

## Cytotoxic effect of triptolide on LPS-treated macrophages through sustained phosphorylation of p38 MAP kinase and apoptosis

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### Running title:

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### Abstract

Triptolide induced the death of cells of lipopolysaccharide (LPS)-treated macrophage-like cell line J774.1/JA-4 cells through apoptosis but not through activation during incubation at 37°C for 4 h. This phenomenon was dependent on the dose and the time of addition of triptolide; and prior or simultaneous addition to LPS was needed, suggesting mal-regulation of LPS-signaling cascades. LPS rapidly induced phosphorylation of p38 MAP kinase, JNK and Erk1/2 within 15 min of its addition, and then dephosphorylation of them followed by 60 min. However, the addition of triptolide, but not endothall, inhibited this dephosphorylation of phospho-p38 and phosphor-JNK in these cells, suggesting that MKP-1 was involved in the dephosphorylation of these phosphoproteins. Triptolide inhibited the synthesis of MKP-1 mRNA and protein induced by LPS. Compared with our previous studies, where a protein synthesis inhibitor, cycloheximide (CHX), induced rapid apoptotic cell death in LPS-treated macrophages through sustained phosphorylation of p38, triptolide seemed to induce cell death of LPS-treated macrophages by inhibition of MKP-1 induction.

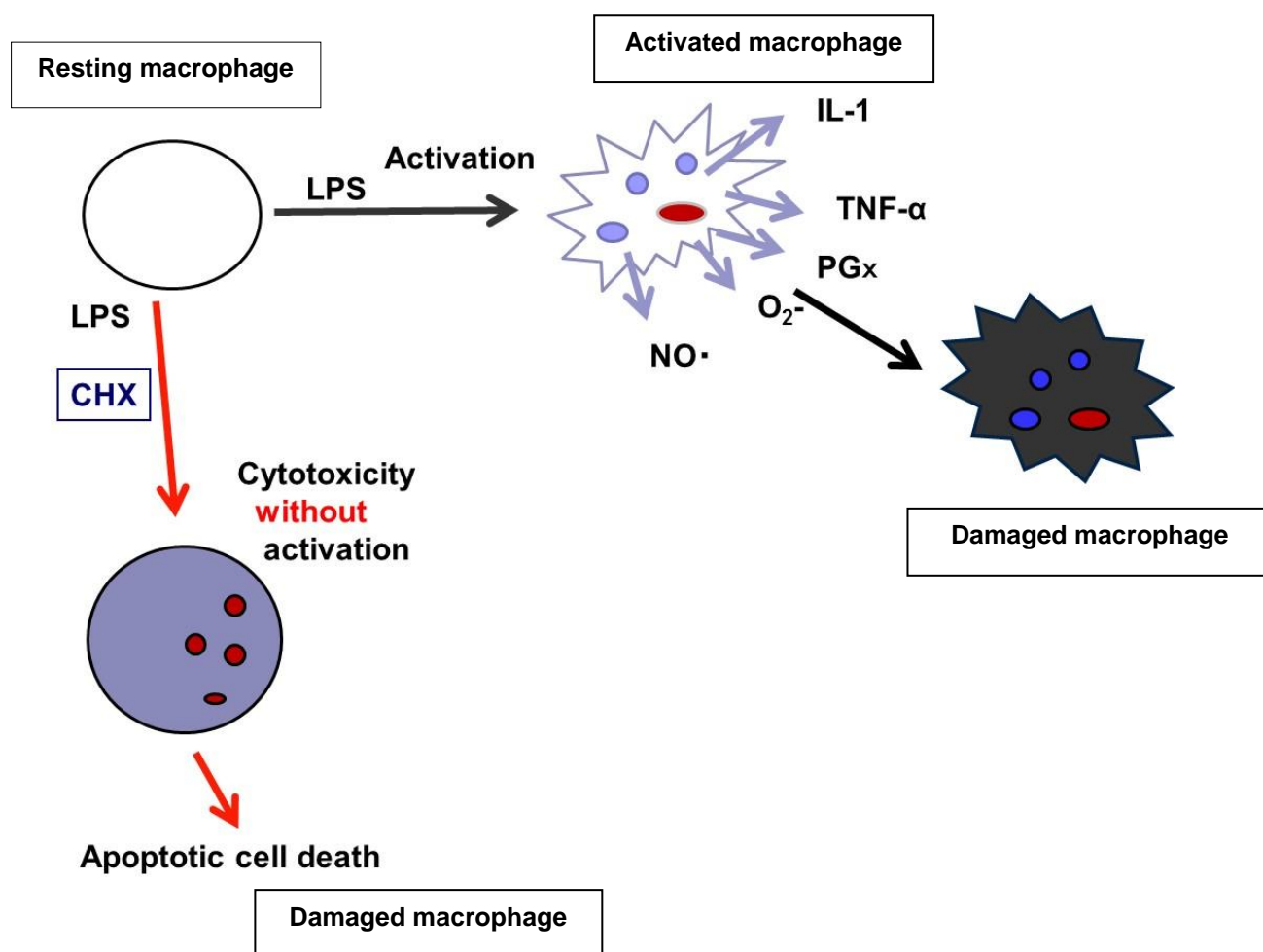
**Key words:** macrophage cell death; triptolide; lipopolysaccharide (LPS); p38 MAP kinase; phosphorylation, apoptosis, MKP-1

## 1. Introduction

Macrophages show a variety of phenotypes in responses to bacterial lipopolysaccharide (LPS), producing inflammatory cytokines, reactive oxygen species (ROS), nitric oxide (NO), and prostaglandins (PGx). These responses are called activated-macrophage phenotypes, and they play important roles in host-defense against microbial infections as well as participate in the regulation of immune reactions.<sup>1</sup> It is well known that macrophages are readily activated by one of the major components of Gram (-) bacteria, lipopolysaccharide (LPS).<sup>2</sup> The LPS-activated macrophages show a variety of characteristic phenotypes including TNF- $\alpha$  secretion and generation of superoxide anion ( $O_2^-$ ) and nitric oxide (NO); and these effector molecules and/or cytokines are cytotoxic not only to pathogens, degenerated cells or tumor cells but also to the macrophages themselves afterwards.<sup>3</sup> However, if the LPS was added to the macrophages in the presence of cycloheximide (CHX), a protein synthesis inhibitor, the cells rapidly become damaged through apoptosis but not through macrophage activation (Fig. 1).<sup>1</sup> Extensive studies of the LPS-induced rapid cell death of the macrophages in the presence of CHX have revealed that CHX seems to affect the early stages of LPS-signaling cascades,<sup>4, 5</sup> ; and inhibition of the synthesis of certain proteins that should be immediately induced by LPS-treatment might be involved in these phenomena including apoptosis.<sup>6, 7</sup> Besides, unusual phosphorylation patterns of MAP kinases have been observed in this early stage of LPS action; and among them, sustained phosphorylation of p38 MAP kinase is remarkable.<sup>7, 8</sup> When a selective inhibitor of p38 MAP kinase, SB202190, is

added prior to LPS addition, not only inhibition of downstream kinases of p38 phosphorylation but also the sustained phosphorylation of p38 MAP kinase is observed together with severe cell damages of the macrophages.<sup>7-9</sup> These studies in our laboratory seem to show that sustained phosphorylation of p38 MAP kinase *per se* is not always linked to activation of p38 MAP kinase, but that impaired dephosphorylation of p38 MAP kinase might be critical to dysregulation of the LPS-signaling cascades and subsequent induction of macrophage cell death.

In order to ascertain the role of sustained phosphorylation of phospho-p38 MAP kinase and the related protein phosphatase activity in the induction of apoptosis in the LPS-treated macrophages, we investigated selective inhibitors of protein phosphatases without inhibitory activity toward general protein synthesis, like CHX, among anti-inflammatory compounds. We found triptolide, a Chinese plant medicine from *Tripterygium wilfordii* Hook F,<sup>10</sup> as an inhibitor of MKP-1,<sup>11, 12</sup> as well as endothall, an inhibitor of PP2A,<sup>13</sup> as a reference compound. The results obtained in our present study showed that triptolide inhibited the induction of MKP-1 mRNA in cells of the LPS-treated macrophage-like cell line J774.1/JA-4 and induced sustained phosphorylation of p38 MAP kinase together with apoptosis. Besides, the data suggested that LPS immediately induced phosphorylation of p38 MAP kinase and almost simultaneously induced MKP-1, implying that MKP-1 was responsible for the dephosphorylation of phospho-p38 MAP kinase and was involved in the regulation of LPS-signal cascades to avoid apoptotic cell death as well as over-activation of the macrophages.



**Fig. 1. Outline and background of this study**

Resting macrophages are readily activated by LPS and become activated macrophages, which show a variety of characteristic phenotypes including secretion of inflammatory cytokines, such as IL-1 and TNF- $\alpha$ , and generation of prostaglandins (PGx), superoxide anion ( $O_2^-$ ), and nitric oxide ( $NO\cdot$ ). These cytokines and/or chemical mediators sometimes are cytotoxic to the macrophages themselves, resulting in damage to them and ceased production of inflammatory cytokines and mediators. On the other hand, in the presence of cycloheximide (CHX), LPS induces rapid cytotoxic changes to the macrophages without macrophage activation. The pathways of this cell death include apoptotic changes such as caspase 3 activation as well as DNA cleavage. It is also remarkable in this apoptotic process that activation of p38 MAP kinase through phosphorylation and sustained phosphorylation of p38 occur. To understand the role of the sustained phosphorylation, we examined protein phosphatases of the macrophages, PP2A and MKP-1 in this study.

## 2. Materials and Methods:

### 2.1 Reagents

*Escherichia coli* (055: B5) LPS was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), and Ham's F-12 and fetal

bovine sera were purchased from Life Technologies (Carlsbad, CA, U.S.A.). Penicillin-streptomycin mixed solution came from Nacalaitesque (Kyoto, Japan); and Triptolide, from *Tripterygium wilfordii*, and Endothall (7-Oxabicyclo [2.2.1] heptane-2,3-dicarboxylic acid) were

obtained from Sigma-Aldrich. Murine interferon- $\gamma$  (IFN- $\gamma$ ) was a generous gift from TORAY (Tokyo, Japan). Primary antibodies, including rabbit anti-p38, phosphor-p38, SAPK/JNK, phosphor-SAPK/JNK, Erk44/42 (Erk1/2) and phosphor-Erk44/42(p-Erk1/2), were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.); and anti-MKP-1 antibody was obtained from Santa Cruz Biotechnology (Dallas, Texas, U.S.A.). Anti-PP2A antibody was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.); and anti-rabbit immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase (HRP), used as a secondary antibody, was purchased from Cell Signaling Technology. An *in situ* cell detection kit for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay was obtained from Roche.

## 2.2 Cell Culture

Culturing of the JA-4 cell line, an LPS-sensitive subline of the murine macrophage-like cell line J774.1 was performed as described previously.<sup>14</sup> In brief, the cells were cultured in Ham's F-12 medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 50 U of penicillin and 50  $\mu$ g of streptomycin per mL, and were maintained in culture dishes (Falcon #1001, Corning, NY, U.S.A.) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The cells were maintained by culturing them as described above, and were used for various assays after several passages and before the 25<sup>th</sup> passage from the time of defrosting of the stored cells in order to keep stable phenotypes of the cells.

## 2.3 Cell Toxicity Assay

Cells were harvested and seeded at  $1 \times 10^5$  cells /0.25 mL per well onto 48-well plates (Corning #3548, Corning, NY,

U.S.A.) and incubated at 37°C for about 4 h. Then the medium was exchanged for 0.25 mL of fresh medium, after which triptolide, endothall or CHX was added to the cultures at appropriate concentrations, followed by incubation with or without 100 ng/mL LPS at 37°C, usually for 4 h; and then the cells were chilled on ice to stop culture. The culture supernatants were collected into microfuge tubes and centrifuged at 10,000 rpm for 1 min at 4°C. The resultant supernatants were used to assess lactate dehydrogenase (LDH) activity, which is a cytosolic enzyme marker whose release into the culture supernatant corresponds to cell damage.<sup>4, 8</sup> The assay was performed by using an LDH-Cytotoxic Test Wako (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's protocol. Absorbance was measured at 450 nm with subtraction at 620 nm by using a MultiSkan FC (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Culture supernatants collected at 0-time incubation were used to determine the background release of LDH. To determine the total LDH activity, we added Triton X-100 at a final concentration of 0.1% to the cultures of non-treated cells at the end of the incubation at 37°C for 4 h, and then incubated them for an additional 30 min at 37°C for complete lysis of the cells. Cytotoxicity was expressed as % of the total activity according to the following formula:

$$\% \text{ of total} = \{(\text{experimental release}) - (\text{background release}) / \text{total activity}\} \times 100$$

## 2.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)/Western Blotting

JA-4 cells were seeded into 60-mm dishes (Corning #430556) at  $2 \times 10^6$  cells/5 mL. The cells were incubated at 37°C for 4 h, and then the culture medium was changed to fresh medium containing 300 ng/mL triptolide or not at 37°C for 30 min, followed by incubation at 37°C for 30 min. Then 100

ng/mL LPS without or with 10 µg/mL CHX was added at the time 0, and the cells were incubated further at 37°C for 15 or 60 min. The cells were subsequently chilled on ice, washed twice with phosphate-buffered saline without divalent cations (PBS (-)), and then detached from the dishes by treatment with 0.25 U/mL trypsin and 0.1 mM EDTA in PBS. The cells were collected by centrifugation at 5000 rpm (2280×g) for 1 min at 4°C, and the resultant cell precipitates were suspended in ice-cold 0.7 mL of buffer A, comprising 10 mM KCl, 0.1 mM EDTA, and 0.4% NP-40 in 10 mM HEPES-KOH buffer, pH7.8. The suspension was mixed 5 times with a vortex mixer for 7 sec each time, and then centrifuged as described above. The final pellets were used as nuclear fractions and treated with 0.2 mL of a 2-fold concentration of SDS-sample buffer comprising 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 8% (v/v) glycerol, and 0.02% (w/v) bromophenol blue in 80 mM Tris-HCl buffer (pH 6.8), and then boiled at 100°C for 5 min. The samples were separated by SDS-PAGE using 5-20% gradient slab gels (ATTO, Tokyo, Japan). They were transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, U.S.A.) for Western blotting. The blots were blocked with Blocking One PTM (Nacalai tesque, Kyoto, Japan), and then reacted with a primary antibody, anti-MKP-1 (1 : 1000), anti-phosphorylated p38 MAPK (1 : 1000), anti-p38 MAPK (1 : 1000), anti-phosphorylated JNK (1 : 1000), anti-JNK (1 : 1000), anti-phosphorylated Erk1/2 (1 : 1000), anti-Erk1/2 (1 : 1000) or anti-PP2A (1 : 1000) at 4°C overnight, followed by reaction with the secondary antibody, anti-rabbit IgG antibody conjugated with HRP (1 : 1000), at the room temperature for 1 h. Chemiluminescence was generated by using Pierce Western blotting Substrate (Thermo Fisher Scientific) and detected by using an LAS 3000 Mini Image Analyzer

(FUJIFILM, Tokyo, Japan). The results were analyzed by using Image J software (Ver. 1.48V).

## 2.5 Morphologic Observation

Cells were observed under an Olympus CKX41 phase-contrast microscope, and photographs were taken with magnification ×200 in random fields of each sample.

## 2.6 Terminal dUTP TUNEL Assay

Approximately  $1.5 \times 10^3$  cells were plated per well of 8-well slide glasses (Thermo Fisher Scientific). After incubation at 37°C for 4 h, the cells were incubated further without or with 300 ng/mL triptolide at 37°C for 30 min. Then 100 ng/mL LPS was added, followed by incubation at 37 °C for 4 h. For the positive control, 10 µg/mL CHX was added; and the cells were then incubated in the presence or absence of 100 ng/mL LPS at 37 °C for 4 h. The cells were fixed for 30 min with 3.6% formaldehyde (pH.7.4) in the culture medium. After the fixed cells had been washed with PBS (-) repeatedly, they were treated with 0.1% (v/v) Triton X-100 in PBS (-) at room temperature for 10 min to render the cell membrane permeable, which procedure was followed by repeated washing with PBS (-). A TUNEL assay using an *in situ* cell death detection kit (Roche) was performed according to the manufacturer's protocol. In brief, the permeabilized cells were treated with the TUNEL reaction mixture and incubated in a humidified dark chamber at 37°C for 1 h. The samples were then washed with PBS (-), followed by incubation with 20 ng/mL 4, 6-diamidino-2-phenylindole (DAPI) for 10 min at the room temperature. After washing of the cells with PBS (-), the cells were mounted by using PermaFluor® mounting medium (Thermo Fisher Scientific). The images were observed under an LSM700 laser-scanning microscope (Carl Zeiss MicroImaging GHBH, Jena, Ger-

many).

## 2.7 RNA Isolation, cDNA Synthesis and Quantitative PCR Analysis

The cells were seeded at  $5 \times 10^5$  cells per mL per well onto 12-well plates (Costar; Corning) and incubated at 37°C for 4 h. They were then incubated without or with 300 ng/mL triptolide at 37°C for 30 min, after which 100 ng/mL LPS was added to the cells, followed by incubation at 37 °C for 0, 15, 30 or 60 min. The cells were next chilled on ice, washed with PBS (-) twice, and harvested by use of a cell scraper. After washing of the cells with PBS (-) by centrifugation, the cellular RNA was extracted by using Tripure Isolation Reagent (Roche) according to the manufacturer's protocol. Reverse transcription was done in 10- $\mu$ L reaction volumes with a ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan) as per our previous studies.<sup>15</sup> Quantification of mRNA levels was performed by use of real-time PCR systems (LightCycler; Roche Diagnostics and Applied Biosystemes 7500 Real-Time PCR System; Thermo Fisher Scientific) and THUNDERBIRD SYBR qPCR Mix (TOYOBO) with gene-specific primer sets. The following primer sets for qRT-PCR were used: MKP-1, 5'-GTGCCTGACAGTGCAGAATC-3' and 5'-CACT-GCCCAGGTACAGGAAG-3'; GAPDH, 5'-GGAAAGCTGTGGCGTGATG-3' and 5'-CCAGTGAGCTTCCCGTTCAG-3'. Transcription levels of the desired genes were normalized to the mRNA level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene used as the internal control.

## 2.8 Statistical Analysis

Results were expressed as the mean  $\pm$ S.E.M. of at least 3 experiments performed using *in vitro* cell preparations. Data for the 2 groups were analyzed by using Student's *t*-test, and data for more than 2 groups were

compared by using one-way ANOVA with Bonferroni multiple comparison as a *post hoc* test (Pharmaco basic software Ver. 15; Three S, Tokyo, Japan). Statistical significance was set at  $p < 0.05$  or  $p < 0.01$ .

## 3. Results

### 3.1 Induction of cell damage in the macrophages treated with triptolide and LPS

When the macrophages were treated with 100 or 300 ng/mL triptolide at 37°C for 30 min, and then with 100 ng/mL LPS followed by further incubation at 37°C for 4 h, about 10-20% of the cells became damaged (Fig. 2, lower 2 rows, right). However, no such damage was observed without LPS addition (Fig. 2, lower 2 rows, left). As the negative controls, the cells without triptolide addition showed an intact morphology, being round (-LPS) or slightly elongated and/or spread (+ LPS) (Fig. 2, top row). As the positive controls, the cells treated with 10  $\mu$ g/mL CHX alone showed round, intact shapes (-LPS), but those treated with CHX and LPS were severely damaged, with evidence of rupture (+ LPS; Fig. 2 second row from top). These results show that triptolide induced macrophage cell death in the presence of LPS, and suggest that the induction of the macrophage cell death after LPS-treatment was not limited by inhibition of protein synthesis by CHX<sup>4-9</sup> or anisomycin (unpublished data), but by certain kinds of anti-inflammatory herbal medicine such as shikonin<sup>16</sup> or triptolide (this study).

To examine a possible correlation between sustained phosphorylation of p38 and induction of cell death of LPS-treated macrophages, we focused on protein phosphatase activity and searched its inhibitors. Among them, triptolide was used as an inhibitor of the phosphatase MKP-1<sup>11, 12</sup> and endothal, as one of phosphatase PP2A<sup>13</sup> in this study, with their doses varied.

Whereas triptolide induced LDH release dose-dependently and significantly at 300 ng/mL (14%) and 1000 ng/mL (11%), endothal did not (Fig. 3A). Besides, neither triptolide nor endothal induced significant release of LDH from the cells without LPS-addition. These results show that induction of macrophage cell death by triptolide depended on biological activities of LPS and suggest that inhibition of MKP-1, but not that of PP2A, was involved in the induction of the aberrant reaction of LPS in the macrophages, leading to cell damage.

Further studies on the timing of triptolide addition to LPS-treated macrophages revealed that triptolide needed to be added 30 or 15 min prior to, or simultaneously with, LPS addition; otherwise there was no observable significant release of LDH if triptolide was added at 15 min or later following LPS addition (Fig. 3B). Under these conditions, no LDH release was observed in the cells without LPS addition. These results show that triptolide did not induce macrophage cell death after certain LPS signals had been transduced to the cells, and suggest that the penetration of triptolide into the cells and subsequent inhibition of MKP-1 required more time than for the transduction of LPS signals into the cells. Therefore, triptolide was added to the cells 30 min prior to LPS addition in most of the experiments in this study, because the highest LDH release was observed at 30 min before LPS addition.

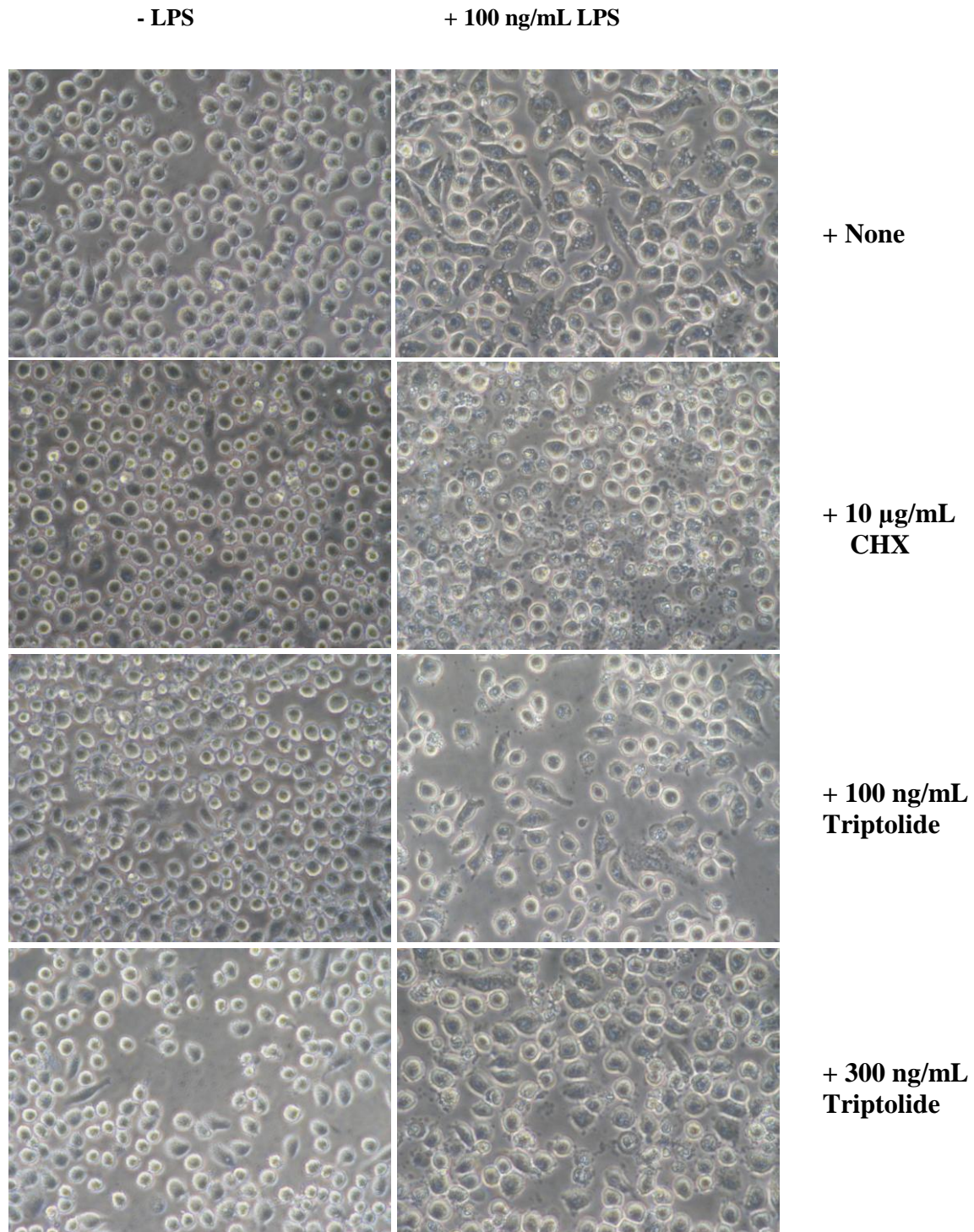
Because we found previously that CHX induces rapid cell death in LPS-treated macrophages after latent periods of incubation before cell damage appears,<sup>4-9</sup> we examined the time-course of induction of LDH release in triptolide and LPS-treated macrophages (Fig. 3C). In the presence of LPS, triptolide induced LDH release slightly, gradually but not significantly during 60-210 min after LPS addition; and then significant release was observed after 240 min or later (Fig. 3C, left graph). In

contrast, CHX showed no release by 180 min after LPS addition, and then slightly but not significantly at 210 min, and then significantly at 240 min and later (Fig. 3C, right graph).<sup>4,6</sup> These results suggest that triptolide gradually changed the integrity of the macrophage cell membrane barriers, whereas CHX did so suddenly; although the morphology of the damaged cells in these 2 groups differed considerably after incubation for 4 h (Fig. 2, right photos).

### 3.2 Induction of apoptosis in the macrophages treated with triptolide and LPS

The results in Figs. 2 and 3 showed that triptolide induced macrophage cell death in the presence of LPS as did CHX, with accompanying apoptosis. In this study, we also examined whether apoptosis was observed in cells treated with triptolide and LPS.<sup>5-9</sup> Triptolide increased the number of TUNEL (+) cells after incubation at 37°C for 4 h only in the presence of LPS, and CHX also induced TUNEL (+) cells only in LPS-treated macrophages (Fig. 4A). No TUNEL (+) cells were observed in the cells without LPS addition, nor in those without CHX nor triptolide but with LPS. This morphological observation was quantified by counting the number of both TUNEL (+) and TUNEL (-) cells in random fields, and the results showed that about 15% of the cells were TUNEL (+) in both LPS + triptolide and LPS+CHX groups (Fig. 4B).

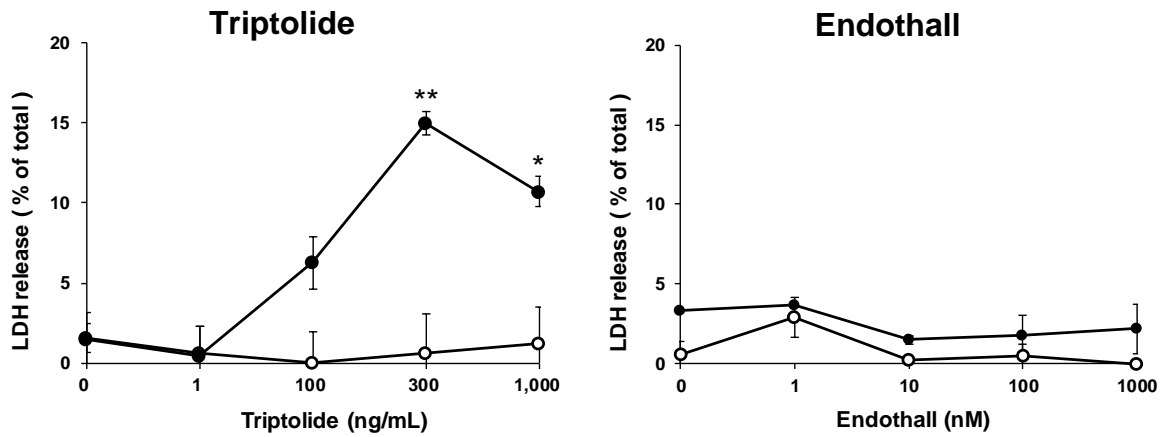
Time-course of induction of apoptosis in LPS+triptolide-treated cells revealed that incubation of the cells at 37°C for 60 min or longer resulted in the appearance of significant numbers of TUNEL (+) cells, whereas in LPS+CHX-treated cells, 180 min or more were required (Fig. 4C). These results seemed to be correlated with the difference in the time-course of LDH release between LPS+triptolide and LPS+CHX (Fig. 3C).



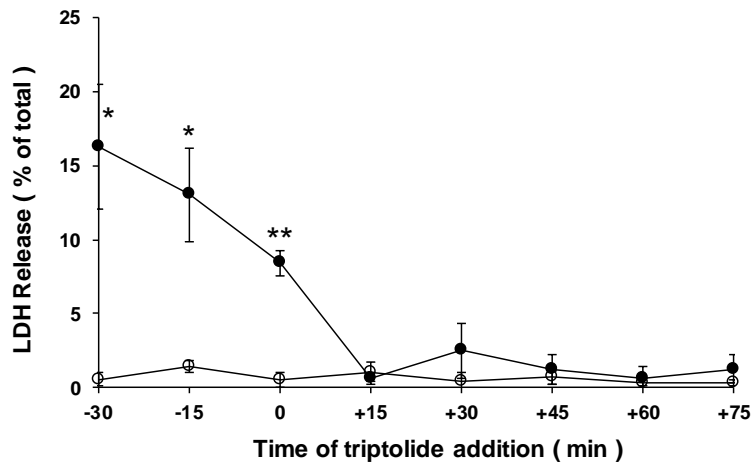
**Fig. 2. Induction of morphological changes in macrophages treated with triptolide and LPS**  
The macrophages were treated with 100 or 300 ng/mL triptolide (TL) 30 min before the addition of 100 ng/mL LPS, followed by incubation at 37°C for 4 h. As negative controls, LPS was not added to each well; and as a positive control, 10 µg/mL CHX was added simultaneously with 100 ng/mL LPS; and then the cells were incubated at 37°C for 4 h. Photographs of random fields were taken, and typical ones are shown. Original magnification, x 200.



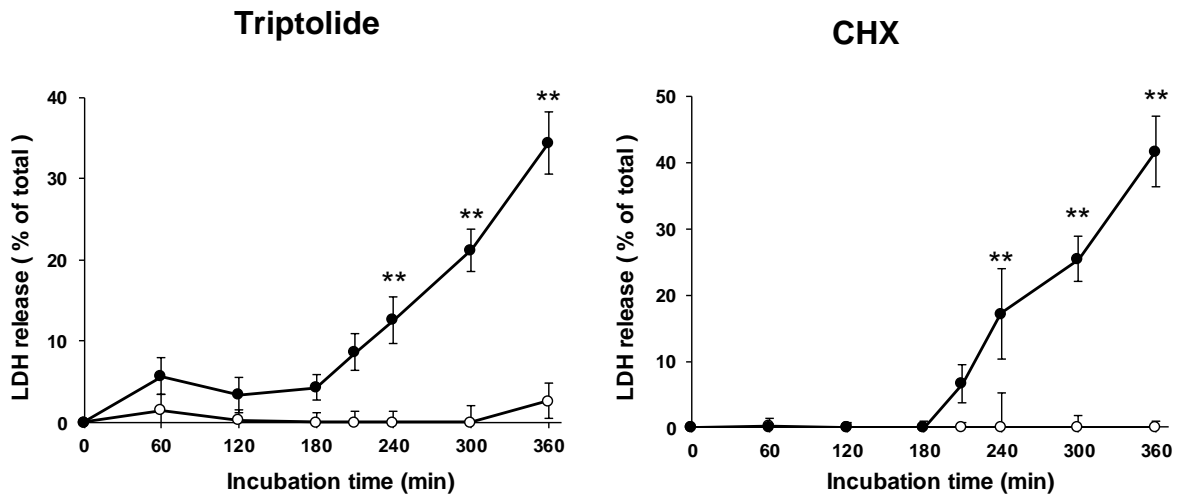
**(A) Dose-dependent induction of macrophage cell death by phosphatase inhibitors**



**(B) Effect of the time of triptolide addition on induction of the cell damage in LPS-treated macrophages**



**(C) Time-course of induction of macrophage cell death by triptolide and CHX**



**Fig. 3. Induction of macrophage cell death in the presence or absence of LPS and/or triptolide, endothall or CHX**

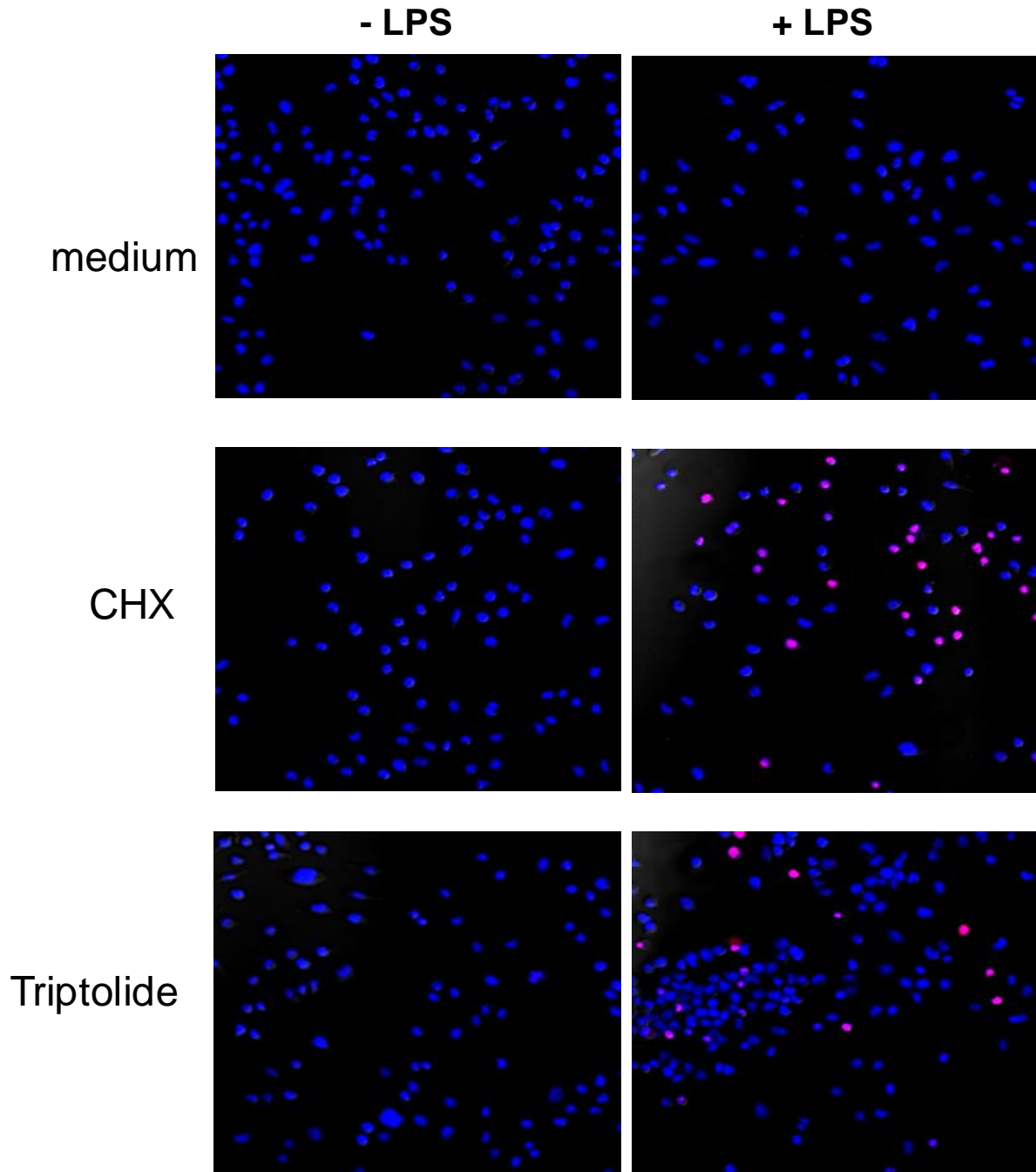
(A), To verify the involvement of protein phosphatases in sustained phosphorylation of p38 MAP kinase in the induction of cytotoxicity of LPS-treated macrophages, we added either triptolide, an MKP-1 inhibitor (left), or endothall, a PP2A inhibitor (right), at 1-300 ng/mL or 1-1000 nM, respectively, to the cells 30 min before addition of 100 ng/mL LPS. The cells were then incubated at 37°C for 4 h. Cell damage of the macrophages was quantitatively estimated in terms of LDH release, as described in the text. (B), To optimize the conditions of triptolide addition to the cells, we added 300 ng/mL triptolide at 30 or 15 min before, simultaneously, or at 15, 30, 45, 60 or 75 min after the addition of 100 ng/mL LPS (closed circles). Cell culture supernatants were then obtained and subjected to the LDH assay, as described above. For the negative controls, the triptolide was added but without LPS addition (open circles). (C), The time-course of induction of macrophage cell death by 300 ng/mL triptolide was examined in the presence or absence of 100 ng/mL LPS (left). Triptolide was added to the cells 30 min prior to addition of LPS at 0 time, as described above in “A” and “B.” For the control, the time-course was also examined when 10 µg/mL CHX was used in the presence (closed circles) or absence (open circles) of LPS similarly as for the triptolide experiment except that CHX was added simultaneously with 100 ng/mL LPS at 0 time (right). The results are shown as the means±SE for independent 3 experiments performed separately on different days. The significance of difference between the groups with and without LPS addition at each time was estimated by using Student’s *t*-test (A, B) and Turkey’s test (C), and is shown as \* P<0.05 and \*\* P<0.01, respectively.

**3.3 Sustained phosphorylation of p38 MAP kinase in LPS- and triptolide-treated macrophages**

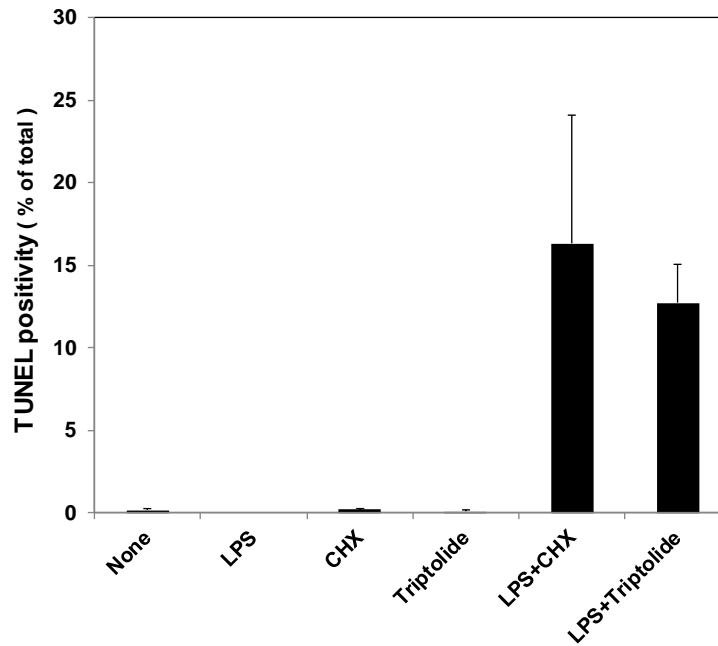
In order to know whether p38 MAP kinase was phosphorylated and its phosphorylated state was being continued after incubation of the macrophages with triptolide and LPS, we treated cells with 300 ng/mL triptolide at 37°C for 30 min, and then with 100 ng/mL LPS at 37°C for 0-60 min. The cell nuclei were isolated, extracted, and examined for pp38, p38, pJNK, JNK, pErk1/2, Erk1/2, MKP-1, and PP2A by SDS-PAGE/Western blotting. When the cells were treated with LPS alone, phosphorylation of p38 MAP kinase, JNK, and Erk1/2 occurred at 15 min after LPS addition, and these phosphorylation levels decreased at 60 min (Fig. 5). However, when the cells were pretreated with triptolide for 30 min followed by incubation with LPS for each time point, the phosphorylation of these 3 kinds of MAP kinases occurred at 15 min,

but the levels of phosphorylated p38 MAP kinase and JNK were sustained at high levels by 60 min, whereas that level of phosphorylated Erk1/2 decreased. Because the total levels of p38, JNK and Erk1/2 did not change in these cells during incubation without (data not shown) or with LPS, and/or triptolide or CHX, the difference in the sustained phosphorylation seemed to be attributed to the activities of protein phosphatases. So next we examined MKP-1 and PP2A levels in these cells. Little or no MKP-1 was detectable at 0 time and 15 min after addition of LPS to the cells; but at 60 min, MKP-1 became detectable in the cells treated with LPS alone. However, the induction of MKP-1 was abolished by the addition of CHX or triptolide (Fig. 5, lower panel). These results suggest that sustained phosphorylation of p38 MAP kinase for 60 min after LPS addition caused by triptolide or CHX seemed to be attributed to impaired induction of MKP-1, but not PP2A.

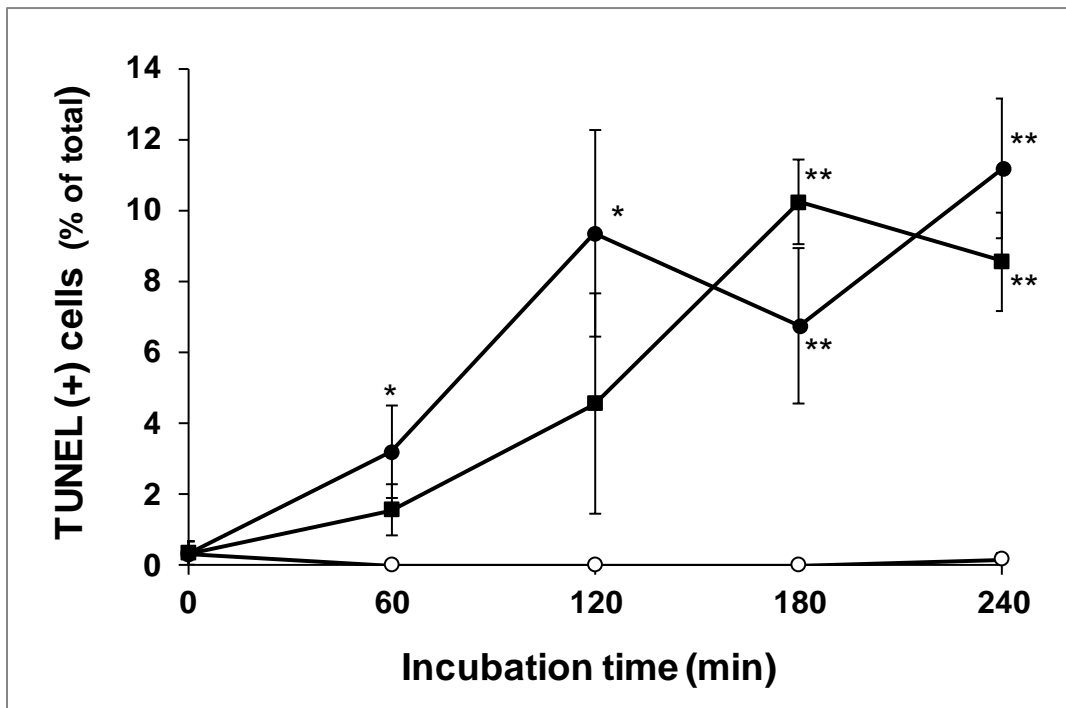
**(A) TUNEL staining of the macrophages treated with or without LPS and/or CHX, triptolide**



**(B) Population of TUNEL (+) cells**



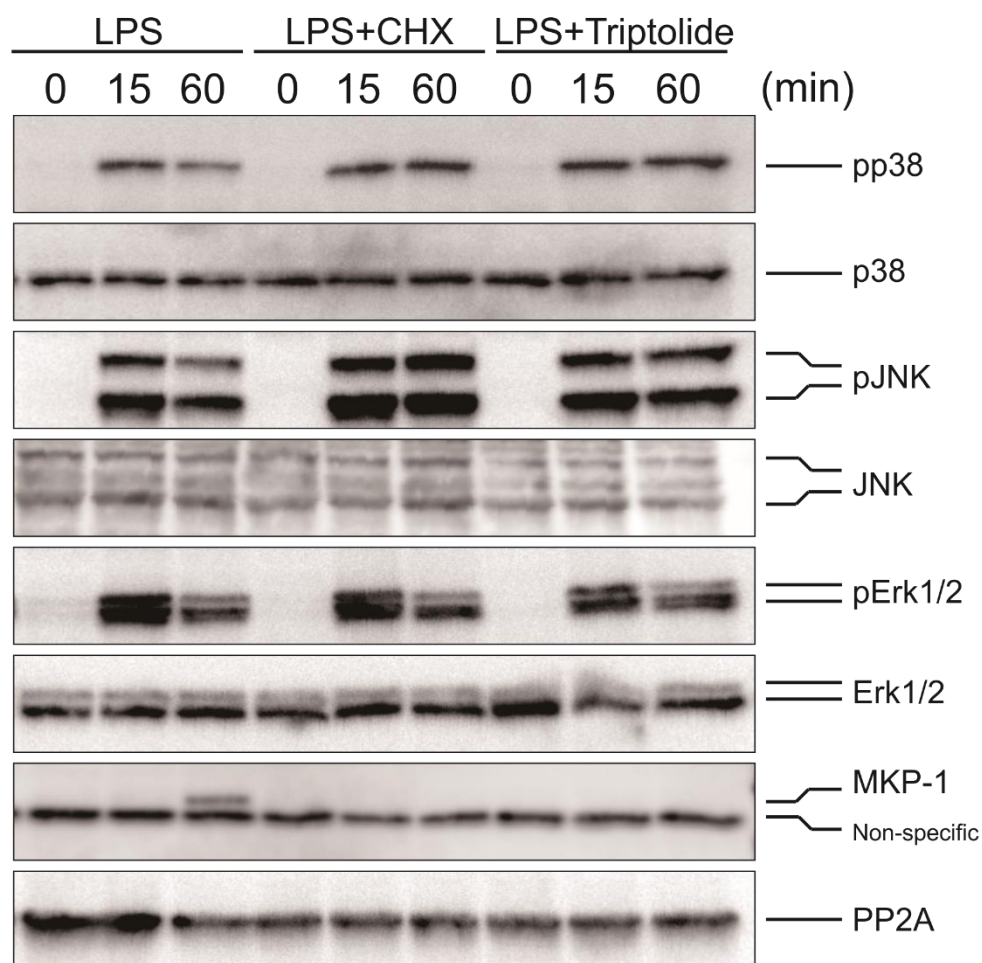
**(C) Time-course of the appearance of TUNEL(+) cell population**



**Fig. 4. Induction of apoptosis in LPS-treated macrophages by triptolide or CHX**

Apoptotic cell death was detected by TUNEL staining as described in the text. The cells were pre-incubated with 300 ng/mL triptolide at 37°C for 30 min followed by treatment with 100 ng/mL , and then incubated at 37°C for 4 h. As for the negative as well as positive controls, the cells were incubated without or with 10 µg/mL CHX in the presence or absence of 100 ng/mL LPS at 37°C for 4 h. Fixation and subsequent processes for TUNEL staining were as described in the text. (A), TUNEL (+) cells are shown

with red fluorescence, and every cell shows blue fluorescence by DAPI-staining. (B), Both TUNEL (+) and (-) cells were photographed and counted in 10 different fields after incubation with or without LPS, and/or CHX or triptolide at 37°C for 4 h; and the population of TUNEL (+) cells was scored in each field. The results are given as the means±SD for 2 independent experiments performed on separate days. (C), Populations of TUNEL (+) cells treated with none (open circles), triptolide + LPS (closed circles) and CHX + LPS (closed squares) are shown as relative value (%) to total cells stained with DAPI, and the results are shown as the means±SE for 3 independent experiments performed separately on different days. The significance of difference between the groups with or without LPS, and/or CHX or triptolide, *versus* the untreated cells at 0 time was estimated by performing Turkey's test and are shown as \* P<0.05, and \*\* P<0.01, respectively.



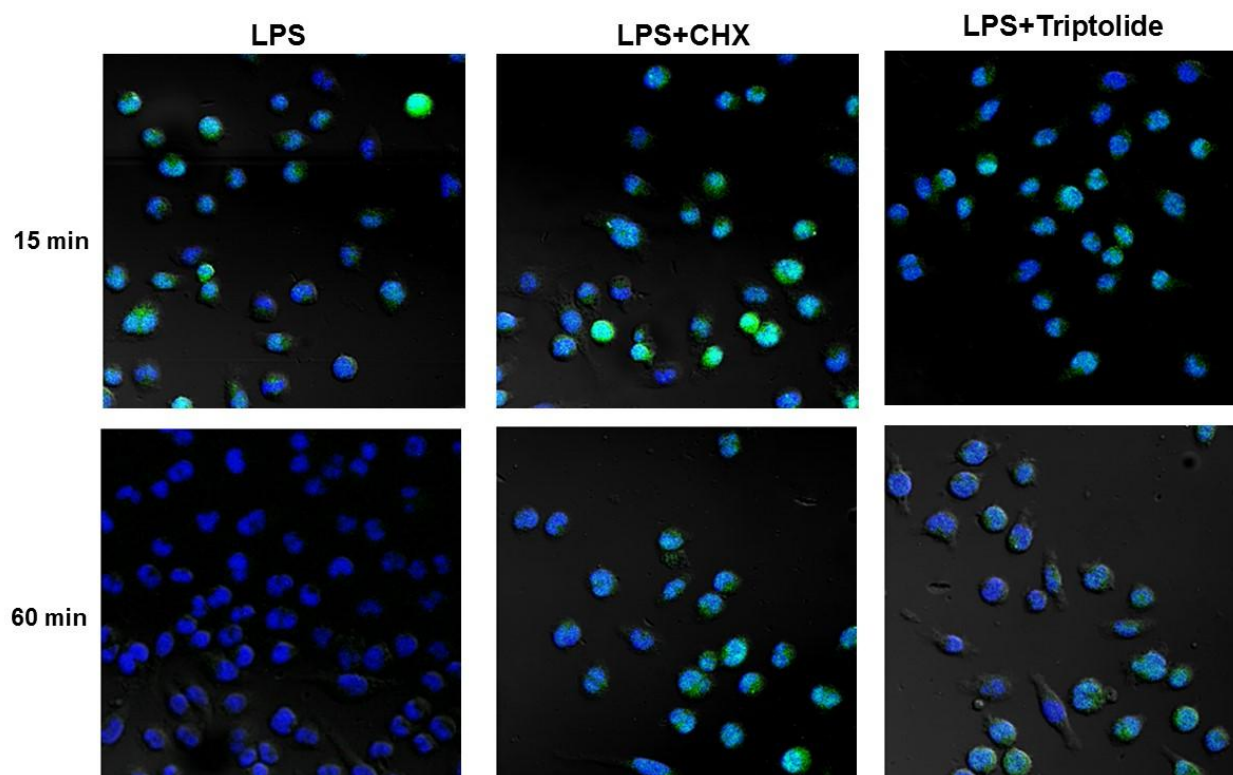
**Fig. 5. Sustained phosphorylation of p38 MAP kinase and mal-induction of MKP-1 by triptolide or CHX in LPS-treated macrophages**

The cells were treated with LPS alone, LPS+CHX or LPS+Triptolide, as described in the legend to Fig. 5; and then they were harvested at 0, 15 or 60 min after LPS addition. After the cells had been washed, their nuclei were obtained as described in the text. The cell nuclei were disrupted by sonication, and then subjected to SDS-PAGE/Western blotting using anti- pp38, p38, pJNK, JNK, pErk1/2, Erk1/2, MKP-1, and PP2A antibodies, as described in the text. Note that the upper thin band only in LPS-treated cells at 60 min corresponds to MKP-1 but the thick bands in every sample, to a non-specific antigen in the photograph (the second line from the bottom).

### 3.4 Changes in localization of phosphorylated p38 in LPS-treated macrophages by CHX or triptolide

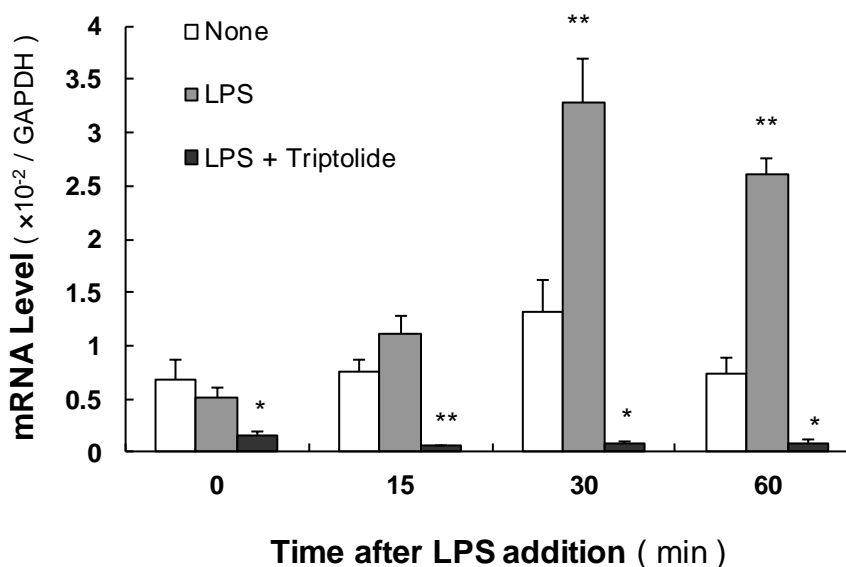
To ascertain the results in Fig. 5 showing that sustained phosphorylation of p38 MAP kinase occurred in the nuclei of the cells treated with triptolide and LPS, we next examined the subcellular localization of phosphorylated p38 by performing immunohistochemistry (Fig. 6). LPS alone induced phosphorylated p38 in the both nuclei and cytoplasm at 15 min after LPS addition (Fig. 6, upper left); but at 60 min

after LPS addition, phosphorylated p38 became non-detectable in the cells (Fig. 6, lower left). However, cells treated with LPS + CHX (Fig. 6, center) or LPS + triptolide (Fig. 6, right) showed both nuclear and cytoplasmic localization of phosphorylated p38 at 15 and 60 min. These results show that sustained phosphorylation of p38 was observed by 60 min after LPS addition, when the cells were treated with LPS together with CHX or triptolide, which finding is consistent with the results in Fig. 5, which showed the sustained phosphorylation of p38 in the nuclei.



**Fig. 6. Elevated and sustained phosphorylation of p38 in the nuclei of LPS-treated macrophages in the presence of CHX or triptolide**

Localization of phosphorylated p38 (pp38) is shown in the nuclei of the macrophages treated with 100 ng/mL LPS alone, LPS and 10  $\mu$ g/mL CHX or LPS and 300 ng/mL triptolide after incubation for 15 or 60 min after LPS addition. Green fluorescence indicates pp38, and blue fluorescence, nuclei, as described in the text. Note that most of the green fluorescence is localized in the nuclei and that the fluorescence has disappeared from nuclei of LPS-treated cells but, instead, is localized in nuclei of the cells treated with LPS+triptolide or with LPS+CHX.



**Fig. 7. Inhibition of MKP-1 mRNA induction by triptolide in LPS-treated macrophages**

The cells were left untreated or incubated with 100 ng/mL LPS or LPS and 300 ng/mL triptolide at 37°C for 0, 15, 30 or 60 min. After the cells had been harvested and washed, the cellular RNA was extracted and subjected to RT-PCR, as described in the text. The MKP-1 mRNA levels relative to the GAPDH mRNA level were estimated in each experiment, and the results are expressed as the means $\pm$ SE for 3 independent experiments performed separately on different days. The significance of difference between the groups with LPS in the presence or absence of triptolide *versus* the untreated cells at each time was estimated by Turkey's test and are shown as \*  $P < 0.05$ , and \*\*  $P < 0.01$ , respectively.

### 3.5 Inhibition of MKP-1 mRNA induction by triptolide in LPS-treated macrophages

The results in Fig. 5 showed that MKP-1 was induced at the protein level by 60 min in LPS-treated cells, which induction was abolished by triptolide or CHX. Next, we examined whether MKP-1 mRNA expression was inhibited by triptolide in LPS-treated macrophages. Quantitative RT-PCR revealed that LPS induced significantly higher levels of MKP-1 mRNA at 30 and 60 min after LPS addition than at 0 time or non-treated control at each time (Fig. 7). However, triptolide completely inhibited this induction of MKP-1 mRNA at each time after LPS addition, suggesting that triptolide seems to inhibit MKP-1 at the transcriptional level.

### 4. Discussion

In this study, we sought to elucidate the mechanisms underlying LPS-induced macrophage cell death not through macrophage activation but through mal-regulation of LPS-signaling cascades (Fig. 1). Our previous studies revealed that the addition of CHX simultaneously with LPS to macrophages induced rapid cell death, whereas neither LPS nor CHX alone did not.<sup>4, 5</sup> This novel pathway was shown to be closely related to LPS-responsiveness of the macrophages, because this cell death was not induced in peritoneal macrophages from C3H/HeJ mice,<sup>17</sup> an LPS-non-responder mouse strain, or in LPS-resistant mutant cell lines derived from J774.1/JA-4 cells.<sup>14, 17, 18</sup> Besides, LPS-tolerance to the cytotoxicity is observed in CHX+LPS-treated macrophages,<sup>8</sup> and structure-activity relationships among LPS, lipid A and analogs are seen between the ability of TNF- $\alpha$  release in the

absence of CHX and that of cell damage in the presence of CHX.<sup>19</sup>

Another remarkable phenotype was that the timing of CHX addition to the macrophages before, simultaneous or after LPS addition was key to determining the extent of cell damage afterwards.<sup>4,9</sup> Because CHX acts on cells immediately and inhibits protein synthesis very effectively and dose-dependently, and because LPS transduces its signals to macrophages very rapidly, the combination of experiments constructed with different times of CHX addition<sup>4</sup> and with that of washing-out CHX<sup>5,9</sup> suggested that this novel cell death was caused by abrupt inhibition of LPS-signaling by 60 min. The cell death was shown to be through apoptosis before LDH release, and activation of caspases activities, PARP cleavage, and TUNEL-positivity were shown to precede LDH release.<sup>9</sup> Therefore some key molecule has been supposed to play pivotal roles in the induction of the cell death pathway, and the proposed properties of this molecule include (1) induction by LPS after 30 min but before 60 min<sup>4,5,9</sup>, (2) a short half-life, and (3) a close linkage to LPS-signaling.<sup>14,17-19</sup> In the course of studies on LPS-induced signals,<sup>9</sup> SB202190, a selective inhibitor of p38 MAP kinase, was found to induce apoptotic cell death of the macrophages only in the presence of LPS,<sup>7</sup> and to sustain phosphorylation of p38.<sup>8</sup>

Based on these studies, we focused on the role of sustained phosphorylation of p38 and sought to ascertain this role in the induction of apoptosis in the LPS-treated macrophages by using protein phosphatase inhibitors. Among them, triptolide is known as an inhibitor of the MKP-1 phosphatase,<sup>11,12,20</sup> and is more widely known as an anti-inflammatory compound that suppresses the production of proinflammatory cytokines<sup>21,22</sup> and expression of nitric oxide synthase in LPS-treated macrophages.<sup>23</sup> So we used triptolide in combination with LPS to treat cells of the LPS-sensitive macrophage-like

cell line J774.1/JA-4.<sup>14</sup> As shown in Fig. 7, triptolide strongly inhibited MKP-1 mRNA synthesis as reported in the case of other cells,<sup>24,25</sup> and Western blot analysis revealed depletion of MKP-1 protein in triptolide-treated macrophages in the presence of LPS (Fig. 5). Because triptolide showed no inhibitory effects on [<sup>35</sup>S]methionine incorporation into macrophages in the presence or absence of LPS (data not shown), these results suggest that triptolide inhibited MKP-1 induction by LPS not by protein synthesis inhibition as CHX did (Fig. 5). In the same cells treated with LPS+triptolide, sustained phosphorylation of p38 and JNK was observed (Fig. 5), suggesting that mal-induction of MKP-1 in LPS-treated cells led to sustained phosphorylation of p38 and JNK, but not to that of Erk1/2. There are arguments as to which kinase, p38 or JNK, is more responsible for the regulation of induction of apoptosis<sup>8,26,27</sup>; and the further studies are necessary to examine how continued localization of the phosphorylated kinases leads to initiation of apoptotic changes in these cells.

In this study, we also showed that the timing of triptolide addition was critical for the induction of cell damage to LPS-treated macrophages (Fig. 3B), and 15 min after LPS addition seemed to be too late to induce this damage. In comparison with CHX, which readily penetrates into cells, triptolide required pretreatment of the cells for 30 min before LPS addition to obtain better effects than simultaneous addition. It seems important to consider the chemical and biological properties of triptolide for permeation across the cell membrane to allow binding to its targets. Besides, the time-course of LDH release (Fig. 3C) and the appearance of TUNEL (+) cells (Fig. 4C) also showed some differences between triptolide and CHX. Although a significant number of the TUNEL (+) cells increased earlier in triptolide + LPS-treated group than in the CHX+LPS-treated one (Fig. 4C),



LDH release in the former was gradual with non-significant background release, whereas that in the latter was sudden and sharp (Fig. 3C). These results seem to reflect differences between the properties of triptolide and CHX. Nevertheless, both inhibited the induction of MKP-1 almost completely (Fig. 5). Besides, both induced sustained phosphorylation of p38 and JNK in nuclear extracts of the LPS-treated cells (Fig. 5), as well as in cell nuclei in the case of phosphorylated p38 (Fig. 6).

Concerning the target molecule(s) of triptolide, the following findings suggest MKP-1 phosphatase as being its target: triptolide inhibited MKP-1 mRNA synthesis as early as at 0-15 min after LPS addition (Fig. 7), inhibited the induction of MKP-1 protein by LPS at 60 min (Fig. 5), induced sustained phosphorylation of p38 in the nuclei of the LPS-treated cells at 60 min (Figs. 5, 6), and increased the number of TUNEL (+) cells at 60 min (Fig. 4C) as well as the amount of LDH release at 240 min (Fig. 3C) significantly. Recently, TAB1, which is transforming growth factor- $\beta$ -activated kinase 1 (TAK1)-binding protein 1, was proposed to be a target of triptolide in macrophages.<sup>28</sup> TAK1-TAB1 interaction induced by 500 ng/mL LPS during incubation for 2 h is inhibited by preincubation with 30 nM triptolide for 1 h in Ana-1 macrophage-like cells. They also showed that triptolide inhibits the kinase activity of TAK1 and DNA-binding activity of AP-1.<sup>28</sup> Because TAK1 is the upstream kinase of MAPK kinases,<sup>29</sup> it seems feasible that the activation of p38 and JNK is regulated by TAK1, although there has been no report showing direct regulation of MKP-1 gene expression by this TAK1-TAB1 system in macrophages. Therefore, at present, it seems likely that MKP-1 was the target of triptolide in this study, exerting perturbation of LPS-signaling cascades, resulting in sustained phosphorylation leading to cell death of the macrophages.

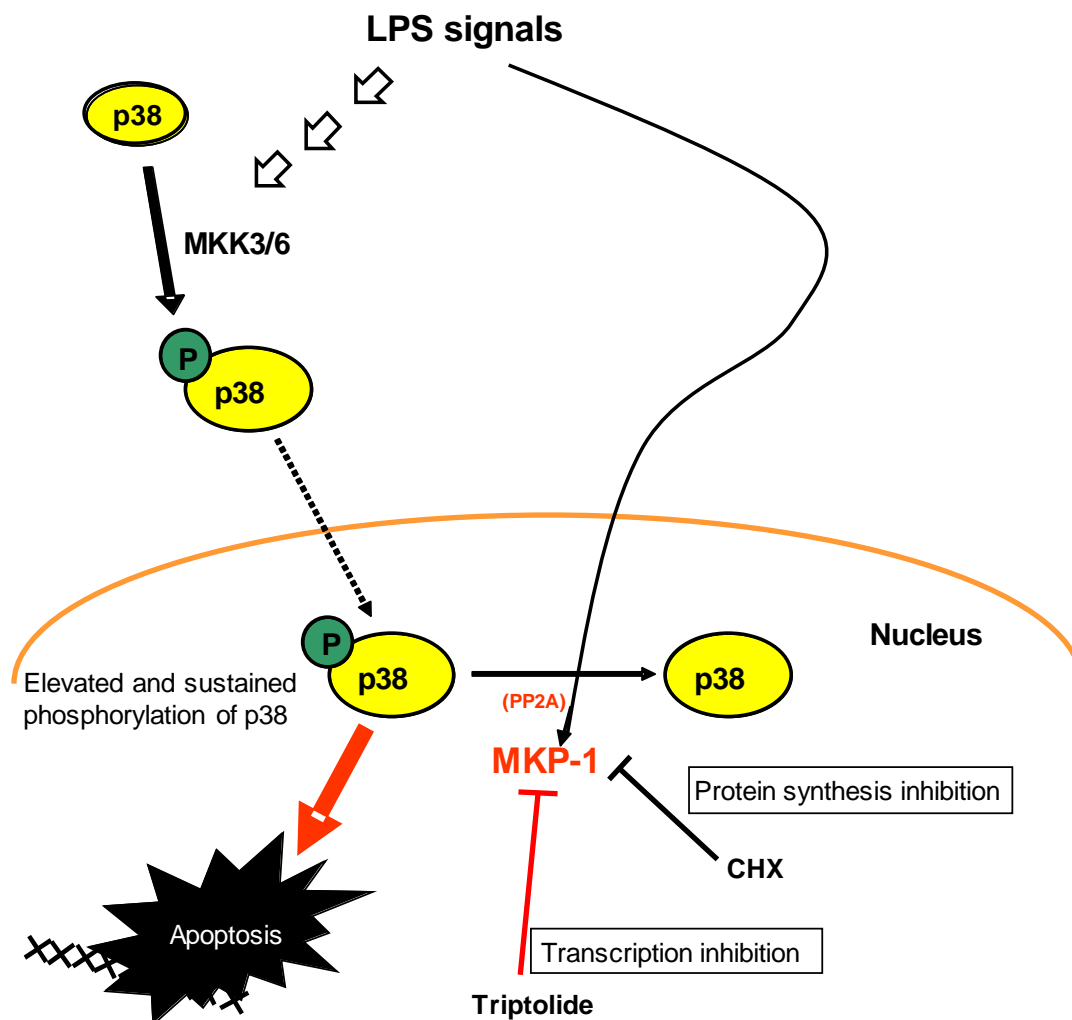
There are some reports describing the effects of triptolide as regards the apoptotic cell death of macrophages: Bao et al. showed that 15-25 ng/mL triptolide increases the generation of reactive oxygen species (ROS) and nitric oxide and induces apoptosis of RAW264.7 cells during incubation at 37°C for 24 h, but they did not add LPS to the cultures.<sup>30</sup> On the other hand, Wu et al. reported that 5-40 ng/mL triptolide attenuates oxidative stress and pro-inflammatory cytokine gene expression in LPS-treated mouse peritoneal macrophages during incubation at 37°C for 24 h, but they provided no description about macrophage cell damage in their experiments.<sup>31</sup> Using human promonocytic U937 cells, Choi et al. reported that 25-75 nM (i.e., 9-27 ng/mL) triptolide induces apoptosis in these cells during incubation at 37°C for 24 h, not through generation of ROS but through activation of caspase-3; but they did not use LPS.<sup>32</sup> These reports described types of cell damage different from those we found, perhaps due to the conditions of the experiments, especially as regards the length of incubation, co-incubation with LPS, and the concentration of triptolide. As to the last point, more than 100 ng/mL triptolide was needed to induce rapid cell damage in LPS-treated J774.1/JA-4 cells, as was shown in Fig. 3A. Furthermore, it should be noted that there are diversities of macrophage phenotypes especially among macrophage-like cell lines and that LPS-induced responses by these macrophages may change according to both incubation time and dose of LPS.<sup>2, 14</sup>

Besides, there are some reports regarding therapeutic application of triptolide; Liu described multiple pharmacological functions of triptolide,<sup>33</sup> and Li *et al* showed anti-cancer activity of triptolide combined with cisplatin in gastric cancer.<sup>34</sup> Based on our present study, triptolide would be more widely used in therapeutic medicine in the future.

## 5. Conclusion

This study shows that triptolide induced apoptotic cell death and subsequent cell damage accompanied by LDH release, inhibited the induction of MKP-1 mRNA and protein, and exhibited sustained phosphorylation of p38 and JNK. These results suggest correlation among

themselves, as illustrated in Fig. 8, implying that MKP-1 was the target of triptolide responsible for regulation of LPS-signaling cascades and that sustained phosphorylation of at least p38 might have led the macrophages to undergo apoptotic cell death for prevention of excessive processes of inflammation.



**Fig. 8. Summary of this study concerning induction of apoptosis and sustained phosphorylation of p38 by CHX or triptolide in LPS-treated macrophages**

LPS rapidly induces MKK3/6 activation, resulting in phosphorylation of p38; and pp38 is translocated into the nuclei. MKP-1, but not PP2A, is also induced transcriptionally by LPS signaling and involved in dephosphorylation of pp38 into p38 by its protein phosphatase activity. This transcriptional induction of MKP-1 mRNA is strongly inhibited by triptolide. The turnover of MKP-1 protein is rather rapid and thus CHX decreases the MKP-1 level by inhibiting protein synthesis. Taken together, CHX and triptolide induce sustained phosphorylation of p38 in LPS-treated macrophages, in which pp38 is accumulated in the nuclei. The pathways for apoptosis induction in these cells, however, remain unclear.

**6. Acknowledgements:** This work was partly supported by JSPS KAKENHI Grant numbers 12672147(FA), 26860072 (AK) and 17K15529 (AK).

**7. Conflict of Interest** The authors declare no conflict of interest.

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