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A synthetic analog of α-galactosylceramide induces macrophage activation via the TLR4-signaling pathways

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ABSTRACT

α-Galactosylceramide (α-GalCer), a bioactive glycolipid isolated from the marine sponge *Agelas mauritianus*, is a potent immunomodulator with therapeutic potential for the treatment of autoimmune diseases and cancer. The Toll-like receptor 4 (TLR4), one of the promising molecular targets for immune-modulating drugs, is commonly expressed in innate immune cells especially macrophages and dendritic cells. Currently, whether α-GalCer can activate TLR4 signaling pathways remains unreported. In this study, we examined the effects of α-GalCer and its various structural analogs, CCL-1∼47, on TLR4 activation. We found that one α-GalCer analog (CCL-34), but not α-GalCer itself, strongly stimulated NF-κB activity in RAW 264.7 cells. CCL-34 activated NF-κB in a TLR4-dependent manner and stimulated TNF-α production in bone marrow cells of TLR4-functional C3H/HeN mice but not in those of TLR4-defective C3H/HeJ mice. Furthermore, CCL-34 treatment stimulated NF-κB activation and IL-8 production in a 293 cell line constitutively expressing human TLR4, MD-2 and CD14. Treatment of RAW 264.7 cells with CCL-34 also activated TLR4-downstream mitogen-activated protein kinases (ERK, JNK and p38), induced expression of TLR4-downstream genes (TNF-α, IL-6, IL-1β and iNOS) and promoted production of cytokines characteristic of activated macrophages. CCL-34-treated RAW 264.7 cells acquired a distinct morphology similar to that of LPS-activated macrophages and exhibited higher phagocytotic activity. Moreover, treatment with a TLR4-neutalizing antibody inhibited the CCL-34-induced morphological alteration. In summary, we identify a novel synthetic compound CCL-34 that can activate macrophages via TLR4-dependent signaling pathways. Our results suggest that CCL-34 is an immune modulator and may serve as a potential drug lead for immunotherapy.

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Abbreviations: α-GalCer, α-galactosylceramide; TLR4, Toll-like receptor 4; ELAM-1, endothelia leukocyte adhesion molecule-1; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; IL-6, interleukin-6; GM-CSF, granulocyte/macrophage colony-stimulating factor; NF-κB, nuclear transcription factor-κB; iNOS, inducible nitric oxide synthase

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1. Introduction

The Toll-like receptor (TLR) family proteins play vital roles in the recognition of pathogens, the initiation of innate immune response and the control of adaptive immunity [1]. To date, twelve TLR family members have been identified and various ligands for the different TLR receptors have been characterized [2]. A report by Medzhitov et al first described the identification of TLR4 and showed that constitutive expression of TLR4 induced activation of the transcription factor NF-κB and up-regulated the inflammatory cytokines [3]. The involvement of the TLR4 receptor in innate immunity was further demonstrated by experiments showing that TLR4 was the receptor for lipopolysaccharides (LPS), glycolipids present on the outer membranes of all Gram^{-ve} bacteria [4]. TLR4 is commonly expressed in monocytes, neutrophiles, macrophages and dendritic cells, but can also be detected in non-immune cell types such as epithelial cells and endothelial cells [5–9]. In addition to the exogenous ligand LPS, several endogenous ligands, such as fibrinogen, hyaluronic acid, heparin sulfate and β-defensin, have been identified to trigger TLR4-mediated inflammatory reactions and/or adaptive immune responses [10–13].

TLR4-mediated signaling pathways have been extensively studied using LPS as the prototype ligand. Stimulation of TLR4 can trigger MyD88 (Myeloid differentiation factor 88)-dependent and the MyD88-independent signaling pathways. In MyD88-dependent TLR4 signaling, MyD88 and TRAP (TIR domain-containing adaptor protein) associate with the cytoplasmic domain of activated TLR4 and then recruit IRAKs [interleukin-1 (IL-1) receptor-associated kinases], which subsequently activates TRAF6 (tumor-necrosis factor-receptor-associated factor 6) leading to activation of nuclear factor-κB (NF-κB) and MAPK (ERK, p38 and JNK). On the other hand, TRIF (TIR–domain-containing adaptor protein including IFN-β) and TRAM (TRIF-related adaptor molecule) can be activated via a MyD88-independent TLR4 signaling pathway and this subsequently mediates activation of NF-κB and IRF-3 (interferon regulatory factor 3). In both pathways, activated NF-κB protein translocates from cytoplasm to nucleus and functions as a transcription factor that induces expression of genes encoding pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 [2,14].

Since TLR4 plays essential roles in the activation of host defense mechanisms and has been considered as a promising molecular target, substantial effort has been made to develop TLR4 agonists for treating diseases. Indeed, many TLR4-activating compounds have been proved to be effective as vaccine adjuvants and/or stand-alone immunomodulators. For example, several medicinal plants-derived natural compounds, purified polysaccharides and purified proteins have been recently shown to stimulate immune responses in a TLR4-dependent manner [15–19]. Furthermore, OK-432 (a penicillin-killed and lyophilized preparation of a low-virulence stain of *Streptococcus pyogenes*) has been used clinically for cancer immunotherapy and this agent's cytokine-inducing and anti-tumor activity is TLR 4-dependent [20]. There is also a considerable amount of evidence demonstrating the therapeutic potential of synthetic TLR4 agonists. For instance, MPL (monophosphoryl lipid A), a chemically modified derivative of the lipid A moiety of LPS, has been used extensively in clinical trials as therapeutic vaccine to fight against cancer, infectious diseases and allergies. Another type of lipid A analogues, the aminoalkylglucosaminide 4-phosphates (AGPs), have been shown to be TLR4 ligands and can function as immunostimulants [21].

Recent studies have revealed that α-galactosylceramide (α-GalCer), a bioactive glycolipid originally isolated from the marine sponge *Agelas mauritianus*, is a potent immune-modulating agent for the treatment of microbial infections, autoimmune diseases and cancer [22,23]. α-GalCer has been identified as a ligand for invariant natural killer T (iNKT) cells. The binding of α-GalCer and the invariant Vα14 antigen receptors on iNKT cells stimulates these cells to produce immunoregulatory cytokines that result in the subsequent activation of other immune cell types (such as NK cells, DCs, B cells, T cells and macrophages) [22]. Currently, there is no report discussing whether α-GalCer can activate other types of receptors on immune cells. In addition, several recent reports have disclosed that α-GalCer derivatives with modification of the phytosphingosine or fatty acid moiety exhibit different immunoactivities from α-GalCer [24–26]. These findings raise the prospect of developing novel glycolipid vaccines/adjuvants based on α-GalCer. Therefore, we synthesized a series of serine-based glycolipid analogs of α-GalCer and compared them with α-GalCer in terms of their immunoactivity. Considering the fact that α-GalCer is a potent immune modulator and several types of glycolipids have been proved to be effective TLR4 activators, we speculated that α-GalCer or the created structurally related analogs of this compound (CCL-1∼47) may be able to activate the TLR4 receptor. In the present study, we investigated this topic and found that CCL-34 (a synthetic analog of α-GalCer) but not α-GalCer itself, is a novel TLR4 agonist. We also used RAW 264.7 macrophages as a cell model to further examine the effects of CCL-34 on the downstream signaling pathways, the expression profile of cytokines and activation of macrophages.

2. Materials and methods

2.1. Synthesis of serine-based lipid analogs of α-GalCer

Synthetic α-GalCer was prepared as described in a previous report [27]. The serine-based lipid analogs of α-GalCer were synthesized by modification of the procedures described in the literature [28]. As shown in Fig. 1A, deprotection of the Fmoc group in 1 was initially carried out and this was followed by amidation with various fatty acids (R_1) to generate compound 2. The allyl group of 2 was then removed using $Pd(PPh_3)_4$ as catalyst and the resulting acid was coupled with lipid amines (R_2) to yield amides. This was followed by deprotection of *para*-methoxyl benzene (PMB) by TFA to produce the various α-GalCer analogs 3. The chain lengths of the fatty acids (R_1) ranged from 6 to 18 carbons and those of lipid amines (R_2) ranged from 4 to 18 carbons. The chemical structures and assigned names of synthetic α-GalCer analogs are shown in Fig. 1B.

2.2. Chemicals and antibodies

Lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4) and polymyxin B sulfate were purchased from Sigma (St. Louis,

MO). Ultra-pure LPS (*E. coli* 0111:B4), a highly purified LPS shown to activate only TLR4 but not other TLRs, was purchased from Invivogen (San Diego, CA). Pyrogen-free water was purchased from Taiwan Biotech (Taoyuan, Taiwan). Anti-ERK1/2 antibody was purchased from BD Biosciences (San Diego, CA) and anti-actin antibody from Sigma Aldrich (St. Louis, MO). Antibodies against phospho-ERK1/2 (Thr202/Tyr204) (clone E10), p38, phospho-p38 (Thr180/Tyr182), JNK and phospho-JNK (Thr183/Tyr185) were purchased from Cell Signaling (Beverly, MA). The MTS510 antibody, shown to recognize the TLR4-MD2 complex [29], was purchased from BioLegend (San Diego, CA).

2.3. Cells and culture medium

The murine macrophage cell line RAW 264.7 was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). RAW264.7/Luc-P1 cells, a LPS-responsive cell line with an integrated reporter gene (pELAM1-Luc), were generated as described previously [19]. The pELAM1-Luc plasmid consists of the NF-κB-responsive region from ELAM1 (endothelial leukocyte adhesion molecule I) followed by the reporter gene firefly luciferase. Both RAW 264.7 and RAW264.7/Luc-P1 were cultured at 37 °C using a 5% CO_2 incubator in DMEM supplemented with 10% heat-inactivated bovine calf serum (HyClone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μM L-Glutamine and 1 mM sodium pyruvate. Hematopoietic cells isolated from bone marrow were cultured at 37 °C using a 5% CO_2 incubator in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries, Kibbutz, Israel), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μM L-Glutamine and 1 mM sodium pyruvate. The 293-hTLR4/MD2-CD14 cells were purchased from Invivogen (San Diego, CA) and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μM L-Glutamine, 10 μg/ml blasticidin, and 50 μg/ml HygroGold. Both blasticidin and HygroGold were obtained from Invivogen (San Diego, CA).

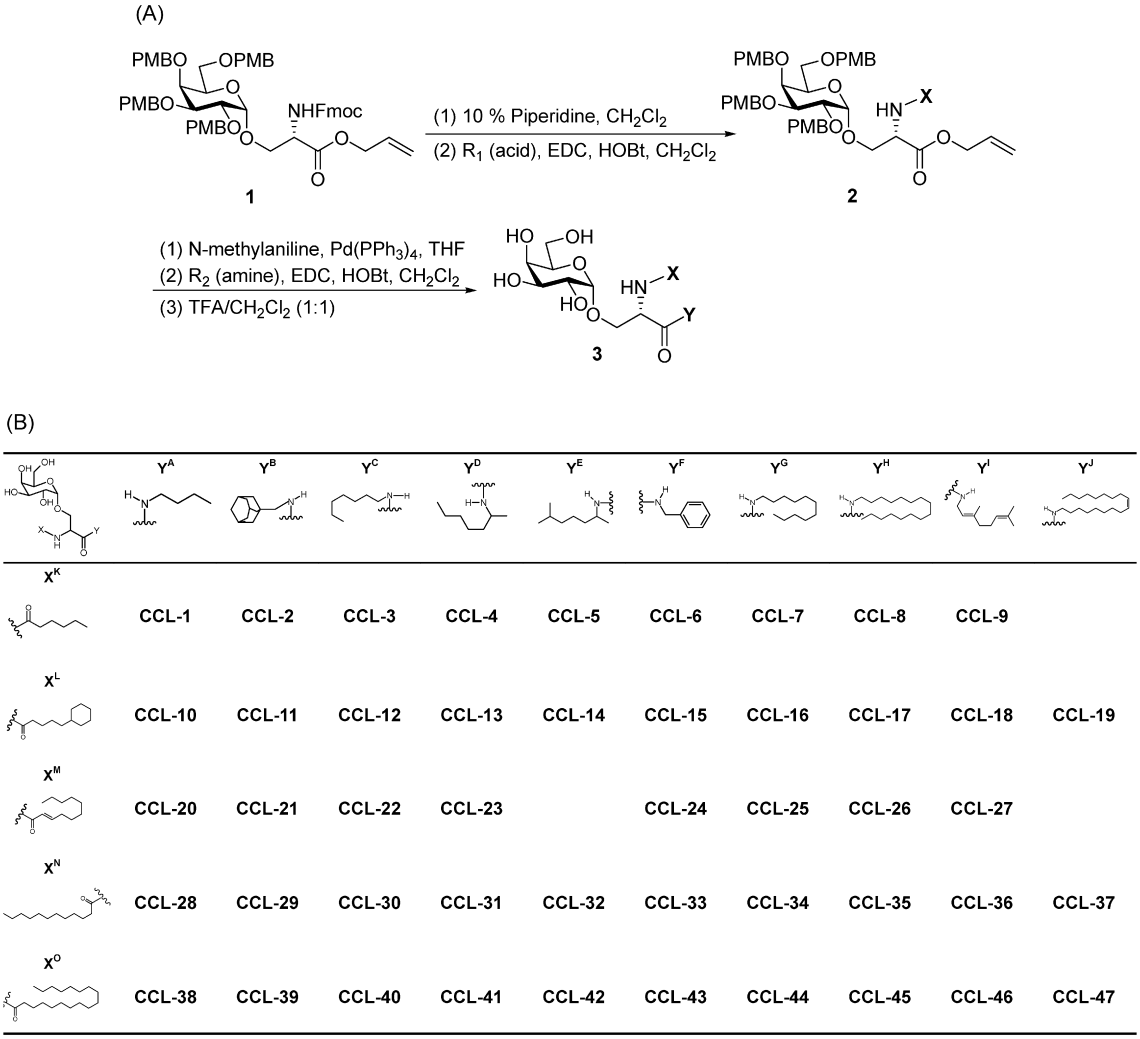

Fig. 1 – Synthesis of α-GalCer analogues used in this study. (A) The synthesis scheme for the α-GalCer analogues (designated as CCL compounds). Reagents and conditions are described in Section 2. (B) The chemical structures and numbering of the CCL compounds.

2.4. Isolation of hematopoietic cells from mouse bone marrow

C3H/HeN and C3H/HeJ mice were regularly maintained in a SPF area at the Animal Center of National Yang-Ming University (Taipei, Taiwan). To isolate bone marrow cells, C3H/HeN or C3H/HeJ mice were sacrificed under aseptic conditions, and the femurs were collected and cut up with scissors. The bone marrow in the femurs were flushed out with medium using a 10 ml-syringe attached to a 25-gauge needle. After centrifugation, the cell pellets were resuspended in growth medium using an 18-gauge needle to produce single-cell suspensions and transferred to 100-mm tissue culture plates. After one week of incubation, non-adherent cells were removed and the adherent cells were maintained for the subsequent experiments.

2.5. Transient transfection

The 293-hTLR4/MD2-CD14 cells (2×10^6) were seeded onto 100-mm plates the day before transfection. The pELAM1-Luc plasmid (3 μg) was introduced into the cells using FuGENE 6 reagent according to manufacturer's instruction (Roche, Basel, Switzerland). Twenty-four hours after transfection, the cells were reseeded in 24-well plates (2×10^5/well) and grown overnight for subsequent drug treatments.

2.6. Luciferase assays

The RAW264.7/Luc-P1 cells or the 293-hTLR4/MD2-CD14 cells transfected with pELAM-1 (described above) were respectively seeded in 24-well plates at the density of 4×10^5 or 2×10^5 cells per well and grown overnight. The cells were treated with LPS (the positive control) or the indicated compound for 5 h, harvested and analyzed using luciferase assays as described previously [19]. The luminescence was measured with an AutoLumat LB953 (Berthold Technologies, Bad Wildbad, Germany). All reagents for luciferase assays were purchased from Promega (Madison, WI).

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RAW 264.7 cells (4×10^6) were seeded into 60-mm tissue culture plates, grown overnight and treated with the vehicle or the indicated drug for 5 h. Total RNA was isolated and treated with a DNA-free™ kit using procedures described previously [19]. One microgram of DNA-free total RNA was reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen, Carlbad, CA) and the synthesized cDNA was further amplified using *Taq* DNA polymerase (BIOTOOLS B&M Labs, Madrid, Spain) with specific primer pairs. The primer sequences and experimental conditions used for the synthesis of the TNF-α, IL-6, IL-1β, and *gapdh* genes have been described previously [19]. The primers and PCR condition used for detecting the mouse GM-CSF gene were ATGTGGCTGCA-GAATTTACTTTTCCT (sense), TGGGCTTCCTCATTTTT-GGCC-TGGT (antisense) and thirty-two amplification cycles (94 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min). The expected PCR products for TNF-α, IL-1β, IL-6, GM-CSF, iNOS and *gapdh* are 331, 287, 343, 435, 306 and 471 base pairs, respectively. The PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

2.8. Western blot

RAW 264.7 cells (10^6) were seeded into 6-well plates for 16 h to 18 h, treated with vehicle or drug, and harvested using the protocols described previously [19]. Aliquots of the protein extracts were quantified by Bradford protein assay (Bio-Rad, Hercules, CA). Protein lysate (50 μg) for each sample was loaded and analyzed using SDS-PAGE gels. Afterwards, the proteins were transferred to PVDF membranes (Millipore, Bedford, MA) and incubated with blocking solution (5% non-fat milk in TBS/0.1% Tween-20) for 1 h. Primary antibody and secondary horseradish peroxidase-conjugated antibody diluted in 1% BSA/TBS/0.1% Tween 20 were sequentially added to and incubated with the membranes overnight and for 1 h, respectively. Protein bands were visualized by chemiluminescence using the ECL detection system (Millipore, Bedford, MA).

2.9. Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells (2×10^5) or bone marrow cells (10^5) were seeded into 24-well plates and grown overnight in 5% CO_2 incubator at 37 °C. The cells were then incubated with vehicle(s) or drugs for various time periods. Culture medium was then harvested for measurement of TNF-α, IL-6 or GM-CSF using the DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN). The ELISA kit for detecting IL-1β was purchased from BioSource (Camarillo, CA). The A_{450} and A_{650} (reference absorbance) were measured using a microplate photometer (Multiskan RC, Model 351, Lab Systems, Stockholm, Sweden). The amount of human IL-8 in culture medium collected from vehicle- or drug-treated 293-hTLR4/MD2-CD14 cells (2×10^5 cells, 5h-treatment) was determined using the DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN).

2.10. Phagocytosis assays

RAW 264.7 cells (2×10^5) were seeded into 60-mm tissue culture plates, grown for 48 h, treated with vehicle or drug for 24 h and then incubated with 200 μg fluorescein-labeled *Escherichia coli* bioparticles (Molecular Probes, Eugene, OR) for 1 h. Next, the bioparticle-containing medium was removed and the cells were washed with 1 × PBS, trypsinized, resuspended in 1ml PBS/1% BSA and analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using WinMDI 2.8 software (TSRI, La Jolla, CA).

3. Results

3.1. Identification of a novel TLR4 activator: CCL-34

The strategy to identify TLR4 activators has been described in a previous study [19]. In brief, candidate compounds that activate NF-κB are initially identified using a NF-κB activity-based reporter assay. Only agents showing activity three-fold

higher than vehicle-only are considered to be NF-κB activators. In the next step, the NF-κB-activating agents are applied to bone marrow cells isolated from wild type or TLR4-defective mice. Those compounds that selectively induce TNF-α expression in the wild-type cells but not in the TLR4-defective cells are considered as TLR4-dependent activators. NF-κB was chosen as the target molecule since it mediates TLR4 downstream signaling in both the MyD88-dependent and the MyD88-independent pathways. Moreover, the NF-κB-responsive region in the reporter construct is derived from the ELAM1 (E-selectin) promoter and this reporter construct has been frequently used to detect TLR4-downstream NF-κB activation [15,30,31]. Initially, the ability of α-GalCer and its synthetic analogues CCL-1∼47 to activate NF-κB signaling were measured. The CCL-1∼47 compounds (shown in Fig. 1B) are designed on the basis of structural similarity to α-GalCer and are composed of a galactose glycosidically α-linked to a serine core carrying various lengths of fatty acid (R_1) and lipid amine (R_2). As indicated in Table 1, α-GalCer did not activate NF-κB in the RAW 264.7 macrophages but two α-GalCer analogs, CCL-25 and CCL-34, did exhibit NF-κB-stimulating activity (4- and 18-fold activation, respectively). Both CCL-25 and CCL-34 carry the same lipid amine (R_2–Y^G), suggesting this specific structure is indispensable to their NF-κB-stimulating activity. Moreover, minor difference in the fatty acid moiety (R_1) seemed to significantly affect the activity of these compounds. For instance, CCL-25 (with R_1-X^M) contains a double bond and shorter fatty acid chain than CCL-34 (with R_1-X^N) and CCL-25 exhibits a weaker activity than CCL-34. On the other hand, CCL-44, carrying the same lipid amine (R_2-Y^G) as CCL-34 but with a longer fatty acid chain (R_1-X^o), does not activate NF-κB (Fig. 1B and Table 1). Since CCL-34 displayed a better NF-κB-stimulating activity than CCL-25, this compound was selected for the subsequent experiments. In addition, CCL-44 (the inactive compound with structure similar to CCL-34) was used in all experiments as a reference control for CCL-34.

Table 1 – The effects of CCL-1∼47 on NF-κB activation in RAW 264.7/Luc-P1 cells

Relative luciferase activity (fold)

Sample name	Mean ± S.E.M.	Sample name	Mean ± S.E.M.
H_2O	1.1 ± 0.04	CCL-23	0.9 ± 0.1
LPS 10 ng/ml	14.6 ± 2.5*	CCL-24	1.0 ± 0.1
0.1% DMSO	1.1 ± 0.0	CCL-25	4.1 ± 1.1*
α-GalCer	1.0 ± 0.1	CCL-26	0.9 ± 0.1
CCL-1	1.0 ± 0.0	CCL-27	0.9 ± 0.1
CCL-2	1.0 ± 0.0	CCL-28	0.8 ± 0.1
CCL-3	1.0 ± 0.0	CCL-29	0.9 ± 0.1
CCL-4	1.0 ± 0.1	CCL-30	0.9 ± 0.0
CCL-5	1.0 ± 0.1	CCL-31	0.9 ± 0.2
CCL-6	1.0 ± 0.0	CCL-32	1.3 ± 0.4
CCL-7	1.3 ± 0.3	CCL-33	0.8 ± 0.2
CCL-8	1.0 ± 0.0	CCL-34	17.8 ± 0.4*
CCL-9	1.0 ± 0.0	CCL-35	0.9 ± 0.0
CCL-10	1.0 ± 0.1	CCL-36	1.8 ± 0.7
CCL-11	1.0 ± 0.0	CCL-37	1.9 ± 0.1
CCL-12	1.0 ± 0.1	CCL-38	0.9 ± 0.2
CCL-13	1.0 ± 0.0	CCL-39	0.8 ± 0.2
CCL-14	1.0 ± 0.0	CCL-40	0.8 ± 0.0
CCL-15	1.0 ± 0.1	CCL-41	0.9 ± 0.0
CCL-16	2.2 ± 1.0	CCL-42	1.0 ± 0.0
CCL-17	1.1 ± 0.0	CCL-43	1.1 ± 0.0
CCL-18	1.0 ± 0.2	CCL-44	1.1 ± 0.1
CCL-19	0.8 ± 0.0	CCL-45	1.1 ± 0.1
CCL-20	1.0 ± 0.2	CCL-46	1.3 ± 0.1
CCL-21	0.9 ± 0.0	CCL-47	1.2 ± 0.1
CCL-22	1.0 ± 0.1		

(1) α-GalCer and CCL-1∼47: 10 μM, (2) LPS is dissolved in pyrogen-free water; α-GalCer and CCL-1∼47 compounds are dissolved in DMSO. (3) * indicates three-fold higher activity than vehicle-treated groups.

We next investigated the concentration-dependent effect of CCL-34 on NF-κB activity. To examine whether these samples were contaminated with LPS, we also measured NF-κB activity in the presence of polymyxin B, a known pharmacological antagonist of LPS. As shown in Fig. 2A, CCL-34 activated NF-κB in a concentration-dependent manner and polymyxin B supplementation did not affect the NF-κB activation induced by CCL-34. We also examined whether TLR4 is involved in CCL-34-induced NF-κB activation by measuring the effect of TLR4-neutralizing antibody (which specifically recognizes the TLR4-MD-2 complex [29]) on CCL-34-induced NF-κB activity in RAW 264.7 cells. To ensure that the inhibition of NF-κB by neutralizing antibody is TLR4-specifc, we used a highly purified LPS (shown to activate TLR4 but not other TLRs) as the positive control in this experiment. As shown in Fig. 2B, as the amount of anti-TLR4 neutralizing antibody added was increased, NF-κB activation by CCL-34 or LPS was accordingly reduced. Furthermore, CCL-34 stimulated the expression of TNF-α (a known downstream target of TLR4) in bone-marrow cells of TLR4-functional C3H/HeN mice but not in those of TLR4-defective C3H/HeJ mice (Fig. 2C). Together, these results suggest that CCL-34 is a TLR4 activator.

Some TLR4 ligands, such as Taxol, are found to activate mouse TLR4 (mTLR4) but not human TLR4 (hTLR4) [32]. To examine whether CCL-34 exhibits species-specific activity, we applied a 293 cell line stably expressing human TLR4, MD-2 and CD14 (designated as 293-hTLR4/MD2-CD14) to measure the ability of CCL-34 on human TLR4 activation. As shown in Fig. 2D, obvious NF-κB activation by CCL-34 was detected in 293-hTLR4/MD2-CD14 cells. Furthermore, CCL-34 treatment stimulated the production of IL-8 (one of the TLR4-downstream cytokines induced via NF-κB activation) in these cells as shown in Fig. 2E. Our results suggest that CCL-34 can activate human TLR4 and its activity on TLR4-depensdent signaling is not species-specific.

3.2. *CCL-34 activates TLR4-downstream signal transduction pathways in RAW 264.7 macrophages*

In addition to NF-κB, MAPK family members (ERK, p38 and JNK) have been previously shown to participate in the TLR4-downstream signaling pathways [33]. We therefore examined whether these signaling pathways are also stimulated by CCL-34 in RAW 264.7 macrophages. Western blots were performed to detect both the total and the active forms of the MAPK proteins (ERK, p38 and JNK) in vehicle-, LPS-, CCL-34- or CCL-44-treated cells. As shown in Fig. 3, there was activation of all MAPK members in the CCL-34-treated macrophages, but not in

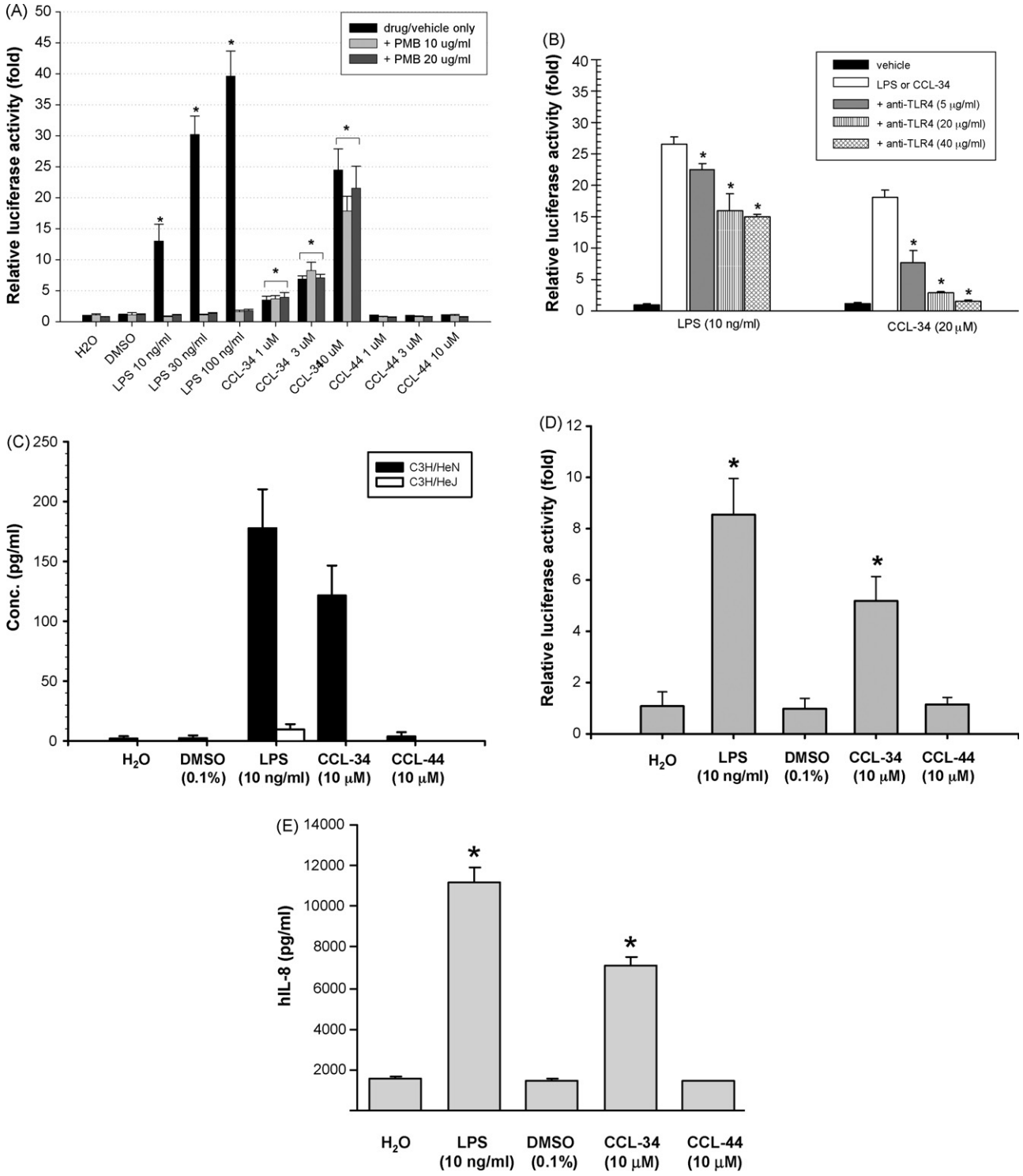

Fig. 2 – CCL-34 is a TLR4 activator. (A) The concentration-dependent effects of CCL-34 on NF-κB activation. LPS, CCL-34, CCL-44 or vehicle (pyrogen-free water for LPS; DMSO for the CCL compounds) were mixed without or with polymyxin B and applied to RAW264.7/Luc-P1 cells (4×10^5) for 5 h. The luciferase assays were performed as described in Section 2. The results are presented as mean ± standard error from three independent experiments. The results were compared by two-sample t-test and * indicates that the value is significantly different from that obtained from the appropriate vehicle-treated group ($p < 0.05$). (B) CCL-34-stimulated NF-κB activation is TLR4-dependent. RAW 264.7 cells (6×10^4 per well in MP-96 plates) were seeded and grown in regular growth medium (DMEM with 10% bovine calf serum) overnight. The medium was replaced with DMEM with 3% bovine calf serum and the cells were untreated or treated with a neutralizing anti-TLR4 antibody (5, 20 or 40 μg/ml) for 1 h, then further incubated with LPS (10 ng/ml) or CCL-34 (20 μM) for 5 h. The NF-κB activity of LPS- or CCL-34-treated RAW264.7/Luc-P1 cells, in the absence or presence of TLR4-neutralizing antibody, was determined by luciferase assays. Vehicles for LPS and CCL-34 are pyrogen-free water and 0.2% DMSO, respectively. The

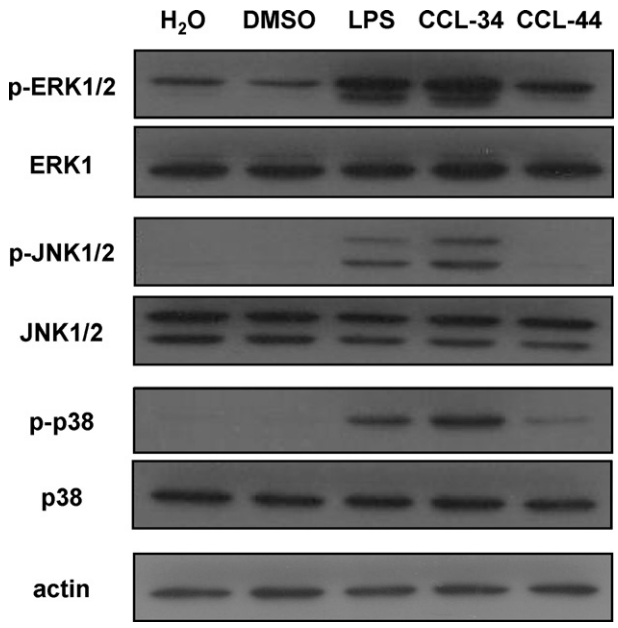

Fig. 3 – CCL-34 activates the ERK-, JNK- and p38-signaling pathways in RAW 264.7 cells. RAW 264.7 cells were treated with pyrogen-free water, DMSO (0.1%), LPS (10 ng/ml), CCL-34 (10 μM) or CCL-44 (10 μM) for 20 min. Expression of the total and activated forms of the MAPK family proteins were determined by Western blot as described in Section 2. Actin expression serves as the loading control. The results are one representative example out of three independent experiments.

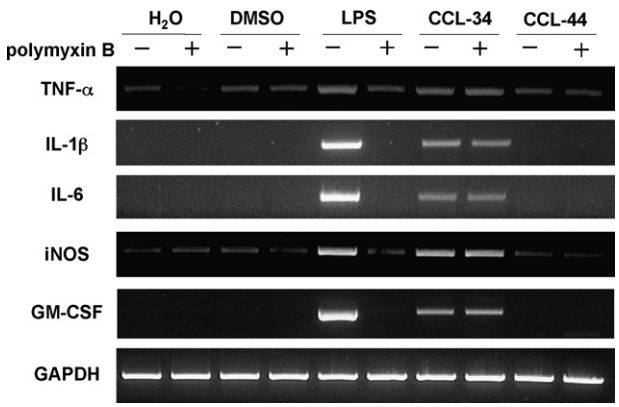

Fig. 4 – CCL-34 induces gene expression of cytokines and iNOS in RAW 264.7 macrophages. RAW 264.7 cells were treated with vehicles, LPS (10 ng/ml), CCL-34 (10 μM) or CCL-44 (10 μM) for 5 h in the absence or presence of polymyxin B. The expression of each indicated gene was measured by RT-PCR. The results are one representative example out of three independent experiments.

the various negative controls. Moreover, the LPS positive control also gave activation of these signaling proteins. This result and Fig. 2A demonstrate that CCL-34 is able to induce activation of the major TLR4-downstream effector molecules including NF-κB, JNK, p38 and ERK1/2.

3.3. *CCL-34 induces expression of TLR4-downstream genes in RAW 264.7 macrophages*

Upon activation by TLR4 ligands, macrophages can become effector cells that release pro-inflammatory cytokines and reactive oxygen species to eliminate foreign antigens. We next applied RT-PCR to measure the expression of the TLR4-downstream cytokines as well as inducible nitric oxide synthase (iNOS) in CCL-34-treated RAW 264.7 macrophages. As shown in Fig. 4, CCL-34 but not CCL-44 stimulated gene expression of TNF-α, IL-1β, IL-6 and iNOS in RAW 264.7 cells. The time-dependent and concentration-dependent induction of TNF-α and IL-6 by CCL-34 was further validated by ELISA (Fig. 5). The induction of TNF-α by LPS and CCL-34 was detected after 2-h treatment (Supplemented data Fig. 1). When RAW 264.7 cells were treated with CCL-34 or LPS for 2 h and subsequently incubated in drug-free medium, substantial secretion of TNF-α in these cells could be detected at 2, 6, 12, and 24 h after removal of the drug (Supplemented data Fig. 2). Unexpectedly, although RNA expression of IL-1β was stimulated by CCL-34, the amount of secreted IL-1β protein after CCL-34 treatment was relatively low (Figs. 4 and 5B). In addition, the induction of GM-CSF in CCL-34-treated cells was also observed (Figs. 4 and 5D). Since TNF-α, IL-1β, IL-6 and iNOS are downstream target genes of TLR4 and elevation of these genes and GM-CSF are characteristic of

results are presented as mean ± standard error from three independent experiments. The data were compared by two-sample *t*-test and * indicates that the value is significantly different from that obtained from LPS- or CCL-34-treated cells ($p < 0.05$). (C) CCL-34 induces TNF-α production in a TLR4-dependent manner. Bone marrow cells isolated from C3H/HeN (wild type) and C3H/HeJ (TLR4-defective) mice were treated with vehicle [pyrogen-free water or DMSO (0.1%)], LPS (10 ng/ml, the positive control), CCL-34 (10 μM) or CCL-44 (10 μM) for 24 h. Culture medium was then collected and assayed for TNF-α production using ELISA. The results are shown as the mean ± standard error from three independent experiments. (D) CCL-34 activates NF-κB in a 293 cell line constitutively expressing human TLR4, MD-2 and CD14 (293-hTLR4/MD2-CD14). The cells (2×10^6) were transfected with 3 μg reporter plasmid pELAM-1. Next day, these transfected cells were seeded (2×10^5/well) in 24-well plates, grown overnight and incubated with vehicles or various compounds for 5 h. The luciferase activity was then measured. The results are presented as mean ± standard error from three independent experiments. The data were compared by two-sample *t*-test and * indicates that the value is significantly different from that obtained from the appropriate vehicle-treated group ($p < 0.05$). (E) CCL-34 stimulates IL-8 production in the 293-hTLR4/MD2-CD14 cells. The cells were treated with vehicles or various compounds for 5 h. The amount of IL-8 in culture medium collected from various treatments was determined by ELISA assays. The results are presented as mean ± standard error from three independent experiments. The data were compared by two-sample *t*-test and * indicates that the value is significantly different from that obtained from the appropriate vehicle-treated group ($p < 0.05$).

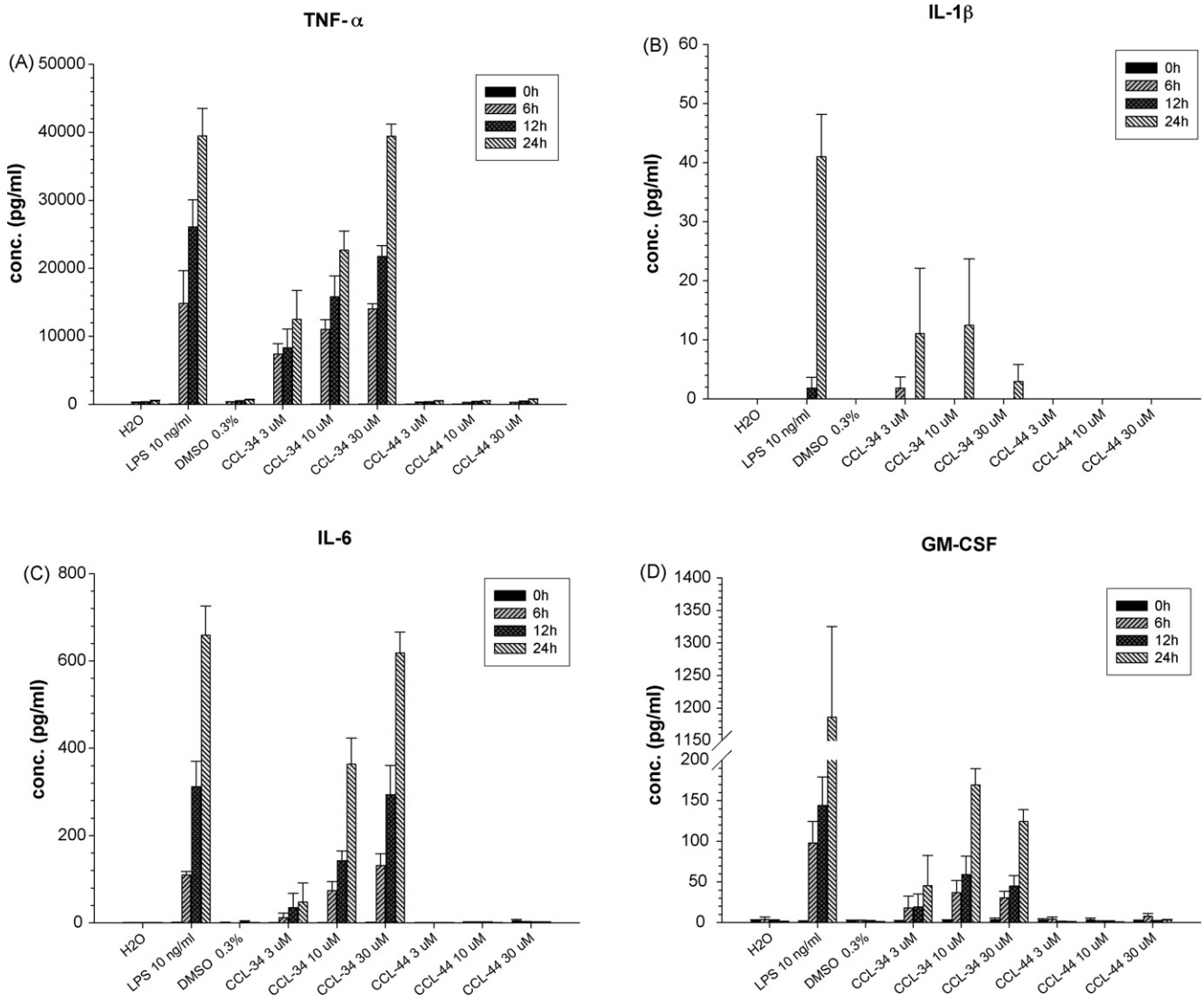

Fig. 5 – Induction of cytokine secretion in CCL-34-treated macrophages. RAW 264.7 macrophages were treated with vehicles, LPS, CCL-34 or CCL-44 at indicated concentrations for 0, 6, 12 or 24 h. Culture medium was then collected and assayed for cytokine production using ELISA. The results are shown as the mean ± standard error from three independent experiments.

activated macrophages, these results indicate that CCL-34 can activate TLR4-downstream target genes in macrophages and induce macrophage activation.

3.4. *CCL-34 induces morphological alteration and increases phagocytotic activity of RAW 264.7 cells*

Activated macrophages usually display a distinct morphology and acquire enhanced phagocytotic activity [34–36]. Therefore, we also examined the morphology and phagocytotic activity of CCL-34-treated RAW 264.7 cells. Untreated RAW 264.7 cells look refractile and rounded and treatment of these cells with vehicle or CCL-44 did not affect the cell morphology [Fig. 6A, a, b, and e]. However, exposure to CCL-34 or LPS (the positive control) induced morphological alteration of the RAW 264.7 cells [Fig. 6A, c and d]. Both LPS- and CCL-34-treated cells became polygonal with pseudopodi and vesicles, which is similar to the morphology of mature macrophages. Under the same treatment conditions, CCL-34 did not cause cytotoxicity of RAW264.7 cells (data not shown). Furthermore, LPS- and CCL-34-treated cells engulfed more fluorescein-labeled *Escherichia coli* bioparticles than vehicle- and CCL-44-treated cells in phagocytosis assays (Fig. 6B), suggesting that these cells had acquired higher phagocytotic activity. Noticeably, treatment with TLR4-neutralizing antibody inhibited the CCL-34-induced morphological alteration (Fig. 7). Taken together, the results described above indicate that CCL-34 is able to activate macrophages via the TLR4-dependent signaling pathways.

4. Discussion

In the present study we identified and characterized a novel TLR4 activator, CCL-34. We showed that CCL-34 induced NF-κB

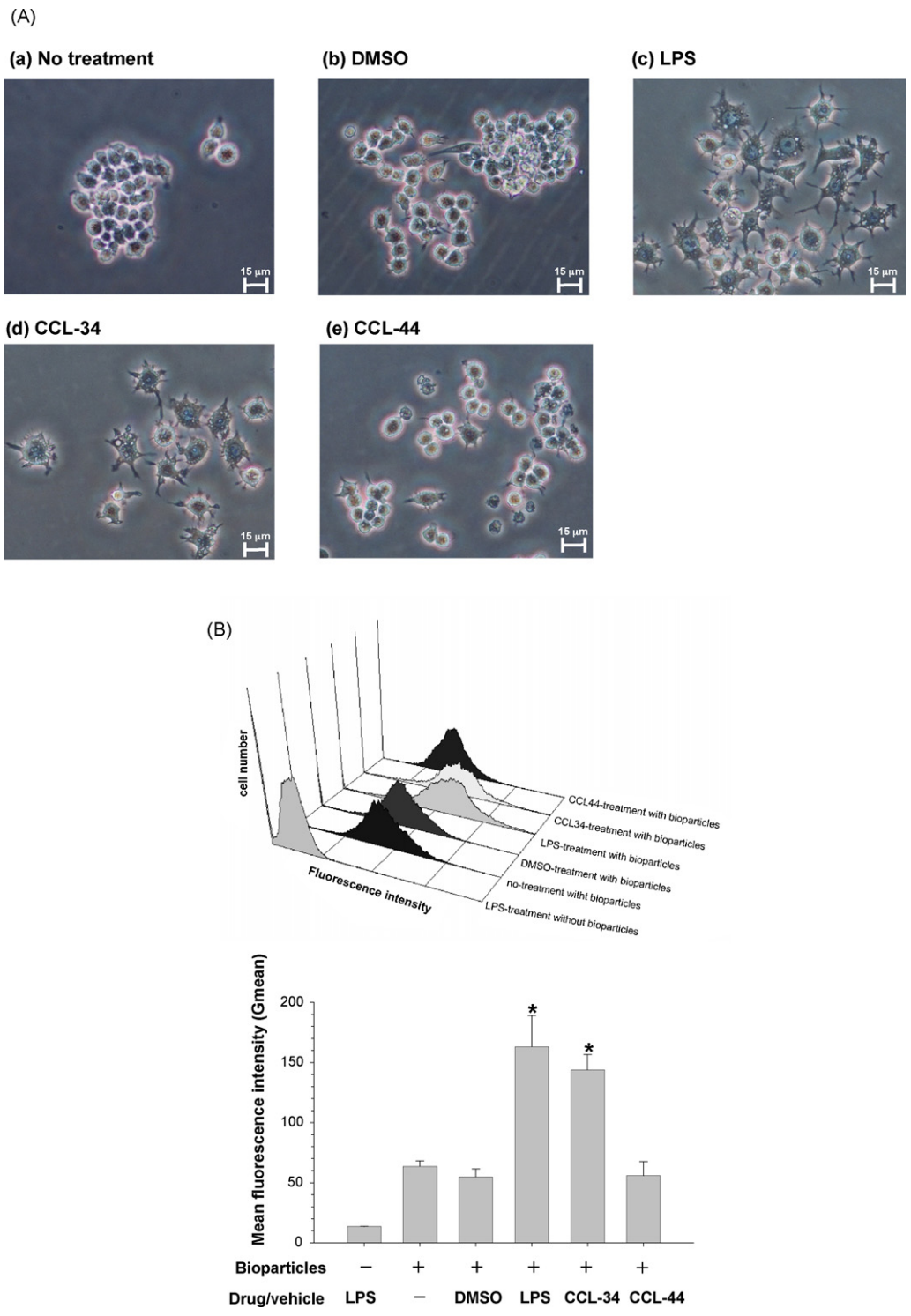

Fig. 6 – CCL-34 induces morphological alteration and increases phagocytotic activity of RAW 264.7 cells. (A) Morphology of CCL-34-treated RAW 264.7 cells. RAW 264.7 cells (2×10^5 in 60-mm plates) were treated with vehicle (0.1% DMSO), LPS (10 ng/ml), CCL-34 (10 μM) or CCL-44 (10 μM) for 24 h and photographed under a phase-contrast microscope. The results are one representative example out of three independent experiments. (B) FACS analysis of phagocytotic activity of CCL-34-treated macrophages. RAW 264.7 cells were treated with vehicle (0.1% DMSO), LPS (10 ng/ml), CCL-34 (10 μM) or CCL-44 (10 μM) for 24 h, incubated with fluorescein-labeled *Escherichia coli* bioparticles for 1 h and then analyzed by FACSort flow cytometer. *(upper panel)* The integrated diagram showing the fluorescent cell population and fluorescence intensity of all treatment groups. The results are one representative example out of three independent experiments. *(Bottom panel)* Mean fluorescence intensity of each treatment group. Averages of Gmean values from FACS analysis of all treatment groups are shown. The results are shown as the mean ± standard error from three independent experiments. The results were compared by two-sample *t*-test and * indicates a value that is significantly different from that obtained from bioparticle alone-treated cells ($p < 0.05$).

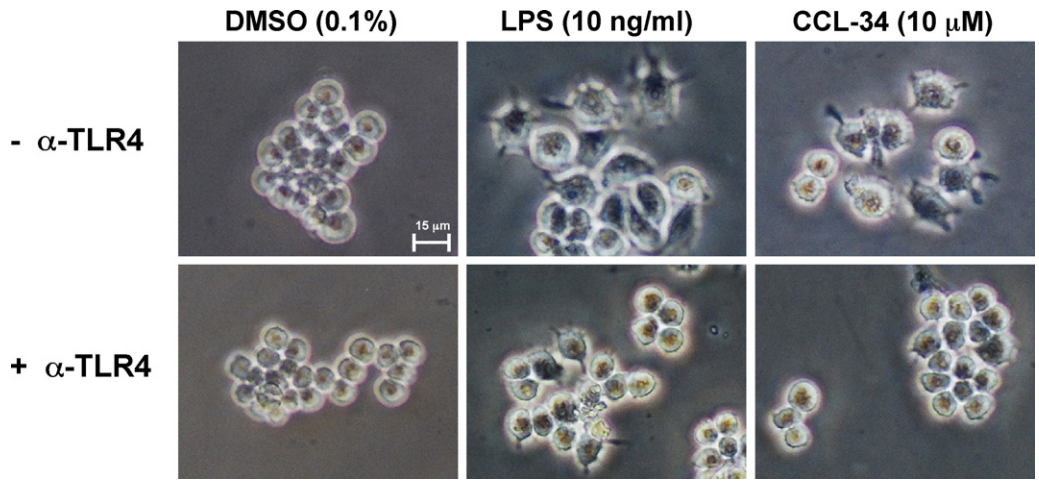

Fig. 7 – Treatment of a neutralizing anti-TLR4 antibody inhibits CCL-34-induced morphological differentiation of RAW 264.7 cells. RAW 264.7 cells (2×10^4 cells in 24-well plates) were untreated or treated with a neutralizing anti-TLR4 antibody (10 μg/ml) for 1 h, then further incubated with vehicle (0.1% DMSO), LPS (10 ng/ml), CCL-34 (10 μM) or CCL-44 (10 μM) for 24 h. Cell morphology was photographed under a phase-contrast microscope. The results are one representative example out of two independent experiments.

activation in a TLR4-depenpent manner in RAW 264.7 macrophages (Fig. 2A and B). Furthermore, CCL-34-stimulated TNF-α production was abrogated in bone marrow cells isolated from TLR4-defective mice (Fig. 2C). We also demonstrated that CCL-34 can activate human TLR4 by showing its ability to activate NF-κB and induce IL-8 production in a 293 cell line constitutively expressing human TLR4, MD-2, and CD14 (Fig. 2D and E). CCL-34 treatment also led to the activation of the TLR4-downstream MAPK-signaling pathways, and the elevated expression of the pro-inflammatory cytokines (TNF-α, IL-6) and iNOS in RAW 264.7 cells (Figs. 3–5). Noticeably, similar signaling pathways and cytokine profiles have been previously observed for TLR4 activation induced with LPS and other glycolipids [37–40]. Based on these observations, we conclude that CCL-34 is able to activate the typical TLR4-signaling pathways.

Recent studies suggest that TLR4 protein alone is not sufficient for LPS-induced TLR4 activation and two additional proteins, CD14 and MD-2, are involved in LPS recognition by TLR4 [41,42]. The CD14 protein concentrates LPS for binding to the MD-2, which is a secreted protein associating with extracellular domain of TLR4 [43–45]. The physical interaction between LPS and TLR4-MD-2 complex at cell surface is essential for optimal LPS-mediated TLR4 activation [46,47]. Accumulating evidence demonstrates that MD-2 is also required for TLR4 activation by other ligands and plays vital roles in species-specific recognition of TLR4 ligands [48–50]. In this study, we demonstrated that the neutralizing antibody against TLR4-MD-2 complex strongly inhibited CCL-34-induced NF-κB activation in RAW 264.7 cells (Fig. 2B), indicating the functional TLR4-MD-2 complex is essential for TLR4 activation by CCL-34. Furthermore, exogenous expression of TLR4, MD-2 and CD14 in human 293 cells are sufficient for CCL-34 to activate TLR4-dependnet signaling pathway as shown in Fig. 2D and E. These results provide molecular evidence that TLR4 recognition by CCL-34 require the assistance from MD-2 (and likely also from CD14), a mechanism similar to that by LPS and other TLR4 ligands. However, our data did not exclude the possibility that CCL-34 may directly bind to and activate TLR4 receptor. Although physical interaction between LPS and TLR4 has been previously suggested [51], whether CCL-34 can directly bind to TLR4 remains further investigation.

Our result indicates that α-GalCer did not activate NF-κB in RAW 264.7 macrophages (Table 1), which is consistent with a previous study showing that α-GalCer did not augment NO production in RAW 264.7 cells [52]. Two α-GalCer analogs (CCL-25 and CCL-34) were identified as NF-κB activators in this study (Table 1). Interestingly, there exists a structure and activity relationship between CCL-25, CCL-34 and CCL-44 (Fig. 8 and Table 1). Although these three compounds all carry the same lipid amine, minor variation in the fatty acid moiety of these compounds seems to significantly affect their NF-κB-activating activity. CCL-34, the fatty acid moiety of which is composed of a 12-carbon fatty acid chain, exhibits the greatest activity. However, CCL-25, which is only one carbon shorter with an 11-carbon fatty acid chain together with a double bond, exhibits only 20% of the activity of CCL-34. Furthermore, CCL-44 with a longer fatty acid chain (18-carbon) than CCL-34 (12-carbon) is completely unable to activate NF-κB. The results suggest that activation of NF-κB is clearly dependent on the length of fatty acid present in the compound. We speculate that the long fatty acid chain that forms part of the α-GalCer molecule may account for its inability to activate NF-κB (Fig. 8).

Recent studies have pointed out that contamination of experimental agents with microbial-derived immunostimulatory molecules such as LPS may lead to false identification of TLR4 agonists [53,54]. In the present study, we also validated that CCL-34 was the activator itself and its activity was not due to LPS contamination because polymyxin B did not block NF-κB activation or the cytokine expression triggered by CCL-34 (Figs. 2A and 4). Furthermore, our results showed that CCL-34 exhibited a similar but less potent immunostimulatory activity compared to LPS in several assays. Like LPS, CCL-34 can activate the TLR4 signaling pathways, stimulate the gene

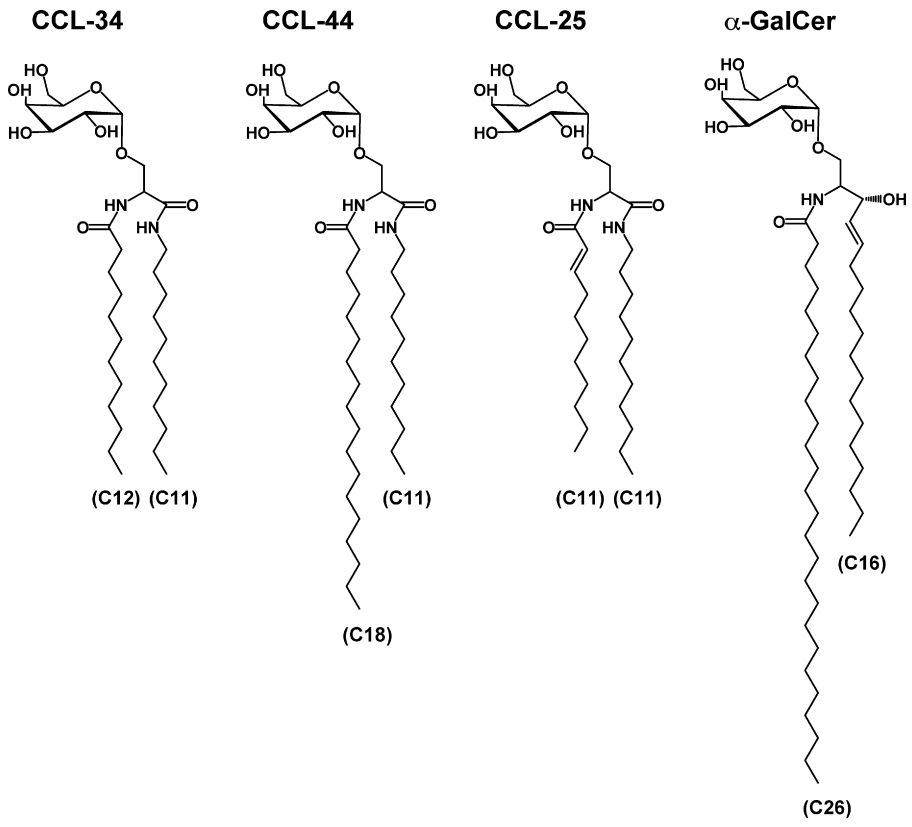

Fig. 8 – The chemical structure of CCL-25, CCL-34, CCL-44 and α-GalCer.

expression of cytokines (TNF-α, IL-1β, IL-6, GM-CSF), switch on the expression of iNOS and induce macrophage activation. However, there exists a discrepancy between these two treatments with regard to IL-1β production. Both CCL-34 and LPS stimulate gene expression of IL-1β but secretion of the IL-1β protein seems to be impaired in the CCL-34 treated cells (Figs. 4 and 5B). Previous studies have shown that IL-1β is first expressed as cytosolic proIL-1β protein and requires proteolytic cleavage by caspase-1 to convert it into a secreted protein [55]. In macrophages, LPS stimulation led to the processing of inactive caspase-1 precursor into active form of caspase-1 [56]. Interestingly, a recent report demonstrates that MPL (a potent TLR4 agonist with much lower toxicity than LPS) strongly stimulates the expression of IL-1β transcripts without production of secreted IL-1β protein. The inability of MPL to stimulate IL-1β secretion is due to the fact that MPL fails to activate caspase-1 [57]. Therefore, it is likely that CCL-34 treatment is not able to activate proteins required for IL-1β maturation (such as caspase-1) and thus impair the secretion of IL-1β. In fact, CCL-34 is structurally distinct from LPS and these two compounds may activate overlapping but non-identical molecular targets. More extensive studies on the gene expression and the biological activity are certainly required to distinguish the biological differences between CCL-34 and LPS treatment. Certainly, such information is crucial for further evaluation of the potential toxicity and therapeutic benefits of CCL-34.

Macrophages play an essential role in host protection against pathogen and tumors. They are important effector cells that eliminate pathogens as part of the innate immune response and activate T cells to induce adaptive immunity [58,59]. Activated macrophages show greater phagocytic activity and an elevated secretion of various anti-pathogen mediators such as cytokines, reactive oxygen species and nitrogen intermediates [58,60,61]. In the present study, we used RAW 264.7 macrophages as a cell model and demonstrated that CCL-34 treatment stimulated cytokine production (ex: TNF-α, IL-6 and GM-CSF) and expression of iNOS (a crucial mediator in NO production), induced a characteristic morphology similar to mature macrophages, and promoted phagocytosis (Figs. 4–6). These results suggest that CCL-34 can induce macrophage activation. Activated macrophages are considered to be potent effector cells exhibiting tumor-killing properties [61,62] and TLR4 agonists, such as OK-432 and synthetic lipid A analogs, have been shown to be immune-promoting adjuvants that have clinical application [18,31]. In the present study, we have demonstrated that CCL-34 can activate human TLR4 (Fig. 2D and E). Moreover, the fact that CCL-34 does not induce IL-1β secretion is beneficial, because compounds that activate TLR4 without inducing IL-1β secretion (such as MPL) have previously been shown to be less toxic and considered as safer and useful adjuvant [57]. The therapeutic potential of CCL-34, as an adjuvant for fighting immune diseases and cancer, remains to be further explored.

Despite many components isolated from natural sources (including plant-derived proteins, polysaccharides and small molecule compounds) have been found to act as TLR4 activators, several limiting factors hinder these components in terms of drug development: (1) during the preparation and storage process, the components may have contamination

with microbial-derived immunostimulatory molecules, such as LPS; (2) the resources for producing and isolating these components are limiting; (3) the structures of these components are complicated and not well-uncharacterized. In the present study we confirmed that the results obtained with CCL-34 are not due to contamination with LPS or bacterial lipoproteins. Indeed, bacterial contamination is very rarely a problem with chemically synthetic compounds. Furthermore, the clear structure and well-established synthesis protocol for CCL-34 make it a more favorable molecule than components identified from natural sources. In addition, the structure of CCL-34 is less complicated than most currently available synthetic TLR4 agonists-lipid A mimics, and thus this compound is considerably easier to be chemically synthesized. Based on the above observations, we think that CCL-34 is an appealing lead compound among currently available TLR4 activators. Certainly, more *in vivo* and *in vitro* experiments are needed to fully measure the therapeutic potential and potential toxicity of CCL-34. Further chemical modification of CCL-34 might also be necessary to enhance efficacy, improve stability and reduce any toxicity of this compound.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.03.006.

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