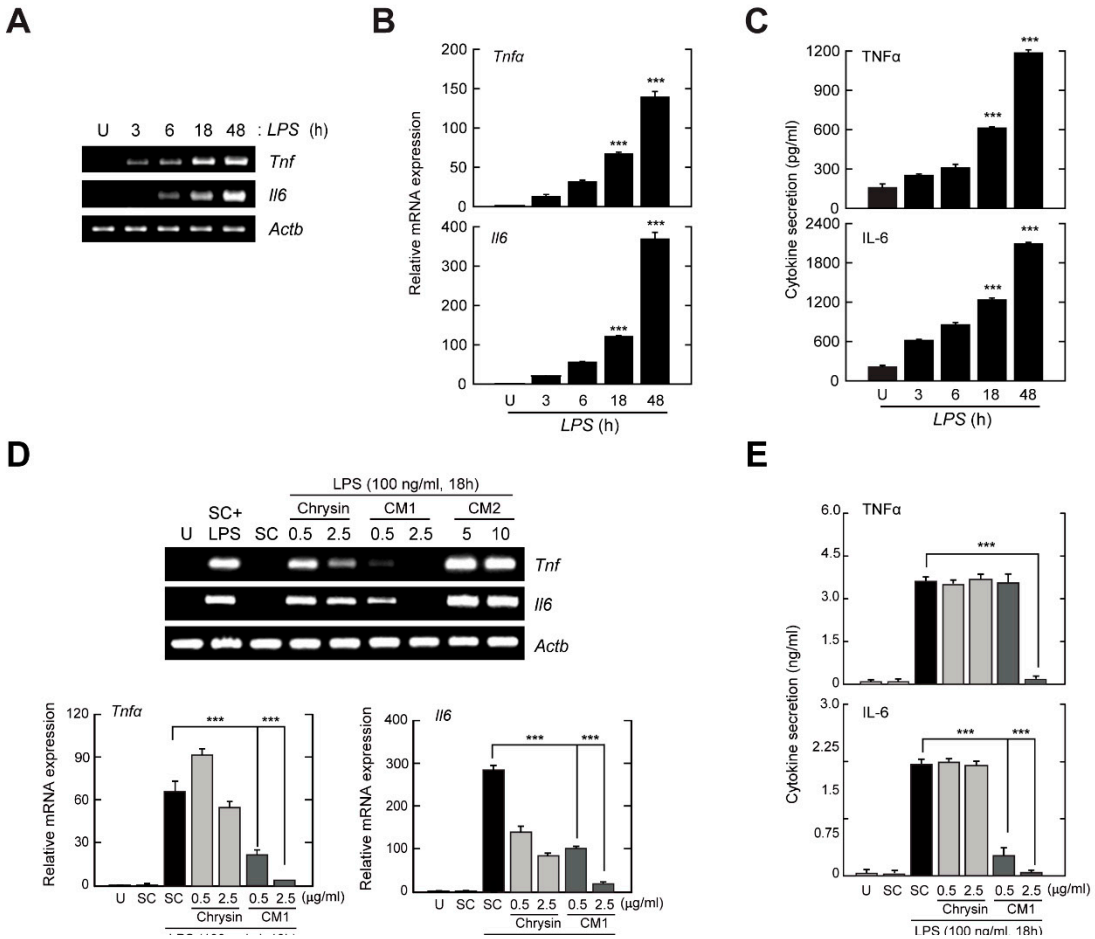

Figure 2. CM1 inhibits LPS-induced inflammatory responses in BMDMs. (A-C) BMDMs were stimulated with LPS (100 ng/ml) for the indicated time periods. (A-B) The mRNA expression of *Tnf* and *Il6* was analyzed using RT-PCR and real-time qPCR. (C) The protein levels of TNFα and IL-6 in culture medium were measured using ELISA. (D-E) BMDMs were stimulated with LPS and then co-treated with chrysin (0.5 or 2.5 μg/ml), CM1 (0.5 or 2.5 μg/ml), or CM2 (5 or 10 μg/ml) for 18 hours. (D) The mRNA expression of *Tnf* and *Il6* was evaluated using RT-PCR (top) and real-time qPCR (bottom). (E) Protein levels of TNFαand IL-6 in culture medium were analyzed using ELISA. Data are representative of three independent experiments and are presented as means ± SD. ***$p < 0.001$, compared with control cells (two-tailed Student's t-test). U, untreated cells; SC, solvent control (0.01% DMSO).

To determine whether polyphenol flavonoids, including chrysin and its derivate CM1 or CM2, inhibited the TLR4-mediated generation of inflammatory cytokines, BMDMs were stimulated with LPS in the presence or absence of each polyphenol flavonoid, and the expressions of TNF-α and IL-6 were evaluated by RT-PCR (Figure 2D), qPCR (Figure 2E), or an ELISA (Figure 2F). We showed that LPS-induced mRNA expression of the *Tnf* and *Il6* genes were preferentially attenuated by the treatment with CM1 in a concentration-dependent manner compared with chrysin (Figure 2D,E). Moreover, the secretions of TNF-α and IL-6 in culture supernatants after LPS stimulation were also

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decreased by treatment with CM1, but not chryin (Figure 2F). However, these inhibitory effects were not detected in the presence of CM2, rather, *Mab*-induced mRNA expressions of the *Tnf* and *Il6* genes were significantly increased under some conditions when treated with CM2 (Figure 2D). These results indicated that CM1 most effectively attenuated TLR4-mediated inflammatory responses in primary macrophages, compared with the chryin and CM2.

3.3. CM1 effectively attenuates TLR4-induced activation of extracellular signal-regulated kinases (ERK) 1/2 MAPK and NF-κB signalling pathway in primary macrophages.

We next investigated whether CM1 modulates the intracellular signaling pathways related in TLR4-mediated the activation of inflammatory responses. In Figure 3A, we showed that LPS stimulation resulted in the rapid activation of all three MAPK subfamilies within 60 minutes, including ERK 1/2, p38, and c-Jun N-terminal kinase (JNK) that required for the activation of inflammatory genes upon diverse TLR ligands through transcription factor complex AP-1 [17]. In particular, LPS-mediated phosphorylation of ERK 1/2, but not p38 and JNK, were more attenuated by pretreatment with CM1in a concentration-dependent manner, compared with chrysin (Figure 3B,C).

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Figure 3. Lee *et al.*

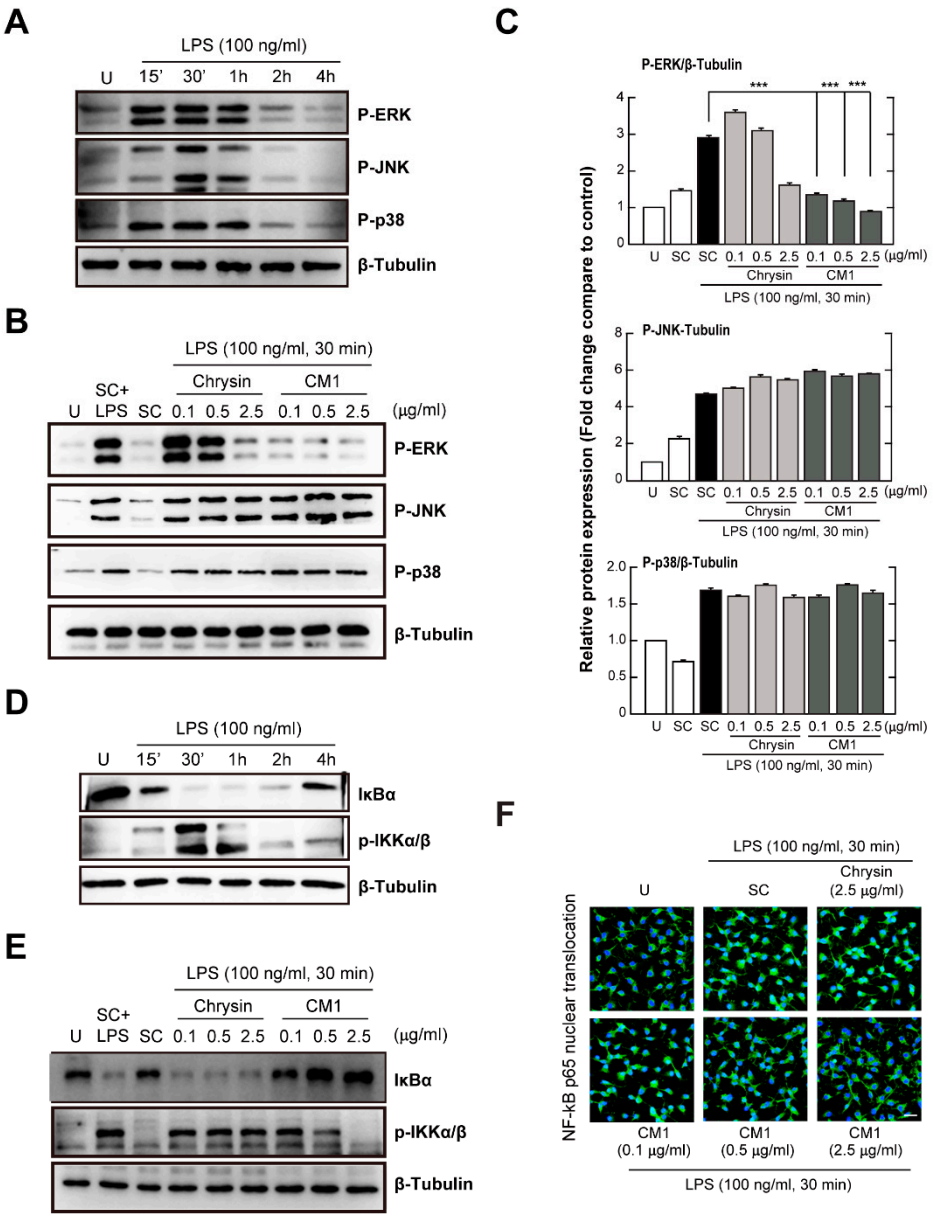

Figure 3. CM1 inhibits LPS-induced ERK phosphorylation and NF-κB activation. (A and D) BMDMs were treated with LPS (100 ng/ml) for the indicated time periods. The both MAPK and NF-κB activation were determined using immunoblot analysis. (B and C) BMDMs were treated with LPS only or LPS and chrysin (0.1, 0.5, or 2.5 μg/ml), or LPS and CM1 (0.1, 0.5, or 2.5 μg/ml) for 30 min. (B) Protein expression was determined using immunoblot analysis. (C) Densitometric analysis of p-ERK, p-JNK, and p-p38 expression was performed and normalized to β–tubulin content. (E and F) BMDMs were treated with LPS only, LPS and chrysin (2.5 μg/ml), or LPS and CM1 (0.1, 0.5, or 2.5 μg/ml) for 30min. (E) Protein expression was determined using immunoblot analysis. (F) Cells were fixed and stained for NF-κB p65 (green) and the nuclei were stained with DAPI (blue). The nuclear translocation of NF-κB p65 was analyzed using confocal microscopy. Scale bar: 20 μm. Data are representative of three independent experiments and are presented as means ± SD. ***$p < 0.001$, compared with control cells (two-tailed Student's t-test). U, untreated cells; SC, solvent control (0.01% DMSO).

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NF-κB signaling is crucial roles in the transcriptional modulation of gene categories regulating TLR4-mediated inflammation [18]. We showed that the incubation with LPS (Figure 3D) lead to the rapid activation of the kinases, IKKα and IKKβ and the degradation of the NF-κB inhibitor, IκB-α. To examine the inhibitory roles of CM1 in LPS-induced activation of NF-κB signaling, BMDMs were incubated with LPS for 30 minutes in the presence of chrysin or CM1, and phosphorylation of IKKα/β and degradation of IκB-α were then evaluated using immunoblot assays. As shown in Figure 3E, CM1 effectively attenuated LPS-induced the activation of NF-κB signaling in primary macrophages. However, these inhibitory effects were not observed in chrysin-treated cells. Moreover, LPS-induced NF-κB nuclear translocation was also attenuated in primary macrophages treated with CM1, but not chrysin (Figure 3F). Taken together, these results showed that CM1 negatively regulated TLR4-mediated activation of the MAPK/ERK 1/2 and NF-κB signaling pathway, which may be essential for the regulation of inflammation in primary macrophages.

3.4. TNFAIP3 is required for CM1-mediated the activation of anti-inflammatory effects in TLR4-stimuated cells.

We previously reported that CM1 exerted the anti-inflammatory action by upregulation of Tollip in LPS-stimulated macrophage-like cell line RAW264.7 [15], indicating CM1 may have a potent to activate other TLR negative regulators. Therefore, we questioned whether TNFAIP3 was required for anti-inflammatory action of CM1 in macrophages as well as Tollip. As shown in Figure 4A,B, CM1 strongly activated the upregulation of TNFAIP3 in LPS-stimulated BMDMs, compared to SC.

Figure 4. Lee *et al*.

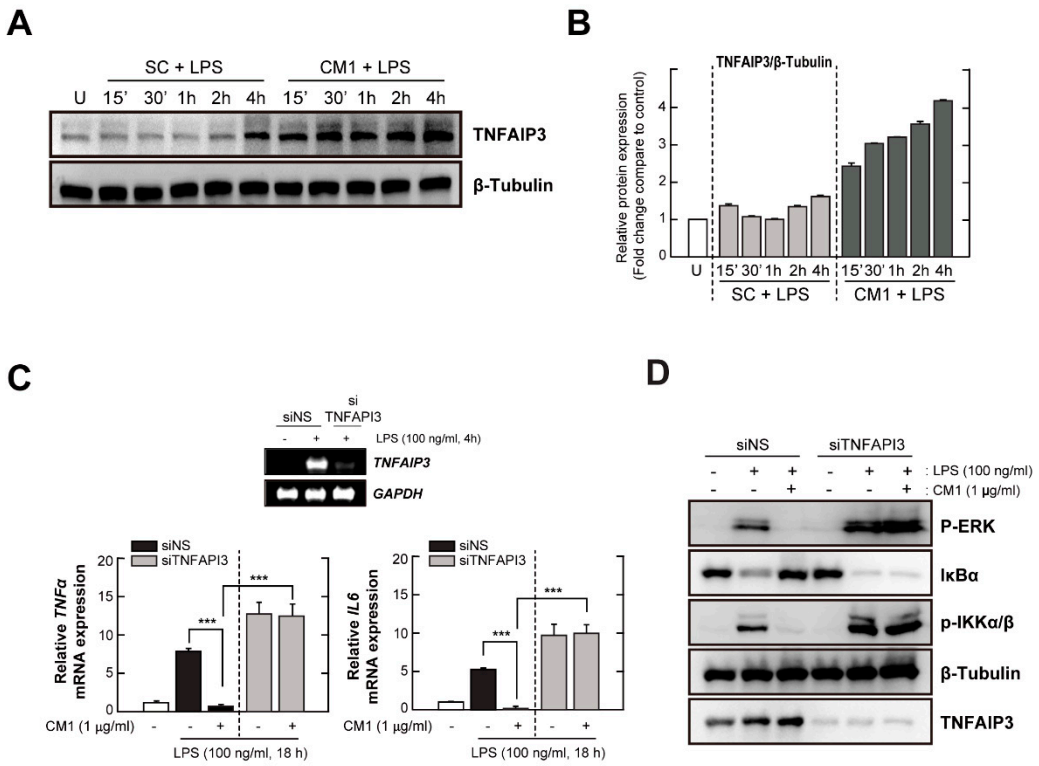

Figure 4. CM1 attenuates LPS-induced inflammatory responses through TNFAIP3 upregulation. (A-B) BMDMs were treated with LPS (100 ng/ml) or LPS with CM1 (1 μg/ml) for the indicated time periods. (A) Protein expression of TNFAIP3 was determined by immunoblot analysis. (B) Densitometric analysis of TNFAIP3 expression was performed and normalized to β–tubulin content.

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(C and D) Hela cells were transfected with control siRNA or siTNFAIP3. (C) The transfected cells were treated with LPS only or LPS and CM1 for 18 hours. The mRNA expression of *TNFα* and *IL6* was determined using real-time qPCR. RT-PCR was performed to assess transfection efficiency (inset). (D) The transfected cells were treated with LPS with CM1 for 30 min. The protein levels of p-ERK, p-IKKαβ, total IκBα and TNFAIP3 were evaluated by immunoblot analysis. Data are representative of three independent experiments and are presented as means ± SD. ***p < 0.001, compared with control cells (two-tailed Student's t-test). U, untreated cells; SC, solvent control (0.01% DMSO); siNS, non-specific siRNA; siTNFAIP3, specific siRNA for TNFAIP3.

To investigate the role of TNFAIP3 in anti-inflammatory functions of CM1 upon LPS stimulation, we transfected HeLa cells with siRNA specific for TNFAIP3 (siTNFAIP3), which effectively silenced the human TNFAIP3 gene, but not housekeeping gene GAPDH (Figure 4C, upper). The TNFAIP3 knockdown in HeLa cells completely inhibited CM1-mediated anti-inflammatory action in LPS-induced the mRNA expression of TNFα and IL-6, whereas HeLa cells transfected with a scrambled control siRNA (siNS) reduced the mRNA expression of these cytokines in CM1-treated HeLa cells, in similar to primary macrophages (Figure 4C, bottom). Moreover, we found that the inhibitory effects of CM1 in LPS-mediated the activation of MAPK/ERK 1/2 and NF-κB signaling was also disappeared by the TNFAIP3 knockdown in HeLa cells (Figure 4D). Collectively, these findings suggested that CM1 effectively attenuated LPS-mediated the activation of pro-inflammatory responses through the upregulation of TNFAIP3.

3.5. CM1 negatively regulates TLR4-induced NF-κB p65 acetylation by activating SIRT1, which contributes to the anti-inflammatory functions in primary macrophages.

Growing evidence suggests that SIRT1 is critically involved in the regulation of protective immune responses, and impairment of SIRT1 resulted in the progression of autoimmune and inflammatory diseases [7]. Moreover, the activation of SIRT1 by naturally occurring dietary polyphenols plays an essential role in the regulation of diverse biological functions, such as oxidative stress, inflammation, and autoimmunity in response to endo- or exogenous stimuli [19]. We found that CM1 treatment resulted in the increase of SIRT1 and the decrease of NF-κB p65 acetylation in LPS-stimulated BMDMs (Figure 5A). However, the inhibitory effects of CM1 in LPS-induced NF-κB p65 acetylation was disappeared by the pretreatment of sirtinol (Figure 5B) or EX-527 (Figure 5C), selective SIRT1 inhibitors. Additionally, the anti-inflammatory effects of CM1 in LPS-mediated the mRNA expression of TNFα an IL-6 was also impaired in a presence of sirtinol (Figure 5D) or EX-527 (Figure 5E).

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Figure 5. Lee *et al*.

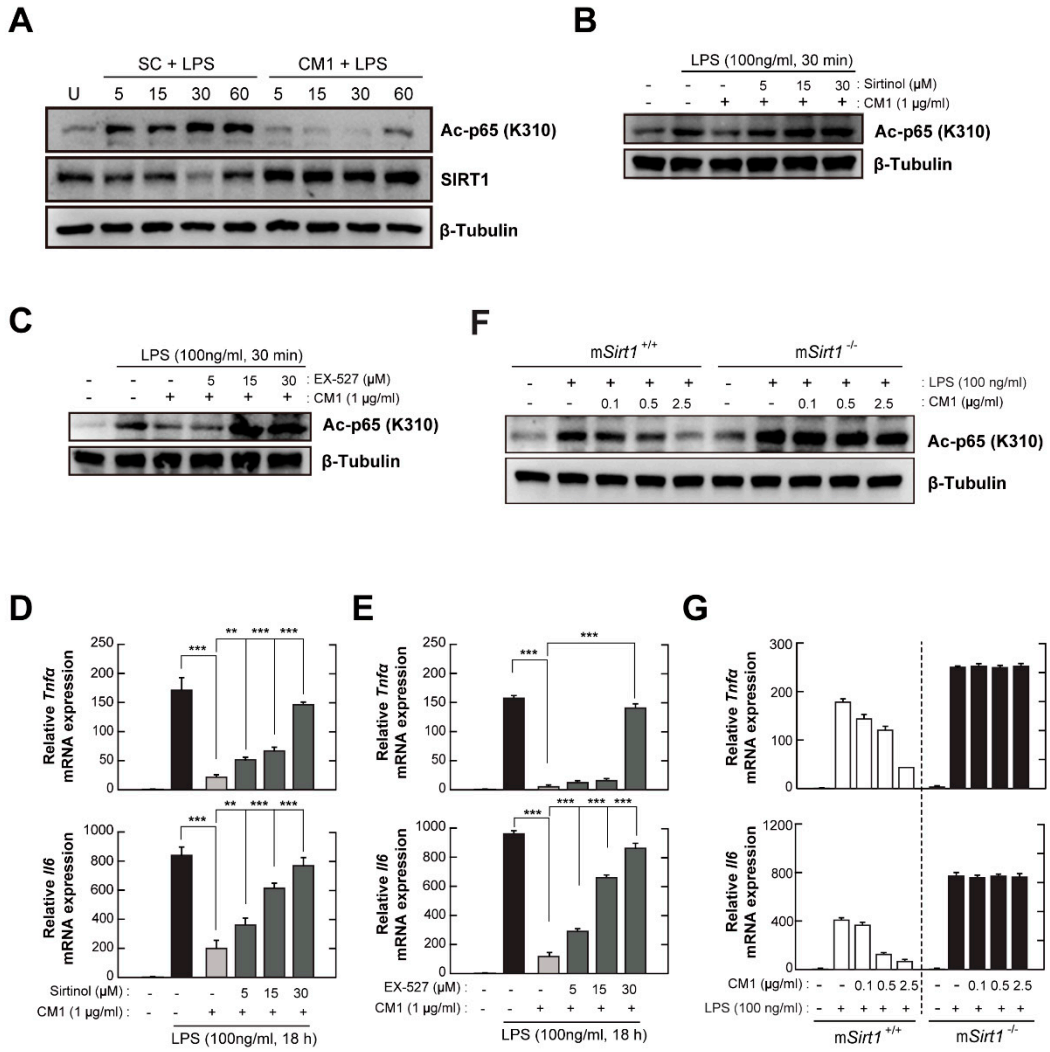

Figure 5. CM1 reduces LPS-induced inflammatory responses by enhancing SIRT1 activity. (A) BMDMs were treated with LPS (100 ng/ml) or LPS with CM1 (1 μg/ml) for the indicated time periods. The expression of NF-κB p65 acetylation and SIRT1 was determined by immune blot analysis. (B-E) BMDMs were pre-treated with increasing concentrations of Sirtinol (5, 15, or 30 μM, for B and D) or EX-527 (5, 15, or 30 μM, for C and E) for 2 hours followed by exposure to LPS only or LPS and CM1 for either 30 min (for B and C) or for 18 hours (for D and E). (B and C) Immunoblot analysis was used to evaluate the acetylation of NF-κB p65. (D and E) The mRNA expression of *Tnfα* and *Il6* was measured using real-time qPCR. (F and G) BMDMs from m*Sirt1*$^{+/+}$ and m*Sirt1*$^{-/-}$ mice were treated with LPS only or LPS and CM1 (0.1, 0.5, or 2.5 μg/ml) for 30 min (for F) or 18 hours (for G). (F) The NF-κB p65 acetylation was determined by immunoblot analysis. (G) The mRNA expression of *Tnfα* and *Il6* was measured using real-time qPCR. Data are representative of three independent experiments and are presented as means ± SD. ***P* < 0.01, ***p < 0.001, compared with control cells (for D and E) or cells isolated from *Sirt1*$^{+/+}$ mouse (two-tailed Student's t-test). U, untreated cells; SC, solvent control (0.01% DMSO).

To assess the role of myeloid-specific SIRT1 in anti-inflammatory effects of CM1, BMDMs were isolated from m*Sirt1*$^{+/+}$ or m*Sirt1*$^{-/-}$ mice and stimulated with LPS in a presence of indicated concentration of CM1 (Figure 5F,G). We showed that LPS-mediated NF-κB p65 acetylation in m*Sirt1*$^{+/+}$ BMDMs was normally attenuated by the treatment of CM1 in a dose dependent manner,

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however, the deficiency of myeloid-specific SIRT1 impaired CM1-mediated de-acetylation of NF-κB p65 (Figure 5F), in similar to selective SIRT1 inhibitors (Figure 5B,C). Consistent of these finding, CM1-mediated anti-inflammatory functions was also disappeared in *Sirt1*-deficient BMDMs, compared to m*Sirt1*$^{+/+}$ BMDMs. These results indicate that myeloid-specific SIRT1 is crucial for the inhibitory effects of CM1 in TLR4-induced inflammation in primary murine macrophages.

3.6. CM1 effectively protects systemic inflammation in vivo, leading to decreased mortality during endotoxin-induced lethal shock.

To investigate the *in vivo* efficacy of CM1, we challenged endotoxin-induced septic shock mice model. As shown in Figure 1A, the endotoxin-induced mice lethality was significantly decreased in CM1-treated mice, when compared with that in SC-treated mice. Consistent with the increased survival rates, the serum levels of TNFα and IL-6 were significantly decreased in CM1-treated mice after LPS challenge (Figure 1B). We further evaluated the mRNA expression of proinflammatory cytokines in lung and spleen tissues in response to LPS injection. The production of TNFα and IL-6 in response to LPS challenge was significantly attenuated in the lung and spleen tissues of CM1-treated mice, compared with those tissues of SC-treated mice (Figure 1C). In addition, neutrophil infiltration was effectively decreased in the lung tissues of CM1-treated mice, compared with SC-treated mice, after LPS challenge (Figure 1D,E). These results indicate that CM1 protects mice lethality by regulating systemic inflammation in septic shock models.

4. Discussion

We recently demonstrated that CM1 exhibited anti-inflammatory effects on TLR4-stimulated primary dendritic cells and DSS-induced colitis mice models and has a potential as the novel therapeutic candidate for inflammatory bowel disease (IBD) [14]. Moreover, we found that CM1 markedly induced the expression of Tollip in macrophage-like cell line, thereby strongly suppressing TLR4-mediated inflammation [15]. These finding may support the possibility that CM1 can modulates the expression of other negative regulators during TLR4 stimulation. Here we demonstrated that CM1 negatively regulates TLR4-induced hyper-activation of inflammatory responses in primary macrophages in vitro, via at least two mechanisms including TNFAIP3-mediated the regulation of ERK/MAPK NF-κB signaling pathway by inhibiting TRAF6 ubiquitination and post-translational modification via SIRT1-mediated the de-acetylation of NF-κB p65 (Figure 7). We further showed that CM1 administration led to increase of mice survival and decrease of inflammatory responses during endotoxin stimulation *in vivo* sepsis mouse model.

TLRs are the best characterized innate immune receptors for the recognition of invading pathogens and their specific ligands, leading to the activation of intracellular signaling cascades that initiate inflammatory responses [18]. Although inflammation is crucial for the host protection in the initial response to various microbial infections, however, excessive and uncontrolled inflammatory responses are harmful by multiple organ dysfunction and failure [20]. In the present study, we demonstrated that the CM1 treatment of BMDMs resulted in the suppression of LPS-induced the generation of inflammatory cytokine TNFα and IL-6 (Figure 2). Moreover, *in vivo* administration of CM1 resulted in the reduction of systemic inflammation and mice lethality in endotoxin-induced septic shock model (Figure 6). Also, we further found that LPS stimulation led to the activation of NF-κB and ERK/MAPK signaling, which is completely abrogated by treatment with CM1. However, LPS-induced the phosphorylation of p38 and JNK was not affected by CM1 treatment (Figure 3). These results partly correlate with recent our studies showing that all MAPKs activation induced by LPS was attenuated by CM1 treatment in macrophage-like cell line RAW 264.7 [15] and primary dendritic cells [14], indicating the mechanism by which CM1 acts may differ depending on the cell type. These results supported that CM1 may solve various human health and diseases arising from these two signal transductions.

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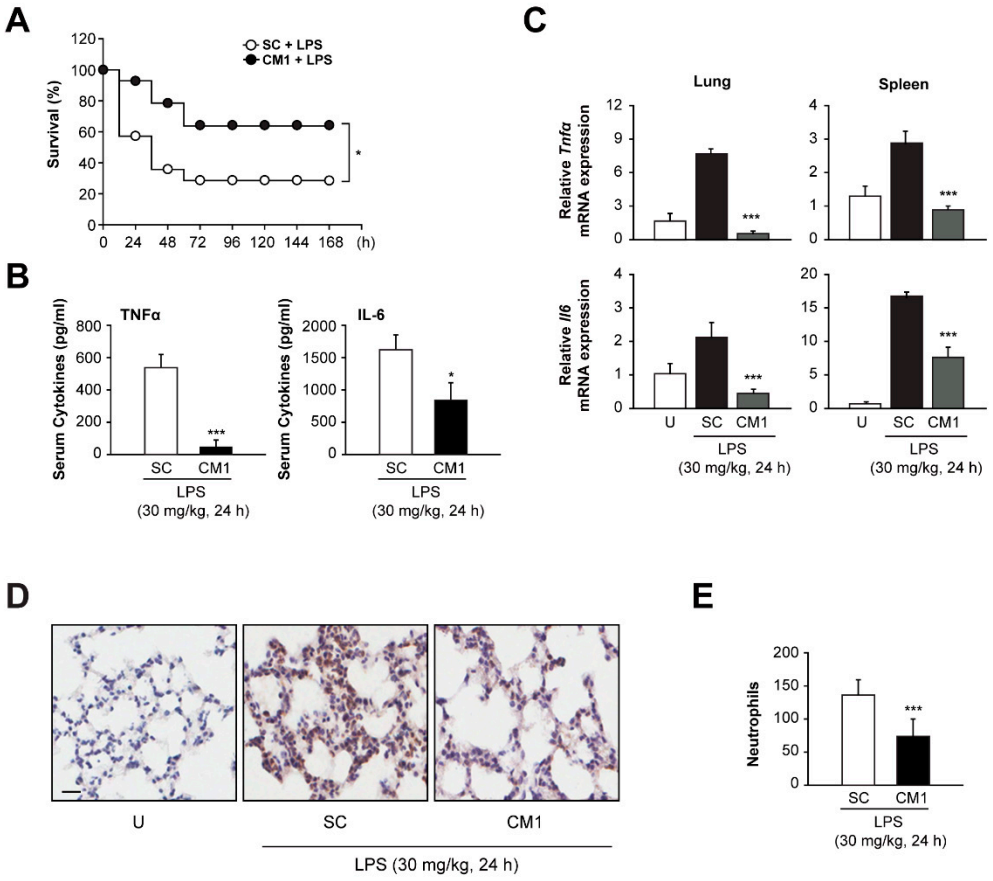

Figure 6. CM1 contributes to mice protection against the lethal shock. (A-E) Mice were intravenously (i.v) injected with either vehicle control or CM1 (10 mg/kg) once daily for 3 days before sepsis induction. Sepsis was then induced via intraperitoneal (i.p) injection of LPS (30 mg/kg). (A) Survival rates of vehicle control-treated or CM1-treated mice were monitored for 168 hours. (B-E) Mice were sacrificed at 24 hours post-LPS injection. (B) Serum samples were collected from vehicle control-treated or CM1-treated mice. The levels of TNFα and IL-6 were measured by ELISA. (C) The expression of *Tnfα* and *Il6* in lung (left) and spleen (right) was analyzed using real-time qPCR. (D) Immunohistochemical analysis of the lung tissue was performed to evaluate neutrophil infiltration. Scale bar: 50 μm. (E) The number of infiltrating neutrophils was counted from 8 random fields. The experiments were conducted in triplicate to ensure reproducibility, with the results expressed as the means ± SD. Statistical significance of mean differences was determined using either a log-rank test (A) or a two-tailed Student's t-test (B, C, E). $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control mice stimulated with LPS. U, untreated; SC, solvent control.

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Figure 7. Lee *et al*.

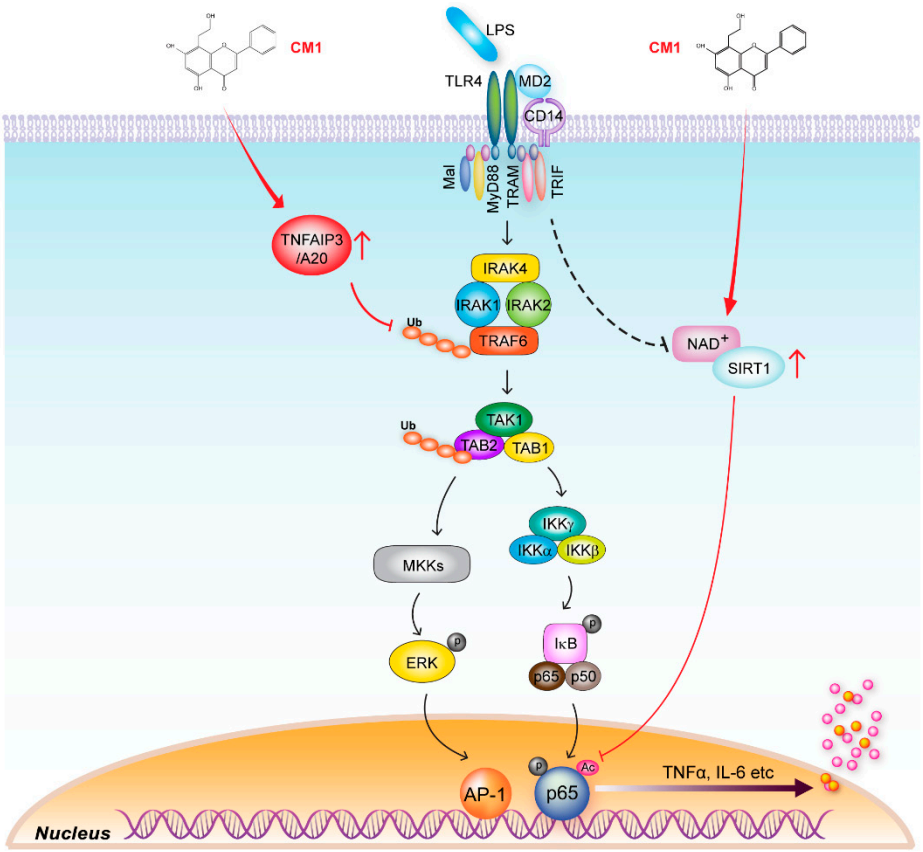

Figure 7. Anti-inflammatory effects and molecular mechanisms of CM1 in response to TLR4. Upon LPS binding to the TLR4/MD2/CD14 complex, the downstream signaling cascade is activated, leading to the ubiquitination of TRAF6 and subsequent activation of TAK1 and the IKK complex, which phosphorylates IκB, leading to the release and nuclear translocation of NF-κB p65. This results in the transcription of pro-inflammatory cytokines such as TNFα and IL-6. CM1 directly upregulates the expression of TNFAIP3/A20, which inhibits the NF-κB p65 pathway, and enhances the activity of SIRT1. Increased SIRT1 activity, in turn, deacetylates p65, suppressing its ability to transcribe pro-inflammatory genes. The dual action of CM1 suggests a potential therapeutic role in controlling LPS-induced inflammation through these molecular targets.

TLR signaling pathways are tightly regulated by multiple steps on transcriptional, epigenetic and post-translational regulation of diverse target genes [21]. Indeed, numerous negative regulators have been investigated to regulate host inflammatory and immune responses [22]. Especially, TRAF6, an essential adaptor molecule of TLR signaling, can be targeted by various negative regulators, including TNFAPI3, estrogen-related receptor α, cylindromatosis, small heterodimer partner, and TANK [6,9,23-27]. Among them, TNFAIP3, also known as A20, is a multifunction protein with dual enzyme activities including an E3 ubiquitin ligase and a deubiquitinase and then crucial for the termination of TLRs-induced NF-κB and MAPKs signaling by the cleavage of K63-linked polyubiquitin chains on TRAF6 [6,28]. Previous study demonstrated that chrysin has anti-neuroinflammatory effects in response to LPS *in vitro* BV2 and primary microglial cells and *in vivo*, which is mediated by the inhibition of TRAF6 polyubiquitination and NF-κB pathways via TNFAIP3

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upregulation [29]. However, the involvement of TNFAIP3 in the molecular mechanisms of anti-inflammatory effects of CM1 remain unclear. Herein, we found that TNFAIP3 protein was induced within 15 min after co-treatment with CM1 and LPS and then maintained for up to 4 hours in primary macrophages, which was produced earlier and stronger and maintained longer than in the cells stimulated with LPS alone (Figure 4A and B). In addition, siRNA-mediated knockdown of TNFAIP3 gene in HeLa cells completely counteracted the inhibitory effect of CM1 in LPS-induced the generation of inflammatory cytokines and to the activation of NF-κB and ERK/MAPK signaling (Figure 4C and D). These results indicated that TNFAIP3 plays an essential role in the antiinflammatory action of CM1 in response to TLR4. Future studies should identify the molecular mechanisms by which CM1 increases TNFAIP3 expression and examine whether it contributes not only to quantitative increase but also to the modulation of enzymatic activity.

Emerging evidence indicates that SIRT proteins act as NAD^+-dependent deacetylase and play an essential role in human health and disease linking infection and inflammation [8,30-33]. During inflammation, SIRT1 deacetylates NF-κB p65 at lysine310 and then promotes its exclusion from the nucleus, leading to inactivation of NF-κB signaling and termination of inflammatory responses [34]. Previous study demonstrated that SIRT1-deficient mice exhibited the increase of NF-κB acetylation and activation, exacerbating pulmonary vascular leakage and lung inflammation after exposure to particulate matter [35]. We previously demonstrated that myeloid-specific SIRT1 is crucial for the activation of host protective autophagic response against *T. gondii* infection by controlling deacetylation of FoxO1 and FoxO3a [32]. In particular, Yang and their collaborators reported that chrysin exhibited the protective effects on carrageenan-induced pulmonary and pleurisy injury in rat and is able to attenuate the activation of neutrophils and oxidative stress by regulating the activity of NF-κB via SIRT1/NRF2 signaling pathway [36], which suggest the possibility that CM1 may regulate the excessive inflammation via SIRT1 signalling. Our data showed that pharmacological inhibition of SIRT1 and myeloid-specific SIRT1-deficient macrophages resulted in the abrogation of the anti-inflammatory action of CM1. Our work is important in elucidating a pivotal role of CM1 in the regulation of SIRT1-meidatd the deacetylation of NF-κB p65, thus affecting inflammatory responses in the primary macrophages (Figure 5).

Our findings implicate CM1 exhibited the anti-inflammatory effects during endotoxin stimulation by regulating NF-κB and ERK/MAPK signalling pathways via upregulation of TNFAPI3 and SIRT1 and will facilitate the development of novel therapeutics for inflammatory-associated diseases.

Author Contributions: Conceptualization, Young Bok Ko , Woo Sik Kim, Eui-Baek Byun, and Jae-Min Yuk; Formal analysis, Jae-Hyung Lee, Jinju Kim and Yong Min Choi; Funding acquisition, Young Bok Ko , Eui-Baek Byun, and Jae-Min Yuk; Investigation, Jae-Hyung Lee, Young Bok Ko, Yong Min Choi, and Jinju Kim; Methodology, Hwan-Doo Cho, Hyeonil Choi, Ha-Yeon Song, Jeong Moo Han, and Jin-Man Kim; Project administration, Jae-Min Yuk; Resources, Ha-Yeon Song, Jeong Moo Han, Woo Sik Kim, and Eui-Baek Byun; Supervision, Guang-Ho Cha and Young-Ha Lee; Visualization, Jae-Hyung Lee and Jae-Min Yuk; Writing – original draft, Jae-Hyung Lee and Jae-Min Yuk; Writing – review & editing, Jae-Min Yuk.

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Institutional Review Board Statement: All protocols involving the use of animals were rigorously reviewed and received approval from the Institutional Animal Care and Use Committee at Chungnam National University College of Medicine (Approval Number: 202012A-CNU-200), underscoring our commitment to ethical research practices. These procedures were meticulously carried out in strict adherence to the prescribed guidelines to ensure the welfare of the animals involved in the study.

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References

1. Cohen, J. The immunopathogenesis of sepsis. *Nature* **2002**, *420*, 885–891
2. Hotchkiss, R.S.; Monneret, G.; Payen, D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nature reviews. Immunology* **2013**, *13*, 862-874, doi:10.1038/nri3552.
3. Hug, H.; Mohajeri, M.H.; La Fata, G. Toll-Like Receptors: Regulators of the Immune Response in the Human Gut. *Nutrients* **2018**, *10*, doi:10.3390/nu10020203.
4. Takeuchi, O.; Akira, S. Pattern recognition receptors and inflammation. *Cell* **2010**, *140*, 805-820, doi:10.1016/j.cell.2010.01.022.
5. Liew FY, X.D., Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* **2005**, *5*, 446-458.
6. Boone, D.L.; Turer, E.E.; Lee, E.G.; Ahmad, R.C.; Wheeler, M.T.; Tsui, C.; Hurley, P.; Chien, M.; Chai, S.; Hitotsumatsu, O.; et al. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nature immunology* **2004**, *5*, 1052-1060, doi:10.1038/ni1110.
7. Kong, S.; McBurney, M.W.; Fang, D. Sirtuin 1 in immune regulation and autoimmunity. *Immunol Cell Biol* **2012**, *90*, 6-13, doi:10.1038/icb.2011.102.
8. Yeung, F.; Hoberg, J.E.; Ramsey, C.S.; Keller, M.D.; Jones, D.R.; Frye, R.A.; Mayo, M.W. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J* **2004**, *23*, 2369-2380, doi:10.1038/sj.emboj.7600244.
9. Yuk, J.M.; Kim, T.S.; Kim, S.Y.; Lee, H.M.; Han, J.; Dufour, C.R.; Kim, J.K.; Jin, H.S.; Yang, C.S.; Park, K.S.; et al. Orphan Nuclear Receptor ERRalpha Controls Macrophage Metabolic Signaling and A20 Expression to Negatively Regulate TLR-Induced Inflammation. *Immunity* **2015**, *43*, 80-91, doi:10.1016/j.immuni.2015.07.003.
10. Bertelli, A.; Biagi, M.; Corsini, M.; Baini, G.; Cappellucci, G.; Miraldi, E. Polyphenols: From Theory to Practice. *Foods* **2021**, *10*, doi:10.3390/foods10112595.
11. Safe, S.; Jayaraman, A.; Chapkin, R.S.; Howard, M.; Mohankumar, K.; Shrestha, R. Flavonoids: structure-function and mechanisms of action and opportunities for drug development. *Toxicol Res* **2021**, *37*, 147-162, doi:10.1007/s43188-020-00080-z.
12. Rodriguez-Landa, J.F.; German-Ponciano, L.J.; Puga-Olguin, A.; Olmos-Vazquez, O.J. Pharmacological, Neurochemical, and Behavioral Mechanisms Underlying the Anxiolytic- and Antidepressant-like Effects of Flavonoid Chrysin. *Molecules* **2022**, *27*, doi:10.3390/molecules27113551.
13. Falbo, F.; Aiello, F. Chrysin: A polyedric flavone as a tool to explore new phytotherapeutic applications and drug design. *Arch Pharm (Weinheim)* **2023**, *356*, e2200347, doi:10.1002/ardp.202200347.

14. Song, H.Y.; Sik Kim, W.; Kim, J.M.; Bak, D.H.; Moo Han, J.; Lim, S.T.; Byun, E.B. A hydroxyethyl derivative of chrysin exhibits anti-inflammatory activity in dendritic cells and protective effects against dextran sodium salt-induced colitis in mice. *International immunopharmacology* **2019**, *77*, 105958, doi:10.1016/j.intimp.2019.105958.
15. Byun, E.B.; Song, H.Y.; Kim, W.S.; Han, J.M.; Seo, H.S.; Park, W.Y.; Kim, K.; Byun, E.H. Chrysin Derivative CM1 and Exhibited Anti-Inflammatory Action by Upregulating Toll-Interacting Protein Expression in Lipopolysaccharide-Stimulated RAW264.7 Macrophage Cells. *Molecules* **2021**, *26*, doi:10.3390/molecules26061532.
16. Song, H.Y.; Kim, H.M.; Mushtaq, S.; Kim, W.S.; Kim, Y.J.; Lim, S.T.; Byun, E.B. Gamma-Irradiated Chrysin Improves Anticancer Activity in HT-29 Colon Cancer Cells Through Mitochondria-Related Pathway. *J Med Food* **2019**, *22*, 713-721, doi:10.1089/jmf.2018.4320.
17. Arthur, J.S.; Ley, S.C. Mitogen-activated protein kinases in innate immunity. *Nature reviews. Immunology* **2013**, *13*, 679-692, doi:10.1038/nri3495.
18. Kawai, T.; Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **2011**, *34*, 637-650, doi:10.1016/j.immuni.2011.05.006.
19. Chung, S.; Yao, H.; Caito, S.; Hwang, J.W.; Arunachalam, G.; Rahman, I. Regulation of SIRT1 in cellular functions: role of polyphenols. *Arch Biochem Biophys* **2010**, *501*, 79-90, doi:10.1016/j.abb.2010.05.003.
20. Ulloa, L.; Tracey, K.J. The "cytokine profile": a code for sepsis. *Trends Mol Med* **2005**, *11*, 56-63, doi:10.1016/j.molmed.2004.12.007.
21. Kondo, T.; Kawai, T.; Akira, S. Dissecting negative regulation of Toll-like receptor signaling. *Trends Immunol* **2012**, *33*, 449-458, doi:10.1016/j.it.2012.05.002.
22. Anwar, M.A.; Basith, S.; Choi, S. Negative regulatory approaches to the attenuation of Toll-like receptor signaling. *Exp Mol Med* **2013**, *45*, e11, doi:10.1038/emm.2013.28.
23. Trompouki, E.; Hatzivassiliou, E.; Tsichritzis, T.; Farmer, H.; Ashworth, A.; Mosialos, G. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. *Nature* **2003**, *424*, 793-796, doi:10.1038/nature01803.
24. Kawagoe, T.; Takeuchi, O.; Takabatake, Y.; Kato, H.; Isaka, Y.; Tsujimura, T.; Akira, S. TANK is a negative regulator of Toll-like receptor signaling and is critical for the prevention of autoimmune nephritis. *Nat. Immunol.* **2009**, *10*, 965-972, doi:10.1038/ni.1771.
25. Yuk, J.M.; Shin, D.M.; Lee, H.M.; Kim, J.J.; Kim, S.W.; Jin, H.S.; Yang, C.S.; Park, K.A.; Chanda, D.; Kim, D.K.; et al. The orphan nuclear receptor SHP acts as a negative regulator in inflammatory signaling triggered by Toll-like receptors. *Nature immunology* **2011**, *12*, 742-751, doi:10.1038/ni.2064.
26. Yoshida, H.; Jono, H.; Kai, H.; Li, J.D. The tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for toll-like receptor 2 signaling via negative cross-talk with TRAF6 AND TRAF7. *J. Biol. Chem.* **2005**, *280*, 41111-41121, doi:10.1074/jbc.M509526200.
27. Xia, M.; Liu, J.; Wu, X.; Liu, S.; Li, G.; Han, C.; Song, L.; Li, Z.; Wang, Q.; Wang, J.; et al. Histone methyltransferase Ash1l suppresses interleukin-6 production and inflammatory autoimmune diseases by inducing the ubiquitin-editing enzyme A20. *Immunity* **2013**, *39*, 470-481, doi:10.1016/j.immuni.2013.08.016.
28. Wertz, I.E.; O'Rourke, K.M.; Zhou, H.; Eby, M.; Aravind, L.; Seshagiri, S.; Wu, P.; Wiesmann, C.; Baker, R.; Boone, D.L.; et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature* **2004**, *430*, 694-699, doi:10.1038/nature02794.
29. Li, Z.; Chu, S.; He, W.; Zhang, Z.; Liu, J.; Cui, L.; Yan, X.; Li, D.; Chen, N. A20 as a novel target for the anti-neuroinflammatory effect of chrysin via inhibition of NF-kappaB signaling pathway. *Brain Behav Immun* **2019**, *79*, 228-235, doi:10.1016/j.bbi.2019.02.005.
30. Liu TF, B.C., El Gazzar M, McPhail L, Millet P, Rao A, Vachharajani VT, Yoza BK, McCall CE. Fueling the flame: bioenergy couples metabolism and inflammation. *J Leukoc Biol* **2012**, *92*, 499-507
31. Liu, T.F.; Yoza, B.K.; El Gazzar, M.; Vachharajani, V.T.; McCall, C.E. NAD+-dependent SIRT1 deacetylase participates in epigenetic reprogramming during endotoxin tolerance. *The Journal of biological chemistry* **2011**, *286*, 9856-9864, doi:10.1074/jbc.M110.196790.
32. Lee, J.; Kim, J.; Lee, J.H.; Choi, Y.M.; Choi, H.; Cho, H.D.; Cha, G.H.; Lee, Y.H.; Jo, E.K.; Park, B.H.; Yuk, J.M. SIRT1 Promotes Host Protective Immunity against *Toxoplasma gondii* by Controlling the FoxO-Autophagy Axis via the AMPK and PI3K/AKT Signalling Pathways. *Int J Mol Sci* **2022**, *23*, doi:10.3390/ijms232113578.
33. Wang, X.; Buechler, N.L.; Woodruff, A.G.; Long, D.L.; Zabalawi, M.; Yoza, B.K.; McCall, C.E.; Vachharajani, V. Sirtuins and Immuno-Metabolism of Sepsis. *Int J Mol Sci* **2018**, *19*, doi:10.3390/ijms19092738.
34. Yang, H.; Zhang, W.; Pan, H.; Feldser, H.G.; Lainez, E.; Miller, C.; Leung, S.; Zhong, Z.; Zhao, H.; Sweitzer, S.; et al. SIRT1 activators suppress inflammatory responses through promotion of p65 deacetylation and inhibition of NF-kappaB activity. *PLoS One* **2012**, *7*, e46364, doi:10.1371/journal.pone.0046364.
35. Wu, Z.; Liu, M.C.; Liang, M.; Fu, J. Sirt1 protects against thrombomodulin down-regulation and lung coagulation after particulate matter exposure. *Blood* **2012**, *119*, 2422-2429, doi:10.1182/blood-2011-04-350413.
36. Yang, Z.; Guan, Y.; Li, J.; Li, L.; Li, Z. Chrysin attenuates carrageenan-induced pleurisy and lung injury via activation of SIRT1/NRF2 pathway in rats. *Eur J Pharmacol* **2018**, *836*, 83-88, doi:10.1016/j.ejphar.2018.08.015.

 doi:10.20944/preprints202401.0121.v1