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Research Article

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(Z)-3-(chloromethylene)-6-methylthiochroman-4-one increased proinflammatory factors production in mice macrophages

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ABSTRACT

(Z)-3-(chloromethylene)-6-methylthiochroman-4-one (CMMT) is a new thiochroman ketone compound. Earlier assessments have verified its probable activation on immunine system. We found that CMMT has lipopolysaccharide (LPS)-like effects in vitro and in vivo. The cultured RAW264.7 macrophages and isolated mice intraperitoneal macrophages were treated with different concentrations of CMMT and 800ng/ml lipopolysaccharide (LPS) respectively, and detected the amount of TNF-α, IL-6, NO in supernatant. CMMT and LPS were injected into the housed mice and detected the amount of TNF-α. The results showed that CMMT, like LPS, concentration-dependently stimulated production of TNF-α, IL-6 and NO.

Keywords: (Z)-3-(chloromethylene)-6-methylthiochroman-4-one; RAW264.7 macrophage; Proinflammatory factors; NO; TNF-α

INTRODUCTION

Thiochromanones are a class of compounds with extensive biological activities. Thiochromanones has gained considerable attention because of its diversity in biological activity, such as antifungal activity[1,2] and anti-platelet aggregative activity[3]. Billich et al found 2t-Butyl-6-hydroxy-4H-thiochromen-4-one to be steroid sulfatase inhibitor, and speculated these agents to be effective on tumor treatment, especially estrogen- and androgen-dependent tumors such as tumors of the breast, endometrium and prostate, and squamous cell carcinoma[4]. (Z)-3-(chloromethylene)-6-methylthiochroman-4-one(CMMT) was a new synthesized thiochromanone compound (Fig.1). Chunna Li etal reported that CMMT could exhibit dramatically antitumor activities at a low concentration (IC50 were 0.41−6.05 μg/mL) in vitro[1]. Recently, we found that CMMT increased the amount of NK cells in tumor-bearing mice. That suggested CMMT might be an immunopotentiator. Further evidence supporting this may lie in the findings of earlier research[5].

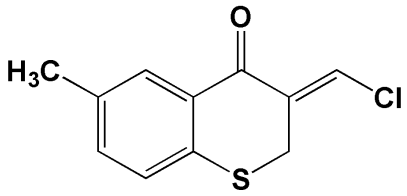

Figure. 1 Chemical structure of (Z)-3-(chloromethylene)-6-methylthiochroman-4-one

As a class of proteins that can consolidate athogen recognition，Toll-like receptors (TLRs) is an activator of innate

immunity distributed in the the surface of different immune cells[6]. TLRs can recognize specific patterns of microbial components by the production of many proinflammatory factors to regulate the acquired immune response.

The expression of certain TLRs in many tumor cell lines has been reported in earlier assessments[7,8], which can lead to tumor progression. Anti-cancer immunity and proteins involved in TLR signaling pathway was also increased by the mediation of TLR[9]. The focus on TLRs has became a new strategy for cancer therapy[10].

Antigen presenting cells (APC), macrophages and cytotoxic T cells can be activated by TLR agonists, which can lead to tumour destruction. TLR agonists can also accelerate tumour cell apoptosis directly. Furthermore recent studies[11] demonstrated that Toll-like receptor agonists have long been used as immunoadjuvants in anti cancer immunotherapy, which can directly promote tumour cell death [12].

Christian Bogdan and Aihao Ding reported Taxol, a microtubule-stabilizing antineoplastic agent, induced expression of TNF-α and IL-1 in macrophages [13]. Some other small molecules were reported to be inducer for inflammatory cytokines [14-16], these small compounds have been confirmed to be the TLR7 / 8 agonist. For example, Loxoribine, a Guanine nucleoside analogues can selectively excite TLR7[16], Imidazoquinoline compounds, imiquimod and resiquimod are TLR7/8 agonist[17]. These reports prompt us to think CMMT maybe also a candidate of TLRs agonist, and the possible cause of its anticancer activity lie in inducing antitumor cytokines production. In this study, we try to verify whether or not CMMT has LPS-like effects on RAW264.7 murine macrophage cells and mice. These results showed that CMMT, like LPS, could induce TNF-α, IL-6 and NO production in murine RAW264.7 macrophages and mice. That provided an experimental basis for further exploring CMMT as a candidate of TLRs agonist.

EXPERIMENTAL SECTION

2.1 Chemicals

LPS (from Escherichia coli, 0111:B4),D-galactosamine (D-GalN) and MTT were purchased from Sigma. DMSO and H_2O_2 were purchased from Tianjin Kermel Chemical Reagent Company. DMEM was purchased from Beijing Solarbio Science & Technology Co. Ltd., Mouse TNF-α, IL-6, IL-1β, IL-12 ELISA kits were purchased from Boster biological technology company.

2.2 RAW264.7 cell culture and treatments

The RAW264.7 cells were maintained at a 37℃ in a humidified atmosphere of 5% CO_2, 95% air, in DMEM supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin and 10% placental bovine serum. For experimental purpose, the cells were harvested in log phase, and were plated at the density of 1×10^6cells/mL medium in 24- or 96-well sterile plate. Then, cells were treated with vehicle, different concentration CMMT solutions(0.0625; 0.125; 0.25; 0.5; 1 and2 μmol/L), and LPS (800 ng/mL). Maintaining DMSO concentration at 1% in every treatment solutions. The supernatants were collected at different time points for NO, TNF-α and IL-6 assay.

2.3 Animals maintenance and treatments

Eight-week male (18–22g) KM mice were obtained from Hebei laboratory animal center (license no: SCXK (ji) 2008-1-003. certificate Numbers: 911098; 912043; 912110; 1206028). The animals were housed in a clean pathogen-free and air-conditioned room with controlled temperature (22±1℃) and 40–70% relative humidity for 3 days and feed on a standard diet and tap water ad libitum, fasted 8 h before the experiment.

Mice were administed three intraperitoneal doses of CMMT (2.5; 5 and 10 mg/kg) in dehydrated castor iol. LPS was single intraperitoneal dose (5 mg/kg) in the positive group. Control group received the same volume of dehydrated castor iol. Mice were euthanized by rapid cervical dislocation. Record the death time and number of mice. The blood was centrifuged 10 min at 3000rpm, 4°C. The serum was collected for TNF-α investigation. The animals were taken care of by our lab. All animal experiments were performed in accordance with institutional guidelines and ethics. College of Pharmaceutical Sciences, Hebei University and Drug Quality Control Key Laboratory of Hebei Province approved all animal experiments performed in this study.

2.4 Isolation and treatments of KM mice intraperitoneal macrophages

The maintenance of mice was same to above to keep KM mice stress-free, clean and uninfected. 1 ml of the NS(dissolved 0.5% starch)solution was injected into peritoneum 2 days prior to cell harvest to increase the yield of macrophages. The abdomen of every mouse was soaked with 75% alcohol for 1 min. 5 ml of RPMI 1640 medium was injected into peritoneum after dry. A small incision along the midline was made by sterile scissors. Then the intact peritoneal wall was exposed. Intraperitoneal macrophages was isolated and cultured according to the former

methods[18]. Cells in log phase were counted and 2×10^5 cells were plated in Costar 24-well flat-bottom plates and incubated for several hours at 37℃. Cells were treated with vehicle, different concentration CMMT solutions(0.25; 0.5 and 1 μmol/L), and LPS (800 ng/mL). maintaining DMSO concentration at 1% in every treatment solutions. The supernatants were collected for TNF-α, IL-1β, IL-6 and IL-12 detection.

2.5 Detection of endotoxin

Probable endotoxin exist in CMMT was detected using endotoxin-specific tachypleus amebocyte lysate under Lipopolysaccharides-free experimental conditions. The experiments were conducted according to the manufacturer's protocol: 2 mg/mL, 1mg/mL, 500μg/mL, 100μg/mL, 10μg/mL and 1μg/mL CMMT (dissolved in Lipopolysaccharides-free DMSO) was added into the detection systems with 100μl tachypleus amebocyte lysate reagent and incubated for 1 h at 37 °C. Vehicle group (Lipopolysaccharides-free DMSO) and control group (Lipopolysaccharides) were conducted in the same conditions and treatment. Each tube was then examined for gelation.

2.6 MTT cell viability assay

Treated the cells which cultured for 12h in 96-well plates with different treatment media, and did MTT cell viability assay following our previously described protocol[19]. The cells in log phase were seeded in 96-well plates and incubated for 12 h, then treated with different treatment media. After 20 h, 10.0μL of MTT (5.0μg/mL) was added, and the cultures were incubated for an additional 4 h. The medium was removed and 100μL of DMSO was added. The absorbance was read at 490 nm with a Bio-Tek microplate Reader.

2.7 Assay of Nitrite Oxide Production

Supernatant was collected from RAW264.7 cells, isolated mice intraperitoneal macrophages after treated with vehicle, LPS or CMMT (different concentrations) for 24 h. The production of NO was determined by measuring the accumulated levels of nitrite in the supernatant with the Griess reagent [19-21].

2.8 Cytokines ELISA Assay

Supernatant was collected from RAW264.7 cells, isolted mice intraperitoneal macrophages after treated with vehicle, LPS or CMMT (different concentrations) for 3 h. Blood from mice treated with vehicle, LPS or CMMT (different concentrations) was centrifuged at 3,000 r/min for 40 min. Supernatant was collected. The release of TNF-α, IL-12, IL-1βand IL-6 in supernatant or serum was measured using ELISA kits according to the manufacturer’s instructions.

RESULTS AND DISCUSSION

We investigated the LPS-like effect of (Z)-3-(chloromethylene)-6-methylthiochroman-4-one in the RAW264.7 cells, isolated mice intraperitoneal macrophages and KM mice. And found that (Z)-3-(chloromethylene) -6-methylthiochroman-4-one, like LPS, could significantly induce the production and release of proinflammatory factors, no matter in isolated mice intraperitoneal macrophages or KM mice. Its anti-tumor activity may lie in this.

3.1 Detection of endotoxin exist in CMMT

Endotoxin-specific tachypleus amebocyte lysate was used to detect the LPS exist in CMMT. The quantity of probable endotoxin exist in CMMT was as clear as that in the vehicle group. Nearly little LPS exist in (Z)-3-(chloromethylene)-6-methylthiochroman-4-one, which excluding the LPS-like effect of CMMT coming from the LPS contamination in CMMT.

3.2 Effects of CMMT on the production of NO, TNF-α and IL-6 in RAW264.7 cells

MTT assay results showed that cell viability was formal in the low concentration of CMMT and LPS treatments, but the high concentrations of CMMT was cytotoxic (Fig. 2), for ruling out the reduction of inflammatory factors coming from the depression of cell viability. Treated the cells with low concentration of CMMT for detecting the NO, TNFα and IL-6 assay.

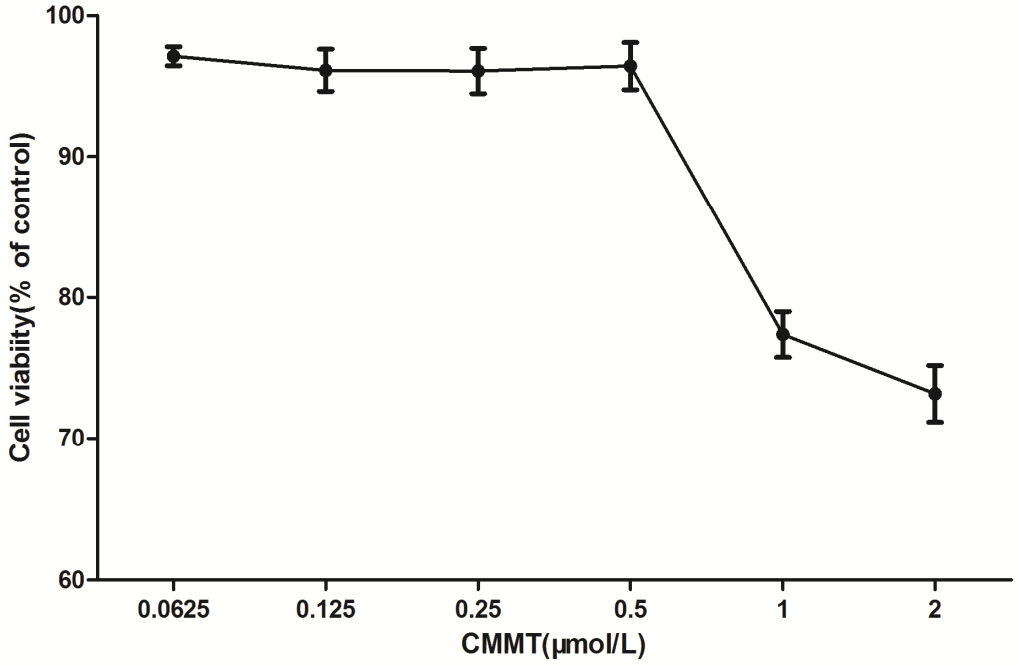

Fig.2 Effects of CMMT(different concentration) on RAW 264.7 cell viability. RAW 264.7 cultures treated with different concentrations of CMMT for 12 h. Cell viability was measured by MTT assay

Results are the mean ± SEM of three independent experiments.

The results showed that low concentrations of CMMT could stimulate RAW264.7 cells to increase production of NO, TNF-α and IL-6 (Fig. 3). When the concentration of CMMT was 0.5μmol/mL, the level of IL-6 and NO was increased by 8.30 and 1.72 folds of the control group respectively, while those in LPS (800 ng/ml) was increased by 4.77 and 1.07 folds of the control group only. CMMT was more potent than LPS in trigger NO and IL-6 production in RAW274.7 cells, but was slightly less potent than LPS in inducing TNF-α release. Even so, we estimate that CMMT had almost the same effects with LPS in stimulating RAW264.7 cells.

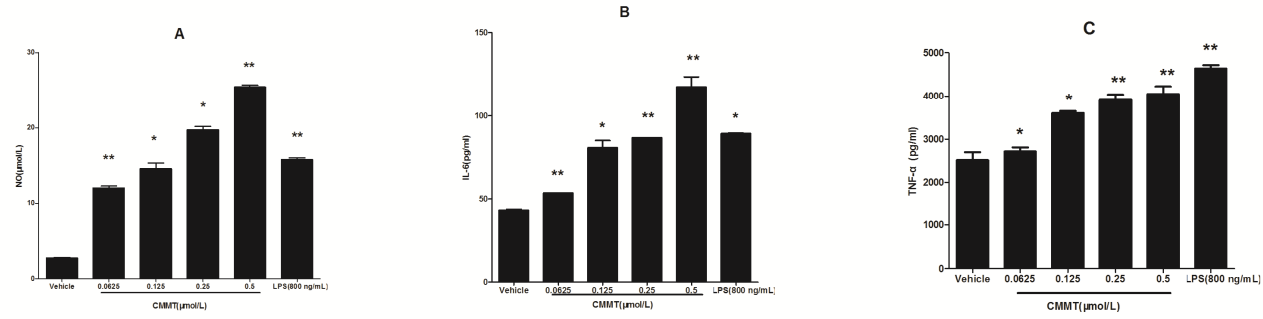

Fig.3 Effects of CMMT(different concentration) and LPS(800ng/ml) on release of NO, IL-6 and TNF-α in RAW264.7 cells. Supernatant was collected from RAW264.7 cells, isolted mice intraperitoneal macrophages after treated with vehicle, LPS(800ng/ml) or CMMT (different concentrations) for 3 h to measurement of NO, 24 h to measurement of TNF-α and IL-6

*The results are the mean ± SEM of three independent experiments. *p<0.05, **p<0.01 compared with vehicle group. (A) NO; (B) IL-6; (C) TNF-α.*

3.3 Effects of CMMT on the release of TNF-α, IL-1β, IL-6 and IL-12 in isolated mice intraperitoneal macrophages

CMMT treatment increased TNF-α release in a concentration-dependent manner from 0.25–1μmol/mL, when the concentration of CMMT was 1μmol/mL, the level of TNF-α, IL-6 and IL-12 was increased by 78.86%, 23.76% and 23.54% compared with vehicle group respectively (Fig. 4B, C and D). As shown in Fig.4, CMMT could induce isolated mice intraperitoneal macrophages to express TNF-α. But the effect of CMMT on expression of IL-1β and in mice intraperitoneal macrophages was not obvious (Fig.4A). LPS was more potent than CMMT in inducing TNF-α, IL-6 and IL-12 release in isolated mice intraperitoneal macrophages. The releases of IL-1β induced by LPS and CMMT were almost the same.

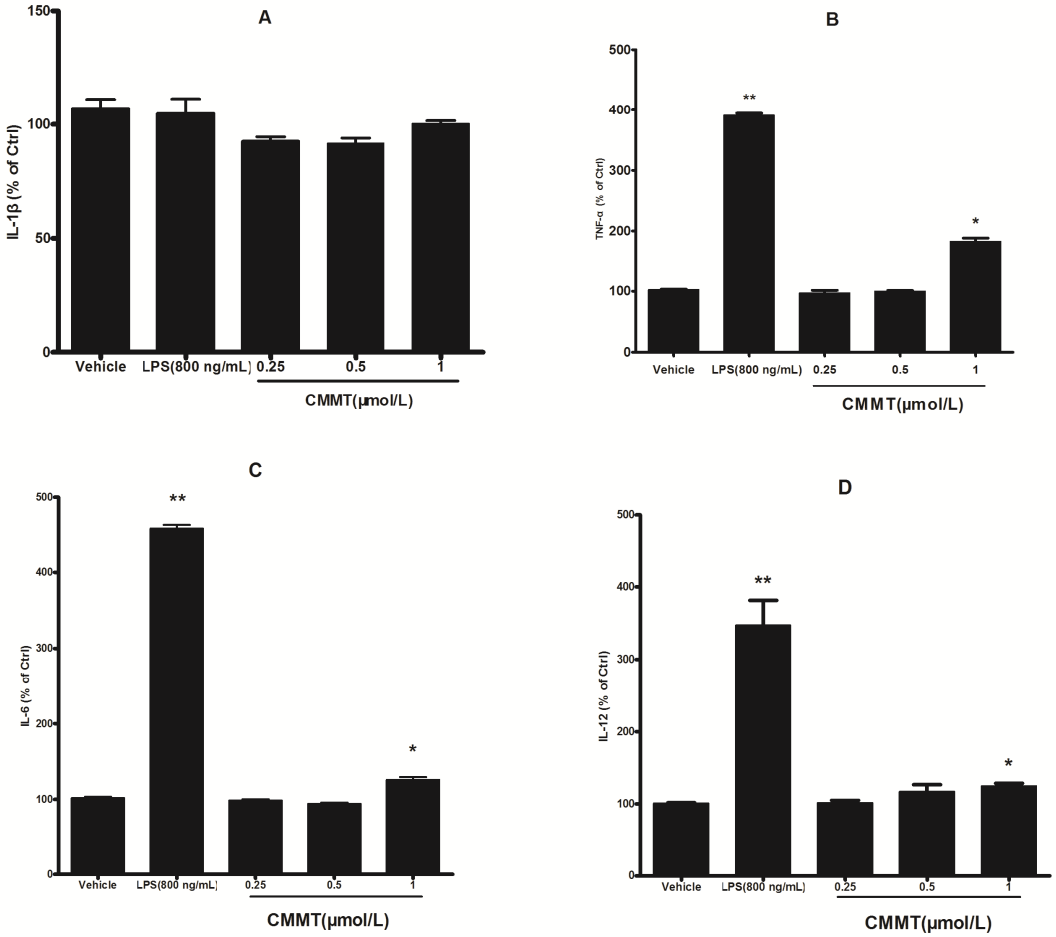

Fig.4 Effects of CMMT(different concentrations) and LPS(800ng/ml) on release of IL-1 β , TNF- α , IL-6 and IL-12 in isolated mice intraperitoneal macrophages. Cultures supernatants were removed at 24 h for measurement of IL-1b, IL-6 and TNF- α

*The results are the mean ± SEM of three independent experiments. *p<0.05, **p<0.01 compared with vehicle group. (A) IL-1 β; (B) TNF- α; (C) IL-6; (D) IL-12.*

3.4 Effects of CMMT on the release of TNF-α in KM mice

To further investigate the effect of CMMT on the release of proinflammatory factors, the release of TNF-α in mice was examined. Fig.5 showed that CMMT could induce KM mice to increase the expression of TNF-α. When the dose of CMMT was 5mg/kg, the level of TNF-α was increased by 392.24% compared with the vehicle group. CMMT was not potent in inducing TNF-α compared with that by LPS in KM mice (Fig. 5).

From these researches did in animal experiment and cell experiment we can find that the increased folds of TNF-α in mice serum is higher than that in mice intraperitoneal macrophages clearly. It indicated that CMMT could stimulate other immune cells, not only intraperitoneal macrophages.

LPS are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond, they are found in the outer membrane of Gram-negative bacteriathey. A lot of proinflammatory factors were producted to defense inflammatory after the stimulation of inflammatory reaction made by LPS.

Why does CMMT have LPS-like effect? Firstly, we can ruled out the possible contamination of LPS, because the purity of used CMMT was more than 98%, even if the 2% impurities were completely LPS, At 0.5μmol/L CMMT, the LPS concentration was < 2.24 ng/ml, and no stimulation effect in our cultured RAW264.7 cells when LPS was <100ng/ml(not shown). And the detection of endotoxin exist in CMMT showed that there was almost little LPS in CMMT. Secondly, There is no the structural similarity between LPS and CMMT(Fig 1). CMMT is a synthesized small molecule compound of thiochromanones. Most agonists of TLRs have been proved to be natural products, but some synthetic small molecules are also ligands of TLRs. The interaction sites of these molecules are TLR7/8. Such as guanine nucleoside analogues Loxoribine and imidazoquinoline compounds imiquimod selective excited TLR7[16,17], while imidazoquinoline compounds resiquimod is TLR7/8 agonists[17]. Recently, Shi et al

Synthesized a TLR7/8 independent imidazolequinoline compound[16], the compound induced IL-12p40; TNF-α and IL-1β secretion.

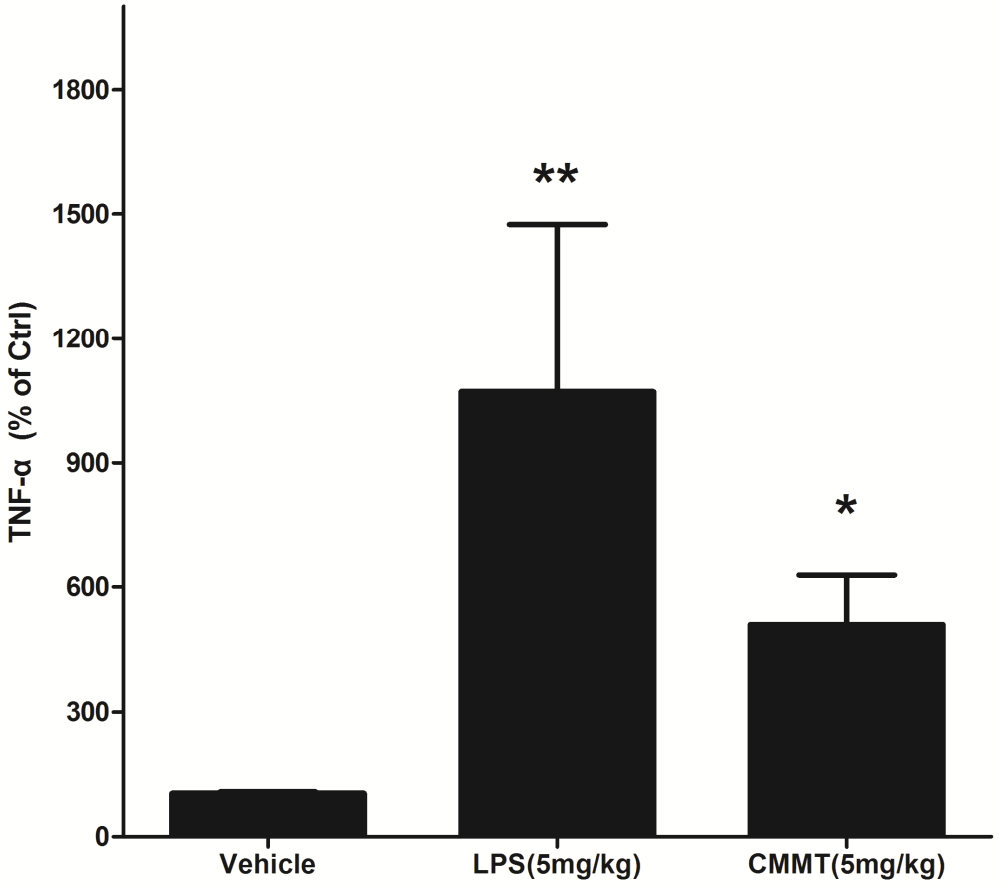

Fig. 5 Effects of CMMT(5mg/kg) and LPS(5mg/kg) on release of TNF- α in the blood of mice. The expression of TNF- α in the blood of mice treated with CMMT (5mg/kg) and LPS (5mg/kg) for 2h, Supernatant of blood was collected. The release of TNF- α in supernatant or serum was measured using ELISA kits

*The results are the mean ± SEM of three independent experiments. *p<0.05, **p<0.01 compared with with vehicle group.*

Current study showed that low concentrations of CMMT had LPS-like role on RAW264.7 cells, isolated mice intraperitoneal macrophages and KM mice, and suggested it to be another small molecule TLRs agonists. CMMT induced cytokine species were almost identical with LPS. But to confirm whether it is a TLR ligand, or the other pathway by which CMMT induced inflammatory cytokines production still need to be lucubrated. From this perspective, it may be an optimistic thing.

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