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Degraded λ-carrageenan activates NF-κB and AP-1 pathways in macrophages and enhances LPS-induced TNF-α secretion through AP-1

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ABSTRACT

Background: Carrageenan (CGN), a high molecular weight sulfated polysaccharide, is a traditional ingredient used in food industry. Its degraded forms have been identified as potential carcinogens, although the mechanism remains unclear.
Methods: The effects of degraded λ-carrageenan (λ-dCGN) on murine RAW264.7 cells and human THP-1-derived macrophage cells were investigated by studying its actions on tumor necrosis factor alpha (TNF-α) secretion, Toll-like receptor 4 (TLR4) expression, and activation of nuclear factor-κb (NF-κB) and activation protein-1 (AP-1) pathways.
Results: We found that λ-dCGN was much stronger than native λ-CGN in the activation of macrophages to secrete TNF-α. Treatment of RAW264.7 cells with λ-dCGN resulted in the upregulation of TLR4, CD14 and MD-2 expressions, but it did not increase the binding of lipopolysacchride (LPS) with macrophages. Meanwhile, λ-dCGN treatment activated NF-κB via B-cell lymphoma/leukemia 10 (Bcl10) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) phosphorylation. In addition, λ-dCGN induced extracellular signal-regulated kinases/1/2/mitogen-activated protein kinases (ERK1/2/MAPK) and AP-1 activation. Interestingly, pretreatment of RAW264.7 cells with λ-dCGN markedly enhanced LPS-stimulated TNF-α secretion. This pretreatment resulted in the enhanced phosphorylation of ERK1/2 and c-Jun N-terminal kinase (JNK) and intensified activation of AP-1.
Conclusions: λ-dCGN induced an inflammatory reaction via both NF-κB and AP-1, and enhanced the inflammatory effect of LPS through AP-1 activation.
General significance: The study demonstrated the role of λ-dCGN to induce the inflammatory reaction and to aggravate the effect of LPS on macrophages, suggesting that λ-dCGN produced during food processing and gastric digestion may be a safety concern.

1. Introduction

Carrageenan (CGN), a sulfated polysaccharide, has been a widely used common food additive as a stabilizer, emulsifier, or thickener. However, there is an intense controversy on its safety. Although the US Food and Drug Administration (FDA) has declared food grade CGN as Generally Recognized As Safe (GRAS) to use as a food ingredient [1], and its safety for adults has also been assured by the Joint FAO/EHO Expert Committee on Food Additives (JECFA) and the National Organic Standards Board (NOSB), there are investigations suggesting that CGN poses a carcinogenic risk to humans [2–4]. Although the controversy is fierce, there is a consensus that degraded CGN (dCGN) has carcinogenic properties in animal models. There are several lines of research evidence sufficiently demonstrating that dCGNs induce colorectal tumors and ulcerative colitis in rats [5], guinea pigs [6], rabbits [7], mice [8] and rhesus monkeys [9]. These studies were extensively performed on animal models, but there were only a few on human cells. Recent studies demonstrated that CGN up-regulated interleukin-8 (IL-8) secretion through Toll-like receptor 4 (TLR4) and activated nuclear factor-κb (NF-κB) in human colonic epithelial cells [10,11]. Benard et al. observed that dCGN induced tumor necrosis factor alpha (TNF-α) production through NF-κB activation in human monocytes [12]. Although secretion of inflammatory cytokines in human cells was increased following CGN or dCGN exposure, the amount of increase was comparatively small, as compared to that observed in animal models. We proposed that the inflammatory response induced by dCGN might be enhanced by some factors such as bacterial lipopolysaccharide (LPS), a major constituent of the outer membrane of the cell wall of all Gram-negative bacteria and one of the most potent inducers of

Abbreviations: CGN, Carrageenan; λ-dCGN, Degraded λ-carrageenan; JECFA, Joint FAO/EHO Expert Committee on Food Additives; NOSB, National Organic Standards Board; CCTCC, China Center for Type Culture Collection

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inflammation [13]. We hypothesized that the inflammatory effect of dCGN in animal models might be partly due to the enhanced effects of LPS by dCGN.

Macrophages are often the first host immune cells that are recruited to the inflammatory site, and are the main source of pro-inflammatory cytokines after activation [14]. Lambda carrageenan (λ-CGN) has been shown to be the most potent CGN of all the CGN forms to cause inflammation [2]. Although dCGN is regarded to have inflammation-promoting activity and carcinogenic potential, there have been no studies on macrophage responses to dCGN exposure. Therefore, we investigated the effect and mechanism of macrophage response to λ-dCGN exposure using both murine-derived macrophage cells, RAW264.7 cells, and human phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 macrophages. Our results showed that λ-dCGN enhanced the inflammatory responses induced by LPS.

2. Materials and methods

2.1. Preparation of degraded CGN

The commercial λ-CGN (Sigma-Aldrich, St. Louis, MO, USA) was re-purified by precipitation with 0.2 mol/l KCl and the soluble fraction was freeze dried to obtain re-purified λ-CGN, which was dissolved in distilled water (1% w/v) with vigorous stirring and adjusted to pH 1.9 using HCl, and then hydrolyzed for 4 h at 37 °C. After being neutralized with NaOH, the solution was filtered through ultra membrane filter with MW cut-off of 50 kDa (Millipore, Bedford, MA, USA) [12], and then the flow-through was ultra filtered again with MW cut-off of 10 kDa to obtain the λ-dCGN with MW cut-off of 10–40 kDa.

2.2. Cell culture

RAW264.7 (murine macrophage cell line) cells were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China) and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) at 37 °C in a 5% CO_2-humidified incubator. In the majority of experiments, cells were plated at a density of 2×10^5 cells/ml and at 60–70% confluence, cells were treated with λ-CGN, λ-dCGN or LPS (from *Escherichia coli* 026:B6) (Sigma-Aldrich) at the indicated concentrations and time periods.

THP-1 (a human acute monocytic leukemia cell line) cells (CCTCC) were maintained in RPMI-1640 supplemented with 10% (v/v) FBS and 0.05 mmol/l 2-mercaptoethanol at 37 °C in a humidified, and 5% CO_2 environment. For experiments, THP-1 cells (5×10^5/ml) were differentiated using 160 nmol/l PMA for 36 h. Differentiation of PMA-treated cells was enhanced by removing the PMA-containing media and the cells were incubated in FBS free, fresh RPMI 1640 for a further 12 h, and then stimulated with λ-CGN, λ-dCGN or LPS at the indicated concentrations and time periods.

2.3. Measurement of TNF-α secretion by ELISA

The secretion of TNF-α was detected by ELISA. RAW264.7 or THP-1 cells were cultured with or without 10 μg/ml λ-CGN and λ-dCGN or the indicated concentrations of λ-dCGN for 24 h or pretreated with the indicated concentrations of λ-dCGN for 1 h and then stimulated with LPS (0.01 μg/ml) for 24 h. In other experiments, RAW264.7 cells were pretreated with 1 μg/ml mouse anti-TLR4 antibody, anti-CD14 antibody (eBioscience Inc., San Diego, CA, USA), 15 μM PD98059, 15 μM BAY 11-7082 and 30 μM PDTC (Sigma-Aldrich) for 1 h, and then stimulated with 10 μg/ml λ-dCGN for 24 h. The expression of TNF-α in RAW264.7 and THP-1 cell culture supernatant was measured with the Mouse TNF-α Immunoassay Quantikine ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) and Human TNF-α ELISA kit (Boster, Wuhan, China), according to the manufacturers' instructions, respectively.

Six cytokines including IL-12p70, TNF-α, IL-10, IL-6, IL-1β and IL-8 in the culture media of RAW264.7 cells treated with 10 μg/ml λ-dCGN or/and 0.01 μg/ml LPS were also determined by flow cytometry using the Mouse Inflammation Cytometric Bead Array (CBA) according to the instruction of the manufacturer (BD Biosciences, San Jose, CA, USA). Cytokine levels were measured on a FACSCalibur flow cytometer (BD Biosciences Pharmingen).

2.4. Flow cytometry analysis

The cell surface expression of TLR4 was detected by flow cytometry. RAW264.7 cells were treated with the indicated concentrations of λ-dCGN for 24 h, then harvested with trypsin–EDTA and incubated with phycoerythrin-labeled antibody against mouse TLR4. After 1 h incubation on ice, cells were centrifuged and washed twice in ice-cold PBS, and then analyzed by using a FACSCalibur flow cytometer. The data were presented as median fluorescence intensity (MFI) in each group.

The effect of λ-dCGN on the binding of LPS on the RAW264.7 cells was also detected by flow cytometry. RAW264.7 cells were treated with the indicated concentrations of λ-dCGN at 37 °C for 24 h, followed by harvesting with trypsin–EDTA, or RAW264.7 cells were harvested with trypsin–EDTA first, followed by treatment with the indicated concentrations of LPS or λ-dCGN at 37 °C for 1 h. The treated cells were then incubated with FITC-LPS on ice for 1 h, centrifuged and washed twice in ice-cold PBS, and finally analyzed on a flow cytometer.

2.5. Real-time quantitative RT-PCR

RAW264.7 cells were cultured in the presence of the indicated concentrations of λ-dCGN for 24 h or pretreated with 10 μg/ml λ-dCGN for 1 h and then stimulated with LPS (0.01 μg/ml) for 24 h. Cells were harvested and total RNA was isolated with TaKaRa RNAiso Plus Reagent (TaKaRa, Dalian, China) according to the manufacturer's protocol. 2 μg of total RNA was employed to synthesize the first strand of cDNA in a 20 μl of reverse transcription (RT) reaction, and 2 μl of RT product was used in PCR amplification using the Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) and the SYBR-Green I monitoring method. Four pairs of specific primers were used in amplification, MD-2-F: CGCTGCTTTCTCCCATA, MD-2-R: TACGCTTCGGCAACTC TA; CD14-F: TTACAACAGGCTGGATAGGA, CD14-R: CTCAGAAACCAGGA GGATG; TNF-α-F: TTGACCTCAGCGCTGAGTTG, TNF-α-R: CCTGTAGCCC ACGTCGTAGC; β-actin-F: TGGAATCCTGTGGCATCCATGAAAC, β-actin-R: TAAAACGCAGCACAGTAACAGTCCG. The fragment of β-actin served as the internal control for qRT-PCR. The concentration of cDNA in each sample was reflected by the threshold cycle (Ct) value, which was compared using the relative quantification method. The relative mRNA expression of each target gene was normalized to that of β-actin.

2.6. Preparation of nuclear extracts and whole cell lysates

RAW264.7 cells were stimulated with 2, 10, 25 and 50 μg/ml λ-dCGN for 24 h or pretreated with 10 μg/ml λ-dCGN for 1 h and then stimulated with LPS (0.01 μg/ml) for 24 h. In other experiments, RAW264.7 cells were pretreated with antibody against mouse TLR4 for 2 h, and then stimulated with 0.01 μg/ml LPS and 10 μg/ml λ-dCGN for 24 h. Nuclear extracts were prepared using the NE-PER nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL, USA) according to manufacturer's protocol. To prepare the whole cell lysate, cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in lysis buffer (Beyotime, Shanghai, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protein phosphatase inhibitor cocktail (Roche, Basel, Switzerland) for 10 min on ice. The homogenates were centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentration was determined using the Bio-Rad DC Protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instruction.

2.7. Western blot analysis

Proteins (30 μg) in the nuclear extracts or in the whole cell lysate were separated by 10% sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and probed with antibodies against TLR4, Bcl10, NF-κB p65, phospho-ERK, phospho-P38, phospho-JNK and c-Jun antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-IκBα, phospho-c-Jun Ser63, and phospho-c-Jun Ser73 (Cell Signaling Technology (CST), Danvers, MA, USA), followed by appropriate horseradish peroxidase (HRP)-linked secondary antibodies (CST). The immunoreactive proteins were detected by WesternBright ECL (Advansta Inc., Menlo Park, CA, USA). The results were quantified by measuring the band intensity and compared to the band intensity of β-actin using AlphaView™ Software (Alpha Innotech, San Leandro, CA, USA) and represented as the relative intensities.

2.8. Nuclear extraction and electrophoretic mobility shift assay (EMSA)

For detection of NF-κB and AP-1 DNA-binding activity, EMSA was performed. Binding reactions containing 10 mM Tris (pH 7.5), 100 mmol/l KCl, 1 mmol/l DTT, 50 ng/μl poly(dI/dC), 2.5% glycerol, 0.05% NP-40, 2.5 mmol/l $MgCl_2$, 1 mmol/l EDTA and 3 μg of nuclear extracts were incubated for 20 min at room temperature with 400 fmol of biotin-labeled double-stranded NF-κB oligonucleotide or AP-1 oligonucleotide in a total volume of 20 μl. The NF-κB consensus oligo was 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and the AP-1 consensus oligo was 5′-CGCTTGATGACTCAGCCGGAA-3′. The bound mixtures were electrophoresed on 5% native polyacrylamide gels in 0.5× Tris–boric acid–EDTA buffer (TBE, 45 mmol/l Tris, 45 mmol/l boric acid and 1 mmol/l EDTA, pH 8.3). After blotting onto a positively charged nylon membrane, the membrane was cross-linked, and the biotin-labeled DNA was detected by LightShift Chemiluminescent EMSA Kit (Pierce), according to the manufacturer's instructions.

2.9. Transient transfection and luciferase reporter assays

To examine the effects of λ-dCGN on NF-κB and AP-1 activation, RAW264.7 cells were transiently co-transfected with 1 μg of firefly luciferase reporter plasmid p-NF-κB-Luc or p-AP1-Luc (Clontech Laboratories, Palo Alto, CA, USA) and 0.1 μg of p-RL, respectively. Transfection was performed using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions. At 24 h after transfection, fresh FBS-free medium was added, then cells were treated with λ-dCGN for 24 h or treated with λ-dCGN for 1 h and then stimulated with LPS (0.01 μg/ml) for a further 24 h. Firefly and *Renilla* luciferase activities were measured in cell lysates using the Dual-Glo® Luciferase Assay System (Promega Corp., Madison, WI, USA). All experiments were repeated three times and the luciferase activity was calculated and normalized using *Renilla* luciferase activity.

2.10. Statistical analysis

Data were expressed as the mean value ± standard deviation (SD) of three independent experiments with technical replicates of each measurement. Statistical analysis was assessed by one-way ANOVA. P value < 0.05 was considered statistically significant.

3. Results

3.1. CGN induced-TNF-α secretion from macrophages

We compared TNF-α production after exposure to native λ-CGN and λ-dCGN. A lower level of TNF-α was induced in macrophages following exposure to native λ-CGN (Fig. 1(A)). In THP-1-derived macrophage cells, secretion of TNF-α increased 1.2-fold after λ-CGN exposure and 2.2-fold following λ-dCGN exposure. In RAW264.7 cells, TNF-α secretion increased 2.7-fold after λ-CGN exposure and 5.1-fold following λ-dCGN exposure. The results indicated that TNF-α secretion was more strongly stimulated by λ-dCGN than by λ-CGN.

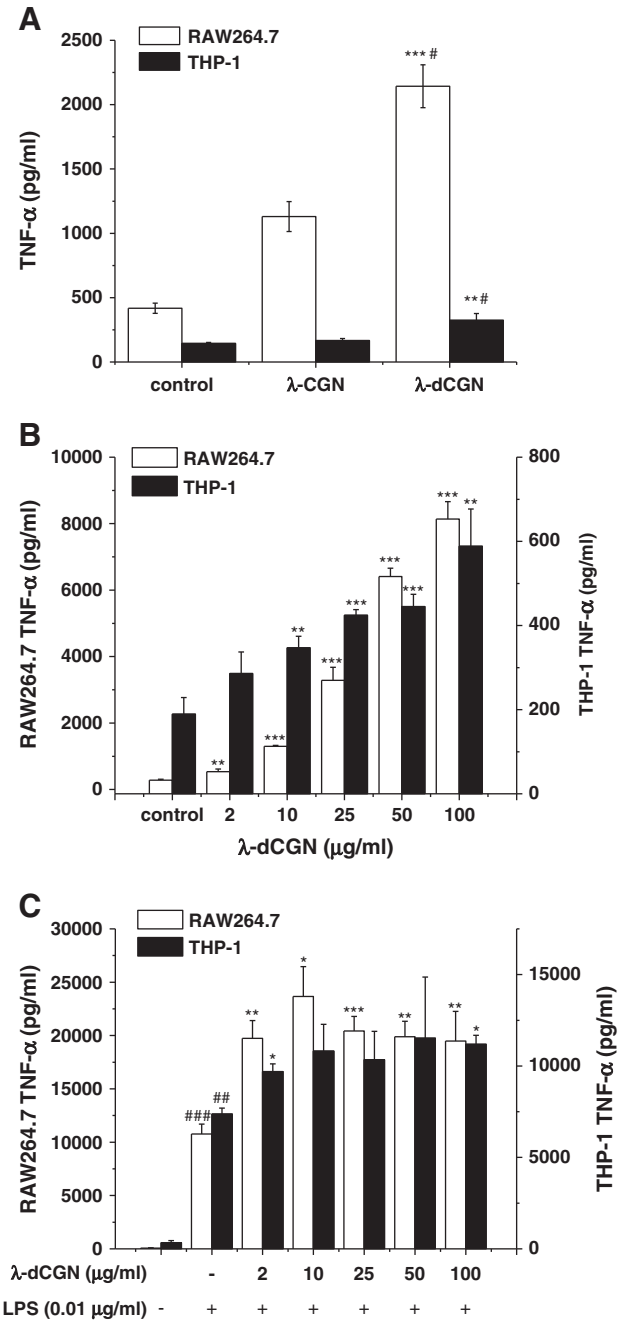

Fig. 1. Carrageenan induces increased TNF-α production in RAW264.7 and THP-1 cells. (A) TNF-α release induced by 10 μg/ml of λ-CGN or λ-dCGN for 24 h. (B) RAW264.7 and THP-1 cells were treated with the indicated concentrations of λ-dCGN for 24 h. Data represent mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (n = 3) compared with the control; $^{\#}P < 0.05$ (n = 3) compared with the λ-CGN-treated. (C) RAW264.7 and THP-1 cells were pretreated with indicated concentrations of λ-dCGN for 1 h and further stimulated for 24 h with LPS (0.01 μg/ml). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ (n = 3) compared with the control; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (n = 3) compared with the LPS treated alone.

As shown in Fig. 1(B), TNF-α production rose linearly in response to increasing λ-dCGN concentrations in both RAW264.7 cells and THP-1-derived macrophage cells. However, the increase of TNF-α production in RAW264.7 cells was significantly higher than that in THP-1 derived macrophage cells. In response to 100 μg/ml of λ-dCGN, TNF-α secretion

increased 3.1-fold in THP-1-derived macrophage cells but 28.9-fold in RAW264.7 cells.

Compared with λ-dCGN, LPS was more potent in inducing TNF-α production in macrophages (Fig. 1(C)). Treatment with 0.01 μg/ml LPS resulted in approximately a 22.1-fold and a 146.3-fold increase in TNF-α secretion by THP-1-derived macrophage cells and RAW264.7 cells, respectively. Interestingly, pretreatment with increasing concentrations of λ-dCGN increased TNF-α expression induced by LPS in macrophages. Data presented in Fig. 1(C) indicated that 10 μg/ml λ-dCGN pretreatment increased TNF-α secretion by about 1.5-fold and 2.2-fold ($P < 0.05$) in THP-1-derived macrophage cells and RAW264.7 cells. This result suggested that λ-dCGN could enhance LPS-induced TNF-α secretion.

At the meantime, the levels of six cytokines including IL-8 in the culture media after treatment with λ-dCGN and LPS were also determined by flow cytometry using the Mouse Inflammation Cytometric Bead Array (CBA) method. As shown in Table 1, the six cytokines showed the similar trends of change, except for the IL-12p70, whose level was below detection when treated with λ-dCGN, and for the IL-10 level, whose level was lower when treated with LPS than with λ-dCGN. The measured cytokine levels were always higher when treated with both 0.01 μg/ml LPS and 10 μg/ml λ-dCGN, which was consistent with the effect on TNF-α as measured by ELISA.

3.2. λ-dCGN increased TLR4 expression but did not affect the LPS binding

Western blot analysis indicated an increase in the protein levels of TLR4 following λ-dCGN exposure in RAW264.7 cells. Fig. 2(A) showed that λ-dCGN pretreatment slightly enhanced the expression of TLR4 induced by LPS. We also detected the surface expression of TLR4 by FACS. In agreement with the Western blot result, the expression of surface TLR4 was increased in a concentration-dependent manner; surface TLR4 was increased by 18.36% following exposure to 100 μg/ml λ-dCGN (Fig. 2(B), (C)). TLR4 is a known receptor for LPS recognition and signaling. To determine whether the upregulation of surface TLR4 induced by λ-dCGN could enhance the response in LPS-induced RAW264.7 cells, we examined the binding of FITC-LPS to RAW264.7 cells after 24 h treatment with λ-dCGN. We found that λ-dCGN treatment for 24 h did not increase FITC-LPS binding to the cells (Fig. 3(A)). The results suggested that λ-dCGN enhanced TNF-α production in LPS-induced RAW264.7 cells by ways other than enhancing the binding of LPS to TLR4. In addition, we considered the possibility that λ-dCGN might also interact with TLR4, and thus competitively block the binding of FITC-LPS. To test this hypothesis, we first treated the cells with LPS or λ-dCGN for 1 h, and then incubated with FITC-LPS and found that pretreatment with increasing concentrations of LPS decreased the binding of FITC-LPS (Fig. 3(B)), indicating LPS and FITC-LPS bind to the same target on the cells. However, pretreatment with increasing concentrations of λ-dCGN did not affect the binding of FITC-LPS (Fig. 3(C)), indicating that λ-dCGN binds to targets that are different from the LPS-binding site on the cells or λ-dCGN, unlike LPS, which cannot rapidly bind to TLR4.

LPS that triggers the innate immune responses through interacting with TLR4 and other factors, including LBP, CD14 and MD-2, is required for interaction with TLR4. Here, we employed real-time quantitative RT-PCR to examine the effect of λ-dCGN on the expression of CD14 andMD-2 genes at themRNA level, and to detect TNF-α mRNA to verify the ELISA results. Fig. 4 revealed that the changes in TNF-α expression after λ-dCGN and LPS treatments were similar to those detected with ELISA

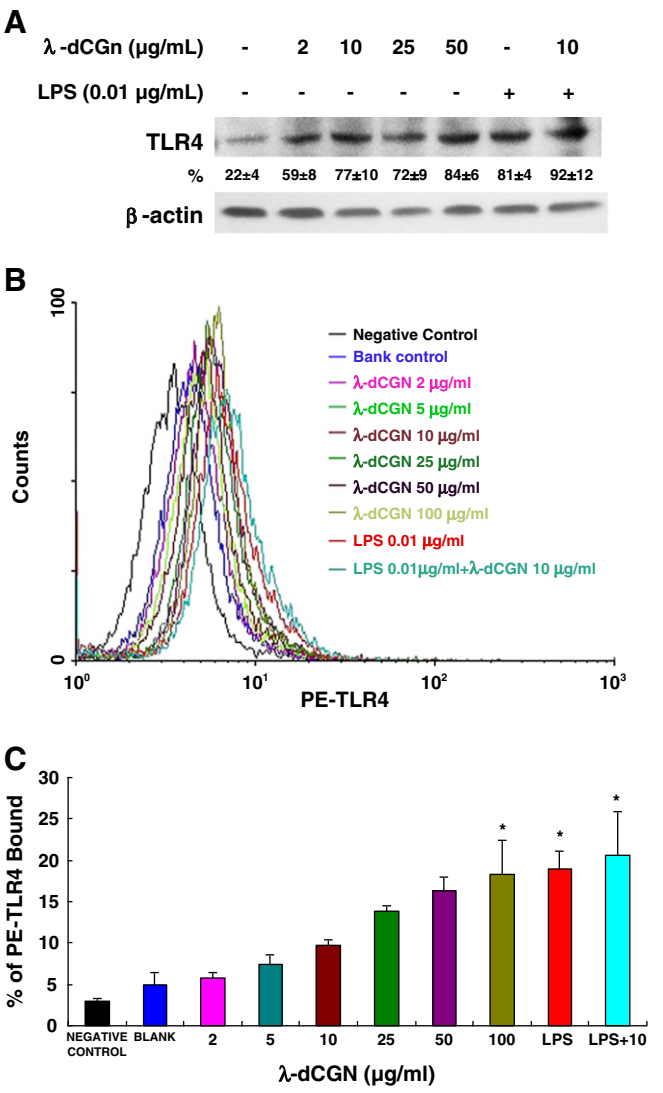

Fig. 2. λ-dCGN increased TLR4 expression in RAW264.7 cells. (A) RAW264.7 cells were treated with different concentrations of λ-dCGN for 24 h or pretreated with λ-dCGN for 1 h and then with LPS (0.01 μg/ml) for 24 h. The whole cell lysates were prepared and analyzed by Western blot using TLR4 antibody. The data were presented as density ratio versus β-actin. (B) RAW264.7 cells were treated with different concentrations of λ-dCGN for 24 h, and then the collected cells were incubated with 1 μg PE-TLR4 for 1 h. Fluorescence intensity was analyzed by flow cytometry. (C) The percentage of fluorescently labeled positive cells is expressed as % of PE-TLR4 bound. Data represent mean ± SD from three independent experiments. $^{*}P < 0.05$ (n = 3) compared with the blank control.

Table 1
Effect of λ-dCGN on secretion of cytokines in RAW264.7 cells (pg/ml).

Sample	IL-12p70	TNF-α	IL-10	IL-6	IL-1β	IL-8
Control	2.4 ± 0.3	109.0 ± 5.2	≤0[a]	4.4 ± 0.4	109.6 ± 10.3	13.4 ± 4.3
λ-dCGN 10 μg/ml	≤0	376.9 ± 13.5*	2.6 ± 0.7	9.6 ± 0.8	321.1 ± 34.6	24.7 ± 6.2
LPS 0.01 μg/ml	1.8 ± 0.2	9121.8 ± 37.5**	1.3 ± 0.3	56.8 ± 6.3**	837.9 ± 19.7**	161.4 ± 10.8**
LPS + λ-dCGN	3.2 ± 0.3	15,447.0 ± 53.2**,#	3.3 ± 0.6	129.3 ± 20.3**,#	1901.3 ± 62.9**,#	173.3 ± 21.3**

[a] Indicates lower than limit of detection.
* $P < 0.05$ compared with those in the control group.
** $P < 0.01$ compared with those in the control group.
# $P < 0.05$ compared with those in the LPS treated group.

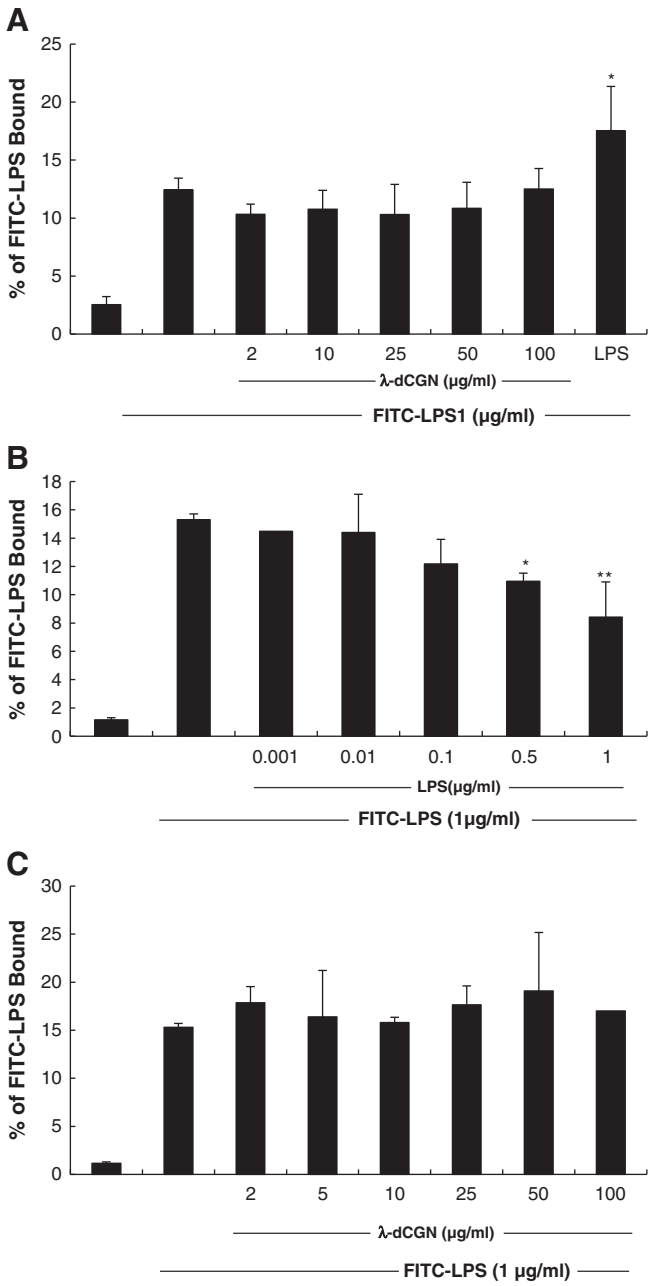

Fig. 3. Effect of λ-dCGN on the binding of LPS to cells. RAW264.7 cells were treated with (A) indicated concentrations of λ-dCGN for 24 h; (B) the indicated concentrations of LPS for 1 h, or (C) the indicated concentrations of λ-dCGN for 1 h, and then after cells were washed with PBS, and then 1 μg/ml FITC-LPS was added for 1 h. Fluorescence intensity was analyzed by flow cytometry. Data represent mean ± SD from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ (n = 3) compared with those in the blank control.

results. CD14 and MD-2 were also up-regulated by treatment with λ-dCGN in a concentration-dependent manner. LPS significantly increased the expression of MD-2 in RAW264.7 cells, but CD14 was only 1.81-fold higher than that of the control. However, no enhanced expression of CD14 and MD-2 was detected with λ-dCGN pretreatment, and their mRNA levels even decreased only slightly.

3.3. NF-κB activation via IκBα phosphorylation was required for λ-dCGN induced TNF-α secretion

NF-κB is one of the most important transcription factors that regulate the expression of numerous pro-inflammatory cytokines. Phosphorylation of IκB leads to release and activation of NF-κB. We examined whether λ-dCGN induces NF-κB activation. As shown in Fig. 5(A), exposure to λ-dCGN increased Bcl10 levels in RAW264.7 cells, and λ-dCGN treatment increased phosphorylation of IκBα in a concentration-dependent manner. We found a significant increase in the nuclear levels of NF-κB following λ-dCGN exposure, suggesting that λ-dCGN induces NF-κB translocation to the nucleus via phosphorylation of IκBα. These results were further confirmed by assaying the DNA binding activity and transcription activity of NF-κB using EMSA and reporter plasmid, respectively. EMSA showed that λ-dCGN treatment enhanced NF-κB DNA binding activity in a concentration-dependent manner (Fig. 5(B)). Reporter plasmid analysis showed that NF-κB transcription activity was increased linearly with increasing concentrations of λ-dCGN (Fig. 5(C)). These results strongly indicate that λ-dCGN induces NF-κB activation via IκBα phosphorylation.

In addition, pretreatment with λ-dCGN markedly increased LPS-stimulated phosphorylation of IκBα. We also found that λ-dCGN slightly increased LPS-induced nuclear levels of p65 subunit of NF-κB. However, no enhancement of NF-κB activation was detected with λ-dCGN pretreatment, instead, λ-dCGN pretreatment attenuated the NF-κB activation in LPS-induced cells. These data suggest that λ-dCGN enhances LPS-induced inflammatory response through other pathways.

3.4. Activation of AP-1 was induced by λ-dCGN and was required in enhancing LPS response in RAW264.7 cells

In addition to the NF-κB signaling pathway, the MAP kinase signaling pathway is also activated by many stimuli including LPS. Thus, we investigated the effect of λ-dCGN on the MAPK pathway. Western blot analysis showed a concentration-dependent increase in phosphorylation of ERK1/2 but changes in p38 and JNK were not detected (Fig. 6(A)), suggesting that MAPK/ERK may also play an important role in TNF-α secretion stimulated by λ-dCGN. We explored the activation of AP-1 following λ-dCGN exposure. The results showed that λ-dCGN treatment concentration-dependently increased the DNA binding activity and transcription activity of AP-1 (Fig. 6(B) and (C)), further confirming that activation of AP-1 is involved in λ-dCGN-induced response.

In addition, pretreatment with λ-dCGN enhanced the phosphorylation of ERK1/2 in LPS-stimulated RAW264.7 cells. Interestingly, it was found that λ-dCGN pretreatment increased LPS-induced phosphorylation of JNK and c-Jun (at Ser 63 and Ser 73), but treatment with λ-dCGN alone had little effect on the phosphorylation of JNK and c-Jun (at Ser 63 and Ser 73). However, the expression level of p-P38 was not up-regulated after treatment of cells with dCGN. dCGN did not enhance LPS-induced p-P38 phosphorylation. A low level of AP-1 DNA binding activity was detected in the nuclear extracts from LPS-treated cells, whereas λ-dCGN pretreatment enhanced AP-1 DNA binding of LPS-stimulated cells (Fig. 6(B)). Consistent with previous finding, reporter plasmid analysis showed that pretreatment with λ-dCGN was able to potentiate LPS-induced transcription activity of AP-1 in RAW264.7 cells (Fig. 6(C)). These results indicate that λ-dCGN may enhance LPS-stimulated inflammatory response through activation of AP-1 in RAW264.7 cells.

3.5. Inhibition of several factors reduced the TNF-α secretion induced by λ-dCGN

RAW264.7 cells were pretreated with anti-TLR4 antibody, anti-CD14 antibody, PD98059 (a selective inhibitor of ERK), BAY11-7082 (an IκB-α phosphorylation inhibitor) and PDTC (an NF-κB activation inhibitor), respectively, and the secretion of TNF-α induced by λ-dCGN was measured (Fig. 7). After these treatments, λ-dCGN-induced TNF-α secretion was reduced, which was basically consistent with the results detected with Western blots. We found that treatment with PD98059 or BAY11-7082 significantly decreased the secretion of TNF-α, with inhibition rates of 93% and 95%, respectively ($P < 0.01$). These results indicate

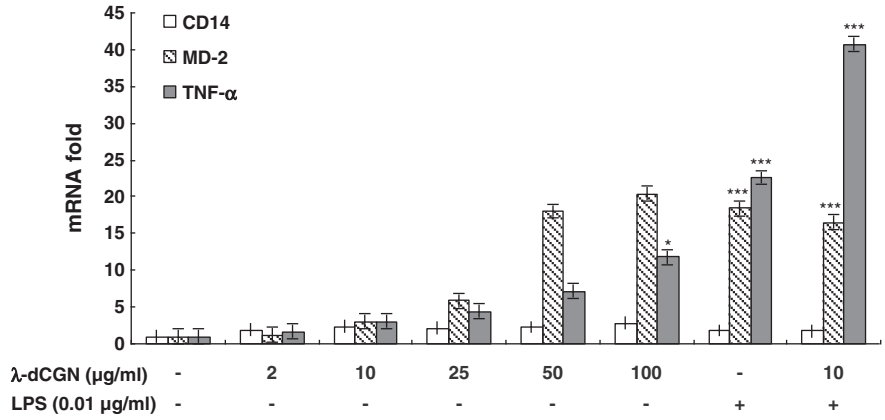

Fig. 4. Effect of λ-dCGN and LPS on mRNA expression of CD14, MD-2 and TNF-α. RAW264.7 were cultured with the indicated concentrations of λ-dCGN for 24 h or pretreated with 10 μg/ml λ-dCGN for 1 h and then stimulated with LPS (0.01 μg/ml) for 24 h. Real-time quantitative RT-PCR was used to measure mRNA expression of CD14, MD-2 and TNF-α. Data represent mean ± SD from three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ (n = 3) compared with those in the blank control.

that λ-dCGN-induced TNF-α secretion is related to the activation of the ERK and the NF-κB pathways. In addition, treatments with anti-TLR4 antibody and anti-CD14 antibody alleviated the λ-dCGN-induced TNF-α secretion, suggesting that both TLR4 and CD14 are involved in the action of λ-dCGN.

4. Discussion

A common feature of the intestinal inflammation is macrophage infiltration and activation [15], which generally results in the secretion of proinflammatory cytokines. The recognized inflammation inducers such as LPS from Gram-negative bacteria can induce the production of these cytokines. In addition, CGN, a common food additive, can also stimulate the production of these cytokines and has been shown to induce ulcerations associated with macrophage infiltration in the animal models [16,17]. Food grade CGN (an undegraded CGN) is considered safe for adults by the FDA and JECFA, but food grade CGN may be contaminated with small amounts of degraded CGN (dCGN), and substantial amounts of dCGN are likely produced in the gastrointestinal tract [18,19]. The recently reported difference in the safety between CGN and dCGN may associate with the difference in their structures. dCGN is a carrageenan that has a molecular weight of 10–40 kDa, whereas the molecular weight of undegraded CGN is >100 kDa [2]. Because of the different molecular weights, viscosity, solubility, and even conformation of dCGN and CGN vary. Undegraded CGN is insoluble in water, but dCGN has good water solubility. Therefore, the ways they contact with, bind to, and enter into the cells are different, which has most likely made the difference in their induced response and signal transduction of macrophages. Therefore, the present study mainly focused on the effect of degraded λ-carrageenan on the inflammatory pathways of macrophage, in order to understand the possible mechanism of dCGN induction of colorectal tumors and ulcerative colitis.

Both λ-CGN and λ-dCGN showed no cytotoxicity to macrophages with concentrations ranging from 2 to 200 μg/ml (data not shown). However, we observed that they induced an increase in TNF-α production in macrophages. Unsurprisingly, λ-dCGN was much stronger than λ-CGN in inducing TNF-α secretion in both RAW264.7 cells and THP-1-derived macrophage cells. Our results support the opinion that dCGN present in undegraded CGN may be associated with the occurrence of intestinal ulcerations and neoplasms [2].

LPS is recognized as a highly proinflammatory molecule and a strong inducer of TNF-α secretion in macrophages. Compared to the LPS-induced increase in TNF-α secretion, exposure of macrophages to λ-dCGN caused a smaller but significant increase in TNF-α secretion, and the amount of TNF-α induced with λ-dCGN appeared to be high enough to trigger a strong inflammatory response. Since the inflammatory property of dCGN is recognized in animal models, we suspected that the inflammatory effect induced by degraded CGN might be due to enhancement of other factors, such as the intestinal microflora or the invading bacteria. We found a strong increase in TNF-α secretion in LPS-induced macrophages pretreated with λ-dCGN, and the level of TNF-α was significantly higher than that produced by λ-dCGN alone and that produced by LPS alone. The result is consistent with a previous report that CGN pre-injection in mice caused a 200-fold increase in LPS-induced TNF-α secretion although CGN by itself did not induce TNF-α secretion [20]. In another report, CGN pretreatment enhanced LPS-induced TNF-α production and increased the mortality rate in mice [21]. Our results demonstrated that dCGN synergistically increased LPS-stimulated TNF-α secretion.

Toll-like receptors (TLRs) are the phylogenetically conserved mediators that are integral to both innate immunity and adaptive immunity. TLRs activate a common signaling pathway that culminates in the activation of a number of transcription factors including the NF-κB, MAPKs and extracellular signal-regulated kinases. Bacterial LPS is a ligand recognized by TLR4. Ligation of LPS to TLR4 results in signaling activation via downstream signaling molecules and elicits a multitude of effects on the immune system. Bcl-10, an intracellular NF-κB activator, is an important signaling molecule that mediates signaling in the TLR4 pathway in the innate immune system. Several reports have indicated that Bcl-10 appears to participate consistently in a TLR4-mediated NF-κB activation pathway in response to LPS stimulation in immune cells, as well as in human intestinal epithelial cells (IECs) [22–24]. It was also found that macrophage cells or IECs treated with LPS and Bcl-10 were upregulated and recruited to TLR4 signaling complexes. Our results indicated that treatment of RAW264.7 with LPS unregulated Bcl-10 expression, which is consistent with the published results. It has been reported that CGN triggers activation of innate immune pathway of inflammatory response, which was also mediated by TLR4 and Bcl10 in human colonic epithelial cells [10,11]. Using Western blot assay and FACS, we found that λ-dCGN increased the expression of TLR4 and Bcl-10 in RAW264.7 cells. The λ-dCGN-induced TNF-α secretion was inhibited by TLR4 antibody, suggesting that TLR4 is involved in the λ-dCGN-stimulated response in macrophages. Moreover, we found that λ-dCGN promoted the increased expression of TLR4 induced by LPS. However, we found that pretreatment with λ-dCGN did not enhance the expression of Bcl-10 induced by LPS. On the contrary, it decreased the expression of Bcl-10 to a level lower than that in the control. Although the effects of λ-dCGN on RAW264.7 cells were similar to that on the TLR4 pathway by CGN in IECs [10] and by LPS in immune cells [23], our competition study with FITC-LPS and λ-dCGN revealed that λ-dCGN, unlike LPS, cannot rapidly bind to TLR4 or bind to the target that is different from LPS-binding site. This is inconsistent with the observation of Bhattacharyya

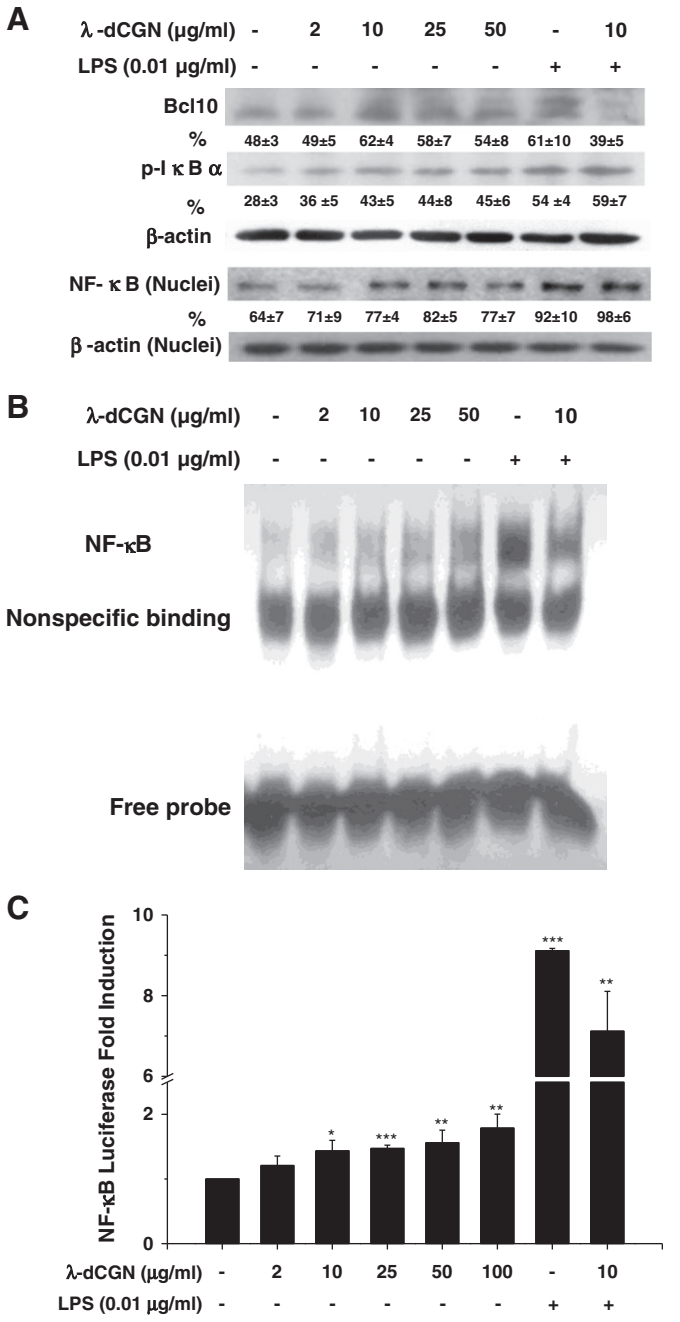

Fig. 5. λ-dCGN induced activation of NF-κB. (A) RAW264.7 cells were stimulated with the indicated concentrations of λ-dCGN for 24 h or pretreated with λ-dCGN for 1 h and then cultured with LPS (0.01 μg/ml) for 24 h. Cell lysates were prepared and the total proteins and nuclear proteins were analyzed by Western blot to analyze the protein levels of Bcl10, p-IκBα and NF-κB p65. (B) Nuclear proteins were prepared from unstimulated cells or cells stimulated with λ-dCGN for 24 h or pretreated with the indicated concentrations of λ-dCGN for 1 h and then with LPS for 24 h, then EMSA analyses were performed to examine the DNA binding activities of NF-κB. The results shown are representative of three individual experiments. (C) RAW264.7 cells were transiently transfected with p-NF-κB-Luc reporter plasmid, then treated with λ-dCGN for 24 h or pretreated with different concentrations of λ-dCGN for 1 h prior to 24 h LPS (0.01 μg/ml) treatment. Firefly and *Renilla* luciferase activities were detected; luciferase fold induction was calculated from the ratio to control by normalizing to *Renilla* luciferase activities. Data represent mean ± SD from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (n = 3) compared with those in the blank control.

et al., who used fluorescence-tagged CGN and proved that TLR4 is the surface membrane receptor for CGN [10], which may imply a difference in binding manner and sites between CGN and dCGN.

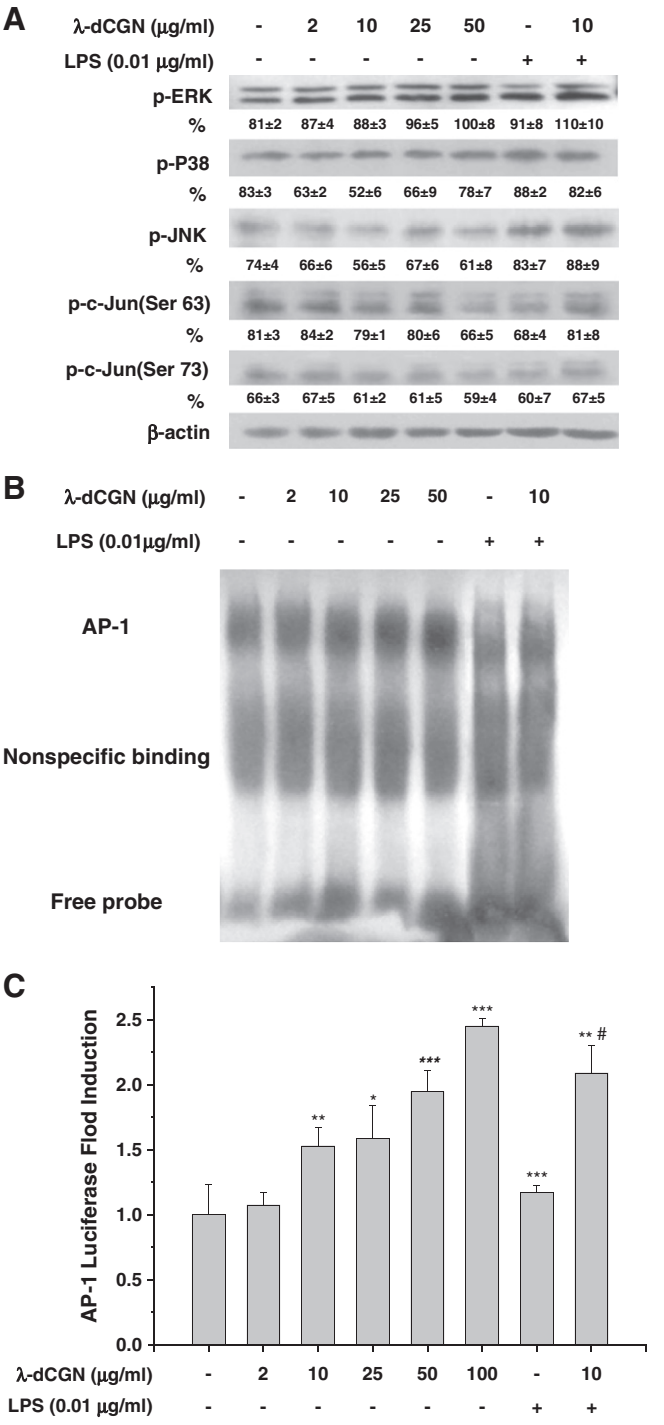

Fig. 6. λ-dCGN induced activation of AP-1 and enhanced LPS-induced inflammatory response through AP-1. (A) RAW264.7 cells were stimulated with the indicated concentrations of λ-dCGN for 24 h or pretreated with λ-dCGN for 1 h and then cultured with LPS (0.01 μg/ml) for 24 h. The levels of p-ERK, p-P38, p-JNK, p-c-Jun (Ser63) and p-c-Jun (Ser73) in the total proteins were detected by Western blot analysis. Data were presented as density ratio versus β-actin. (B) Nuclear proteins were prepared from unstimulated cells or cells stimulated with λ-dCGN for 24 h or pretreated with different concentrations of λ-dCGN for 1 h and then with LPS for 24 h, then EMSA analyses were performed to examine the DNA binding activities of AP-1. The results shown are representative of three individual experiments. (C) RAW264.7 cells were transiently transfected with p-AP-1-Luc reporter plasmid, then treated with λ-dCGN for 24 h or pretreated with the indicated concentrations of λ-dCGN for 1 h prior to the 24 h LPS (0.01 μg/ml) treatment. Firefly and *Renilla* luciferase activities were detected; luciferase fold induction was calculated from the ratio to the control by normalizing to *Renilla* luciferase activities. Data represent mean ± SD from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (n = 3) compared with those in the blank control. $^{\#}P < 0.05$ (n = 3) compared with those in the LPS-treated alone.

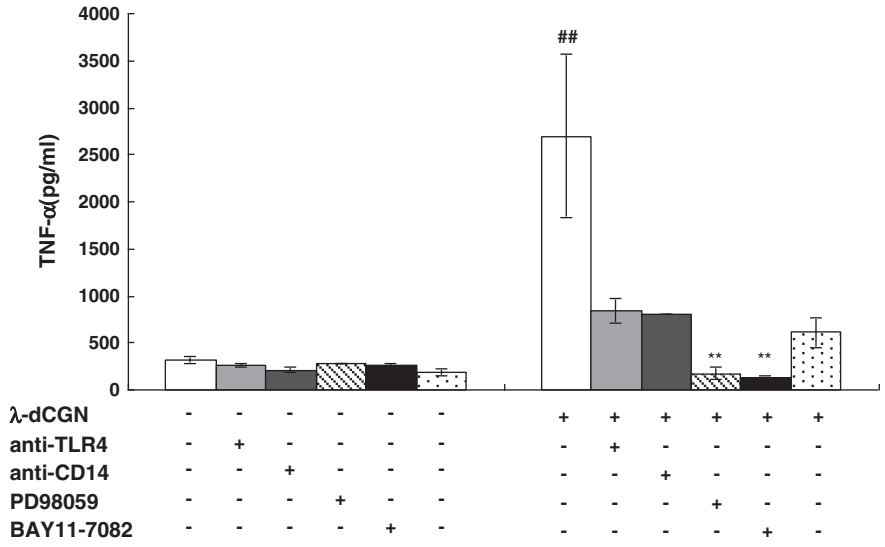

Fig. 7. Inhibition of several factors reduced the TNF-α secretion induced by λ-dCGN. RAW264.7 cells were pretreated with 1 μg/ml mouse anti-TLR4 antibody, 15 μM PD98059, 15 μM BAY 11-7082 and 30 μM PDTC for 1 h, and then 10 μg/ml λ-dCGN was added and incubated for 24 h. The expression of TNF-α was measured by ELISA assay. $^{\#\#}P < 0.05$ compared with the λ-dCGN untreated group; $^{**}P < 0.01$ compared with that in the group treated with the λ-dCGN alone.

NF-κB plays a central role in inflammatory response through induction of proinflammatory cytokines [25]. Previous reports showed that CGN-induced inflammatory response in human colonic epithelial cells was associated with activation of NF-κB, including canonical and non-canonical pathways [11,26]. In canonical NF-κB pathway, NF-κB activation was associated with the phosphorylation and degradation of IκBα [27]. Indeed, λ-dCGN exposure of RAW264.7 cells increased phosphorylation of IκBα and induced NF-κB activation evidenced by increased nuclear level of p65 subunit, enhanced DNA binding activity and increased transcription activity of NF-κB in a concentration-dependent manner. These results have demonstrated that NF-κB activation via IκBα phosphorylation is required for λ-dCGN-induced TNF-α secretion.

In addition to NF-κB signaling pathway, activation of members of AP-1 transcription family such as Jun and Fos through the MAP kinase signaling pathway is also required for the transcription of proinflammatory genes [28]. We found that λ-dCGN treatment of RAW264.7 cells caused increased phosphorylation of ERK1/2, leading to the activation of AP-1. Thus, our results have indicated that activation of AP-1 is also involved in λ-dCGN induced immune response in macrophages.

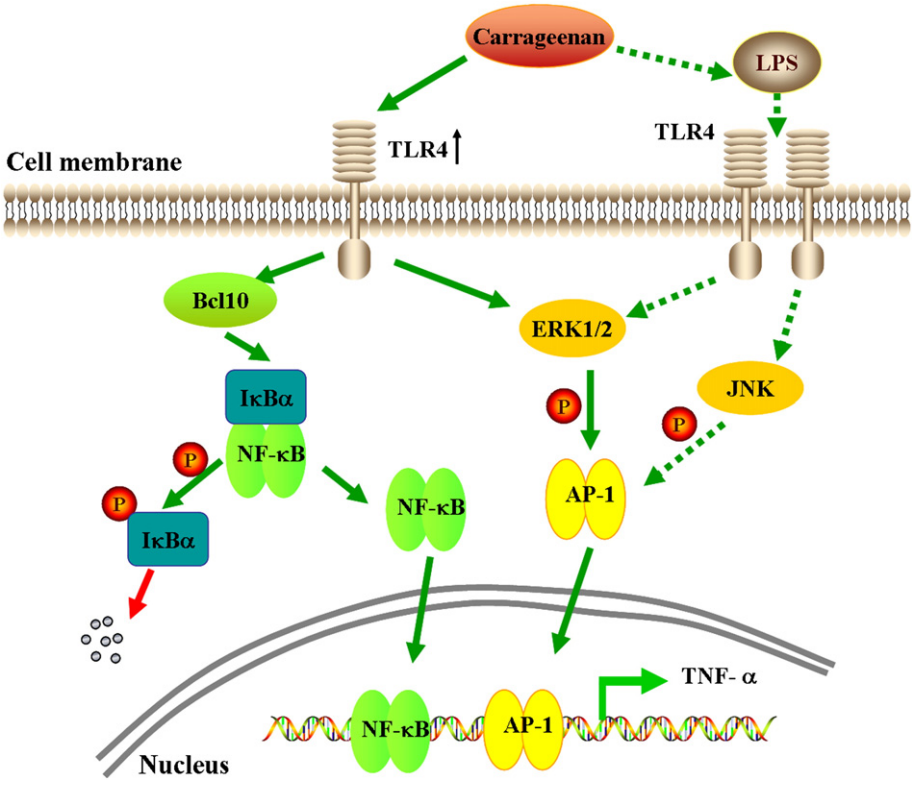

Fig. 8. A proposed model for TNF-α activation by λ-dCGN in macrophages. λ-dCGN alone induces secretion of TNF-α through the TLR4–Bcl10–NF-κB pathway, and also involves ERK1/2–AP-1 pathway. Furthermore, λ-dCGN stimulated the LPS induced TNF-α secretion through the ERK1/2–JNK–AP-1 pathway.

Since TLR4 is a crucial receptor for LPS signal recognition and transduction [29], and pretreatment with λ-dCGN caused a strong increase in TNF-α secretion in LPS-stimulated macrophages, we hypothesized that the upregulation of TLR4 expression induced by λ-dCGN may enhance the binding of LPS to macrophages. We found that λ-dCGN treatment had no effect on the binding of FITC-LPS to macrophages, suggesting that the enhancement of λ-dCGN on LPS-induced inflammatory response is not related to its enhancement on the affinity of LPS binding to TLR4. The current view on the LPS signaling is that MD-2 plays an essential role in LPS responsiveness, and LBP and CD14 also increase the sensitivity of LPS–TLR4 signaling [30,31]. The increased mRNA levels of CD14 and MD-2 as determined by real-time PCR indicated that λ-dCGN enhanced the transcription of CD14 and MD-2, and LPS showed stronger effects to increase their transcription. However, pretreatment with λ-dCGN did not increase the effect of LPS. Therefore, our results indicated that the increased expression of TLR4 in λ-dCGN-induced macrophages alone was insufficient to enhance the binding of LPS to macrophages. Increased TLR4 only, but with insufficient MD-2 or CD14, may not be strong enough to facilitate LPS binding to TLR4. Thus, λ-dCGN-induced effects in macrophages are likely mediated by the interactions of a number of factors including TLR4, MD-2 or CD14 but the precise underlying mechanisms require further studies.

Without LPS exposure, λ-dCGN pretreatment did not induce JNK and c-Jun phosphorylation. Interestingly, with LPS exposure, λ-dCGN pretreatment increased the phosphorylation of JNK and c-Jun. Our data showed that λ-dCGN pretreatment increased the phosphorylation of ERK1/2, and JNK, leading to phosphorylation of c-Jun, increased activation of AP-1, and upregulated expression of TNF-α in LPS-stimulated RAW264.7 cells. These results have demonstrated that activation of AP-1 is involved in the enhancement of LPS-induced inflammatory response to λ-dCGN in RAW264.7 cells.

In conclusion, λ-dCGN induced a stronger secretion of TNF-α in macrophages compared to λ-CGN. This induction was mediated through both the TLR4–Bcl10–NF-κB pathway and ERK1/2–AP-1 pathway. Furthermore, λ-dCGN enhanced LPS-induced secretion of TNF-α, through the ERK1/2–JNK–AP-1 pathway (Fig. 8). The inflammatory effects induced by λ-dCGN in macrophages and perhaps in other types of cells may be partially responsible for its potential carcinogenicity.

Acknowledgements

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