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Triptolide attenuates oxidative stress, NF-κB activation and multiple cytokine gene expression in murine peritoneal macrophage

YUANYUAN WU*, JUN CUI*, XIAOFENG BAO, SIOPENG CHAN, D. OWEN YOUNG[1], DAN LIU and PINGPING SHEN

State Key Laboratory of Pharmaceutical Biotechnology, Department of Biochemistry, Nanjing University, Nanjing 210093, P.R. China

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Abstract. Triptolide is a natural, biologically active compound extracted from the Chinese herb *Tripterygium Wilfordii* hook, that possesses potent immunosuppressive properties. In the course of our study, we examined the effects of triptolide on stimulated murine macrophages and demonstrated that triptolide inhibited superoxide anion production, NO production, and the expression of iNOS mRNA and the mRNA of some key inflammation-related cytokines, namely TNF-α, IL-1ß, IL-6 and IFN-γ. This inhibition was accompanied by the inhibition of nuclear transcription factor NF-κB activation. We further developed a mathematical model to simulate interplay between triptolide, ROS, NF-κB, iNOS and related cytokines. Our results suggest that triptolide acts to regulate inflammation through NF-κB-mediated inhibition of inflammation-related cytokines and the production of superoxide anion and NO.

Introduction

Derived from the Chinese herb *Tripterygium Wilfordii* hook f (TWHf), triptolide (PG-490) is a diterpene triepoxide originally isolated from TWHf on the basis of its cytotoxicity against human nasopharyngeal carcinoma cells *in vitro* and murine leukemia cells *in vivo* (1). Triptolide has subsequently been shown to have myriad other biological effects, including an anti-fertility function (2) and immunosuppressive effects, and has been used to treat patients with autoimmune diseases such as rheumatoid arthritis (3). According to Lu *et al*, triptolide has stronger immunosuppressive capability than cyclosporine and can significantly attenuate the extent of graft coronary arteriosclerosis after heart transplantation (4). The study of triptolide's immunosuppressive properties has uncovered some of its specific molecular effects. Triptolide was found to inhibit mitogen- or antigen-induced proliferation of human peripheral blood T cells (5) and to down-regulate T cell expression of IL-2 at the level of NF-κB transcriptional activation (6). In human peripheral blood B cells, triptolide inhibited immunoglobulin production (5). Triptolide was also found to suppress the activity of NF-κB and c-Jun NH2-terminal kinase, thus inhibiting transcription of the iNOS gene in macrophage cell line RAW 264.7 (7). Despite these breakthroughs, the mechanisms of triptolide's immunosuppressive effects on macrophage phagocyte systems are still unclear.

Macrophages execute important functions in the immune system. Activated macrophages play a role in arthritis by: a) processing and presenting antigens to T cells; b) producing a variety of inflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12, nitric oxide (NO) and other free radicals (eg. superoxide anion); and c) secreting tissue-degrading enzymes (8). Production of inflammatory cytokines and free radicals is considered to be an important parameter of the mononuclear phagocyte system. Nuclear transcription factor (NF-κB) plays an important role in controlling inflammatory gene activation (9), and is maintained in the cytosol as a heterodimer complexed with its inhibitory protein, IκB. When macrophages are stimulated with LPS (lipopolysaccharide), phorbol ester or inflammatory cytokines, IκB is phosphorylated by IκB kinase and degraded. This phosphorylation dissociates NF-κB from IκB and allows NF-κB to translocate to the nucleus, where it activates its target genes (10).

Our present study combines experimental data with mathematical simulation. We first performed a series of *in vitro*

Correspondence to: Dr Pingping Shen, State Key Laboratory of Pharmaceutical Biotechnology, Department of Biochemistry, Nanjing University, Nanjing 210093, P.R. China
E-mail: ppshen@nju.edu.cn

Present address: [1]Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT 84602, USA

Abbreviations: TP, triptolide; NO, nitric oxide; iNOS, inducible NO synthase; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; ROS, reactive oxygen species; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; LDH, lactate dehydrogenase

*Contributed equally

Key words: triptolide, macrophage, superoxide anion, NF-κB, cytokine, simulation

experiments to determine the effects of triptolide on superoxide anion production, NO release and NF-κB activation in primary murine peritoneal macrophages. We also analyzed the expression of iNOS mRNA and the mRNA of some key pro-inflammatory cytokines. The experimental results showed that triptolide had an inhibitory effect on macrophages. Furthermore, based on these experimental results, we developed a mathematical model to validate our conclusions. This combined strategy allowed us to construct a potential triptolide pathway in macrophages. Our findings provide a foundation for future exploration of the immunosuppression mechanism induced by triptolide in macrophages and also introduce a new approach to the study of the anti-inflammatory effects of this traditional herbal medicine.

Materials and methods

Chemicals. Crystalline triptolide was obtained from Alexis (Carlsbad, CA). RT and PCR reagents were purchased from Promega Inc. (Madison, WI). RPMI-1640 medium was supplied by Hyclone (Logan, UT). Other chemicals were purchased from Sigma Chemicals Inc. (St. Louis, MO) unless otherwise mentioned.

Animals. Virus-free 6-week-old male BALB/c mice weighing 18-22 g were purchased from the Animal Center of Nanjing General Hospital of Nanjing Command. Mice were randomized and transferred to plastic cages containing sawdust bedding (five mice per cage) which was changed every third day. They were given food and water *ad libitum* and were acclimated to an environment of 21-24˚C, 40-60% relative humidity, and a 12-h light-dark cycle for a minimum of 1 week prior to study.

Cell culture and treatment. Peritoneal cells were harvested as described (11), and cultured in RPMI-1640 medium containing 10% fetal calf serum supplemented with 100 unit/ml penicillin and 100 μg/ml streptomycin. The macrophages collected above were co-stimulated with 100 ng/ml LPS and triptolide at doses of 5, 10, 20 or 40 ng/ml for 24 h according to the kinetic titration curve studied before the experiment. Macrophages incubated in the culture medium alone acted as the negative control and those incubated with both culture medium and 100 ng/ml LPS acted as the positive control.

Cell viability assay. Trypan blue exclusion was performed to assess percent cytotoxicity of triptolide treatment. Cell cultures were established in 96-well culture plate *in vitro*. Aliquots of a suspension of 1.0×10^6 peritoneal lavage cells/ml were co-stimulated with 100 ng/ml LPS and triptolide at doses of 5, 10, 20 or 40 ng/ml. After 24-h incubation, cell injury was quantitatively assessed by counting macrophages stained with trypan blue. We added 10 μl 0.4% sterile trypan blue per well and thoroughly mixed it with the cells for 30 min. Cells were counted under an inverted microscope. Percent cytotoxicity was calculated from the means of values of three independent experiments using the following equation:

$$\text{Percent cytotoxicity} = \left(1 - \frac{\text{number of live unstained cells incubated with triptolide}}{\text{total number of live unstained cells and damaged stained cells}}\right) \times 100\%$$

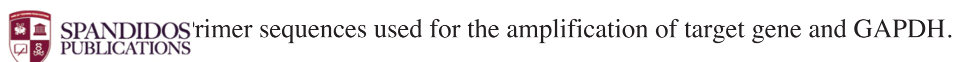
Cell injury caused by triptolide was also quantitatively assessed by measuring the lactate dehydrogenase (LDH) released by damaged or destroyed cells. We tested the extracellular fluid 24 h after incubation using a quantitative Sigma colorimetric kit (Sigma Diagnostics, No. 500-c). Briefly, cellular supernatants (50 μl) were removed from each well and transferred to a 96-well plate and combined with the bathing media containing NADH and pyruvate solutions. LDH activity is proportional to the rate of pyruvate loss. Colorimetric changes were read at 450 nm. Values of LDH release were expressed as Berger and Broida unit/ml of sample (BBU/ml). Tests were performed in triplicates from three experiments.

Neutral red uptake (NRU) assay. The NRU assay was performed according to the method of Borenfreund and Puerner to determine the phagocytic activity of macrophages (12). After 4 h of incubation with serum-free medium containing neutral red (50 mg/l), the cells were washed quickly with PBS and then 0.1 ml of acetic acid at 1% (v/v) in a solution of ethanol 50% (v/v) was added to each well to extract the dye phagocytized by macrophages. After rapid agitation on a microtitre plate shaker, the absorbance was read at 540 nm. Tests were performed in triplicates from three experiments.

Measurement of superoxide anion generation. Superoxide radicals are generated by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiments, the superoxide radicals were generated in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 mM) solution, 1 ml NADH (78 mM) solution and sample solution of cell culture supernatants (200 μl) were mixed. The reaction started by adding 1 ml of phenazine methosulphate (PMS) solution (10 mM) to the mixture. The reaction mixture was incubated at 25˚C for 5 min, and the absorbance at 550 nm in a spectrophotometer was measured against blank samples. L-ascorbic acid was used as a control.

Nitrite assay. Macrophage suspensions from mice were prepared as above. NO production was determined by an assay of the cell-free culture supernatant for nitrite (NO_2^-). Supernatant (100 μl) was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). The absorbance at 550 nm was read after incubation at room temperature for 10 min and nitrite concentration was measured by interpolation of the curve calibrated from sodium nitrite standards.

Preparation of nuclear extracts. Murine peritoneal macrophage were grown in 6-well plates and co-stimulated with LPS (100 ng/ml) and triptolide at doses of 5, 10, 20 or 40 ng/ml for 2 h. Nuclear extracts were prepared as described in ref. 13. Briefly, cells were washed with PBS, resuspended in 400 μl hypotonic buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.9; 10 mM KCl; 0.1 mM ethylenediaminetetraacetic acid (EDTA); 1 mM dithiothreitol (DTT); 0.5 mM phenylmethyl sulfonyl fluoride (PMSF)] and were allowed to swell on ice for 15 min. Nonidet P-40 (10%, 25 μl) was added followed by 10 sec of vigorous



rimer sequences used for the amplification of target gene and GAPDH.

Target gene	Primer sequence	Annealing temp. (˚C)	Size (bp)	Cycles
iNOS	(+) 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3' (-) 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3'	55	311	30
IL-1ß	(+) 5'-TTG ACG GAC CCC AAA AGA TG-3' (-) 5'-AGA AGG TGC TCA TGT CCT CA-3'	57	204	30
TNF-α	(+) 5'-GAG TGA CAA GCC TGT AGC C-3' (-) 5'-GGT TGA CTT TCT CCT GGT AT-3'	57	450	30
IL-6	(+) 5'-TTC TTG GGA CTG ATG CTG -3' (-) 5'-CTG GCT TTG TCT TTC TTG TT-3'	54	380	30
IFN-γ	(+) 5'-TGG AGG AAC TGG CAA AAG GAT GGT-3' (-) 5'-TTG GGA CAA TCT CTT CCC CAC-3'	56	336	35
GAPDH	(+) 5'-AAC GAC CCC T TC ATT GAC C-3' (-) 5'-TCA GAT GCC TGC TTC ACC-3'	57	701	30

(+), sense; (-), anti-sense.

vortexing and centrifugation at 12,000 x g for 30 sec. The supernatant was removed and the nuclear pellet was extracted with 50 μl of hypertonic buffer B (20 mM HEPES pH 7.9; 0.4 mM NaCl; 1 mM EDTA; 1 mM DTT; 1 mM PMSF) by shaking at 4˚C for 30 min. The extract was centrifuged at 12,000 x g for 10 min and the supernatant was frozen at -70˚C. The protein concentration was determined by the Lowry method.

Electrophoretic mobility shift assay (EMSA). Two double-stranded oligonucleotide probes containing a consensus binding-sequence for NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') were 3' end-labeled with DIG. EMSA experiments were performed as described previously (13,14). Briefly, 10 μg nuclear extracts obtained above were incubated with the labeled probe for 1 h on ice and resolved in a 6% non-denaturing polyacrylamide gel. Specificity of the DNA-protein complex was confirmed by competition with a 100-fold excess of unlabeled NF-κB sequences.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from incubated cells by using TriPure isolation reagent kit (Roche, IN, USA) according to the manufacturer's instructions. The yield and quality of the RNA were assessed by measuring absorbance at 260 and 280 nm followed by electrophoresis on 1% agarose gels. One microgram of RNA was reverse-transcribed into cDNA by using oligo $(dT)_{15}$ primers and AMV reverse-transcriptase at 42˚C for 1 h in standard buffer. Sequence-specific primers for iNOS, the pro-inflammatory cytokines and house-keeping gene GAPDH were used to cDNA amplification. GAPDH, iNOS and cytokine gene primers are shown in Table I and RT-PCR was performed as follows: cDNA amplification was initiated after heat denaturation at 95˚C for 5 min. Thermal cycling conditions were as following: 95˚C for 25 sec; various annealing temperatures for each gene for 45 sec (Table I); 72˚C for 30 sec, with a final extension step at 72˚C for 7 min. PCR products were electrophoresed through 1.0% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. Band intensity was calculated densitometrically using Gelwork 1D Intermediate, UVP software. Levels of mRNA were expressed as the ratio of band intensity for target genes to that for GAPDH.

Statistical analysis. Statistical analysis was performed using SPSS software. All values were expressed as the mean ± standard error (SE). Data for cytokine mRNA levels were tested by the Student's t-test. $^{*}P<0.05$ or $^{**}P<0.01$ (as indicated in figures) was considered statistically significant.

Mathematical modeling. Previously, mathematical models have been successfully applied to simulate the dynamic behavior of the NF-κB pathway (15). In this study, we developed a theoretical model based on our hypothesis to describe a possible mechanism of interplay between ROS, NF-κB and several cytokines. The possible interactions of ROS, NF-κB and cytokines in the model are shown in Fig. 10. It includes: a) triptolide attenuates the production of ROS while LPS plays the opposite role; b) ROS could initiate the process of activation and translocation of NF-κB, also it could inhibit the DNA-binding ability of NF-κB (16); c) NF-κB could initiate the transcription of TNF-α, IL-1ß and iNOS in heterodimer form (17); d) triptolide could inhibit the DNA-binding ability of NF-κB (18); e) NF-κB could be activated by TNF-α and IL-1ß via receptor pathway. The differential equations for this model are given:

$$\frac{dROS}{dt} = \frac{p_{ros} + k_{ros1} \cdot lps(t)}{k_{ros2} \cdot tp(t) + c_{e1}} - u_{ros} \cdot ROS(t) \quad [1]$$

$$\frac{dNF\kappa B}{dt} = p_{NF\kappa B} + k_{NF1} \cdot ROS(t) + \frac{k_{NF2} \cdot TNF(t)}{c_{e2} + TNF(t)} + \frac{k_{NF3} \cdot IL(t)}{c_{e3} + IL(t)} - u_{NF\kappa B} \cdot NF\kappa B(t) \quad [2]$$

$$\frac{dTNF}{dt} = p_{TNF} + k_{TNF1} \cdot (\frac{NF\kappa B(t)}{NF\kappa B(t) + c_{e4}})^2 \cdot \frac{1}{k_{TNF2} \cdot ROS(t) + c_{e5}} \cdot f(tp) - u_{TNF} \cdot TNF(t) \quad [3]$$

$$\frac{dIL}{dt} = p_{IL} + k_{IL1} \cdot (\frac{NF\kappa B(t)}{NF\kappa B(t) + c_{e6}})^2 \cdot \frac{1}{k_{IL2} \cdot ROS(t) + c_{e7}} \cdot f(tp) - u_{IL} \cdot IL(t) \quad [4]$$

$$\frac{diNOS}{dt} = p_{iNOS} + k_{iNOS1} \cdot (\frac{NF\kappa B(t)}{NF\kappa B(t) + c_{e8}})^2 \cdot \frac{1}{k_{iNOS2} \cdot ROS(t) + c_{e9}} \cdot f(tp) - u_{iNOS} \cdot iNOS(t) \quad [5]$$

The definitions of state variables and parameters are given in Tables III and IV. The variable tp(t) and lps(t) in equations 1, 3, 4 and 5 denote the concentration of triptolide or LPS in the cells. They could be written as:

$$tp(t) = k_{tp} \cdot (TP - tp(t)) - w_{tp} \cdot tp(t) \quad [6]$$

$$lps(t) = k_{lps} \cdot (LPS - lps(t)) - w_{lps} \cdot lps(t) \quad [7]$$

The function f(tp) describes the effect of triptolide to inhibit DNA binding ability of NF-κB. We postulate the effect of triptolide as the equation below:

$$f(tp) = \frac{q_1 \cdot tp(t) + c_{e10}}{q_2 \cdot tp(t) + c_{e11}} \quad [8]$$

All the model equations described above were solved mathematically using the ODE23s routine of Matlab 6.5 (The Mathworks Inc., Natick, USA). The simulation programs were written in M files using commonly used Matlab subroutines and all the graphics were performed using Matlab 6.5.

Results

Effect of triptolide on cell viability. Because an increase in cell mortality would also result in lower levels of superoxide anion and NO, we tested cell viability at the four different concentrations of triptolide used in our experiment using the trypan blue exclusion method and LDH releasing assay. As shown in Fig. 1 and Table II, cells treated with triptolide at concentrations of 5, 10, 20 and 40 ng/ml did not demonstrate any change in percent cytotoxicity or statistical elevation in LDH release compared to the negative control (cells incubated without triptolide or LPS). Cells remained attached to the incubation plate and showed no morphologic changes when observed under an inverted microscope. Taken together, these results led us to conclude that exposure to triptolide at concentrations of 5, 10, 20 and 40 ng/ml was not cytotoxic and could not trigger cell death and that variations in cellular production were not a phenomenon of cell death.

Triptolide inhibits phagocytic activity and superoxide anion production in murine peritoneal macrophage. Phagocytosis is the most representative function of macrophages. We determined the effect of triptolide on phagocytic activity in LPS-stimulated murine peritoneal macrophages by neutral red uptake assay. Cells were treated with triptolide at doses of 5, 10, 20 or 40 ng/ml and 100 ng/ml LPS for 24 h. As shown in Fig. 2, phagocytic activity increased following 100 ng/ml LPS stimulation, but decreased by 7.2%, 15.4%, 16.7% and 18.6% 24 h after triptolide addition at doses of 5, 10, 20 and 40 ng/ml, respectively.

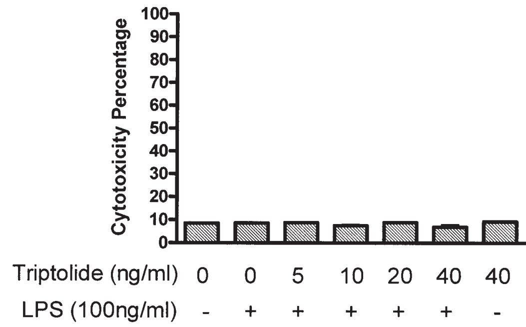


Figure 1. Cytotoxicity of triptolide for murine peritoneal macrophages. Larvage macrophages, 1.0x10^6 cell/ml, diluted with RPMI-1640 were incubated with triptolide at dose of 5, 10, 20, 40 ng/ml, respectively, and co-stimulated with 100 ng/ml LPS or not for 24 h.

Table II. Amounts of LDH released by murine peritoneal macrophages after incubation with triptolide and/or LPS for 24 h.

Triptolide (nmol/l)	LPS (100 μg/l)	LDH activity (BBU/ml)
0	-	195.2±22.8
0	+	150.5±11.9
5	+	207.8±14.9
10	+	209.5±19.4
20	+	190.9±22.1
40	+	217.3±25.3
40	-	203.0±21.8

Cells maintained in suspension in growth medium without triptolide nor LPS served as control group. Values (BBU/ml) are means ± SE.

Table III. The biological meanings of the abbreviations in equations.

Abbreviations	Meaning
tp	Triptolide
lps	Lipopolysaccharide (LPS)
ROS	Superoxide anion
NFκB	Active NF-κB
TNF	TNF-α
IL	IL-1ß
iNOS	Inducible NO synthase

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Biological interpretation of the parameters in equations.

Parameter	Biological interpretation
p_i (i=ROS, NFκB, TNF, IL, or iNOS)	Normal production or activation coefficient of mRNA or protein related
u_i (i=ROS, NFκB, TNF, IL, or iNOS)	Degradation coefficient of mRNA or protein related
k_{NFi} (i=1, 2, 3)	Activation coefficient of NF-κB by ROS, TNF-α or IL-1ß
k_{i1} (i=ros, TNF, IL, or iNOS)	Activation coefficient of ROS, TNF-α, IL-1ß or iNOS by LPS or NF-κB
k_{i2} (i=ros, TNF, IL, or iNOS)	Inhibition coefficient of ROS, TNF-α, IL-1ß or iNOS by triptolide or ROS
c_{ei} (i=1, 2, ... n)	The significant effect constant of related variable
k_j (j=tp, lps)	Absorbing coefficient of triptolide or LPS
w_j (j=tp, lps)	Loss coefficient of triptolide or LPS
q_i (i=1, 2)	The significant effect coefficient of triptolide to reduce NF-κB DNA-binding ability
TP	The initial concentration of triptolide incubated with cells
LPS	The initial concentration of LPS incubated with cells

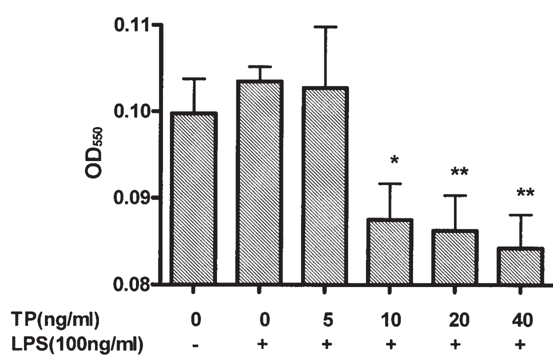

Figure 2. Phagocyte ability for murine peritoneal macrophages evaluated by NRU assay. Cells were incubated for 24 h with the indicated concentration of LPS and triptolide.

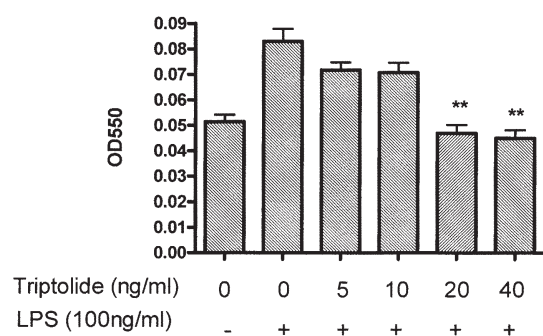

Figure 3. Triptolide inhibits superoxide anion production in murine peritoneal macrophages during the respiratory burst. Cells were incubated for 24 h with the indicated concentrations of LPS and triptolide. Data are expressed as mean ± SE of three experiments with quadruple cultures. Asterisks denote a response that is significantly different from the positive control (*P<0.05, **P<0.01).

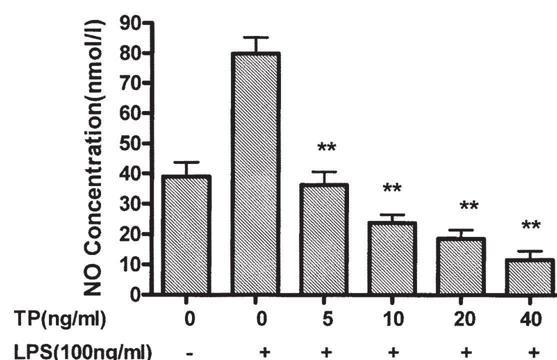

Figure 4. Triptolide inhibits the production of NO in murine peritoneal macrophages. Cells were stimulated with LPS in the presence or absence of triptolide at indicated concentration. The difference between the positive control (LPS treatment only) group and treated group were determined by Student's t-test. Data are expressed as mean ± SE of three experiments with quadruple cultures. Asterisks denote a response that is significantly different from the positive control (*P<0.05, **P<0.01).

We next examined the effects of triptolide on superoxide anion production in murine peritoneal macrophages. It is generally accepted that superoxide anion production can be taken as an index of oxidative metabolism during respiratory burst in phagocytic cells (19). We stimulated the macrophages with 100 ng/ml LPS to mimic inflammation and induce respiratory burst and then treated those macrophages with triptolide at various concentrations of either 5, 10, 20 or 40 ng/ml, respectively, for 24 h. As shown in Fig. 3, triptolide inhibited LPS stimulated superoxide anion production in peritoneal macrophages in a dose-dependent manner. The inhibition reached a maximal value (54.2% of control group value) at a triptolide dose of 40 ng/ml.

Triptolide inhibits NO production and abundance of iNOS mRNA. To investigate the effects of triptolide on NO production, we used a Griess reagent to measure the accumulation of nitrite (the stable end product of NO) in the macrophage culture media. Adherent peritoneal cells were isolated from male BALB/c mice and were treated with 100 ng/ml LPS and triptolide at doses of 5, 10, 20 or 40 ng/ml for 24 h. As shown in Fig. 4, the NO produced by the macrophages treated with triptolide was lower than those incubated with LPS alone. When macrophages were treated simultaneously with

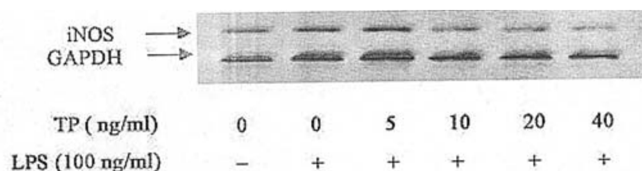

Figure 5. Abundance of iNOS mRNA in macrophages stimulated with LPS and triptolide at indicated dose for 24 h by RT-PCR determination. PCR products for iNOS and GAPDH were run on a 1.0% agarose gel and the relative intensities of the bands corresponding to the target and internal standard (GAPDH) PCR products were visualized in UV light. GAPDH expression was used to confirm equal loading of RNA in each lane. Only the representative gels are shown.

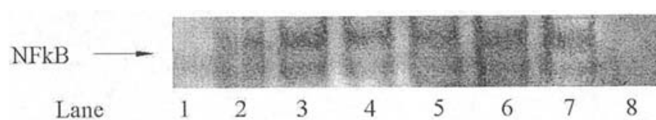

Figure 7. Triptolide inhibits LPS induced NF-κB activation in murine peritoneal macrophages. Cells were cultured for 2 h with LPS (100 ng/ml) and triptolide at indicated dose respectively. Electrophoretic mobility shift assay (EMSA) results (1 of 5 experiments) were obtained as described in Materials and methods. No nuclear extracts were added in lane 1 to serve as a negative control. Lane 2, untreated cells; lane 3, LPS alone; lane 4, LPS plus triptolide at 40 ng/ml; lane 5, LPS plus triptolide at 20 ng/ml; lane 6, LPS plus triptolide at 10 ng/ml; lane 7, LPS plus triptolide at 5 ng/ml. As a specificity control, nuclear extracts in lane 8 were pre-incubated with a 100 molar excess of cold non-labeled probe.

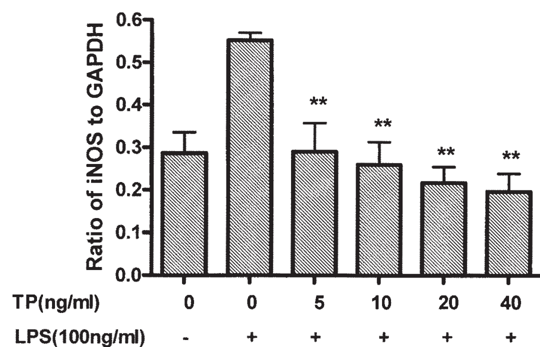

Figure 6. Inhibitive effect of iNOS mRNA expression by triptolide in macrophages. Cells were cultured for 24 h with LPS and triptolide at indicated dose respectively. Total RNA was prepared and analyzed by RT-PCR. The difference between the positive control group (LPS treatment only) and treated group were determined by Student's t-test. Data are expressed as mean ± SE of three experiments with quadruple cultures. Asterisks denote a response that is significantly different from the positive control ($^{*}P<0.05$, $^{**}P<0.01$).

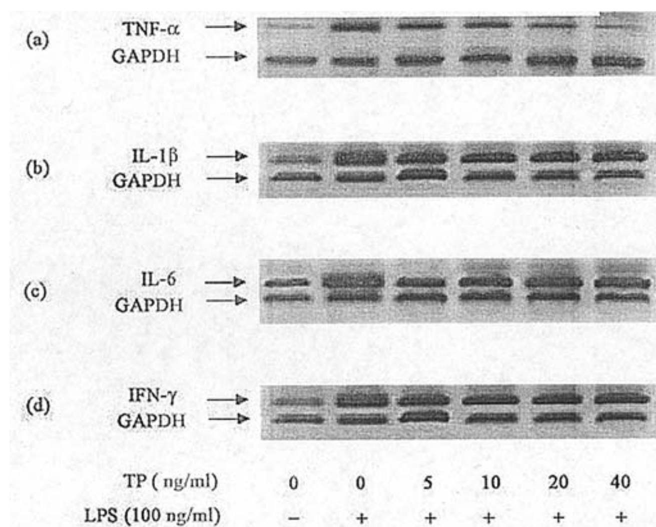

Figure 8. Abundance of (a) TNF-α, (b) IL1-ß, (c) IL-6, (d) INF-γ and GAPDH mRNA in macrophages stimulated with LPS and triptolide at indicated dose for 24 h. PCR products for each cytokine gene and GAPDH were run on a 1.0% agarose gel and the relative intensities of the bands corresponding to the target and internal standard (GAPDH) PCR products were visualized in UV light. GAPDH expression was used to confirm equal loading of RNA in each lane. Only the representative gels are shown.

triptolide and LPS, a significant decrease in NO production was noted. Upon triptolide administration (5, 10, 20, 40 ng/ml, respectively), NO decreased by 54.6%, 70.2%, 76.7% and 85.4% in contrast to the LPS-only group. Since NO production is catalyzed by iNOS, we assessed iNOS mRNA levels in order to determine whether triptolide could also interfere with iNOS gene transcription. As shown in Figs. 5 and 6, a suppressive effect of triptolide on iNOS mRNA levels was observed. The dose-dependent inhibition was statistically significant. The down-regulating extent was 47.4%, 53.0%, 60.7% and 64.4%, respectively at doses of 5, 10, 20 and 40 ng/ml triptolide.

Effects of triptolide on LPS-induced activation of NF-κB. NF-κB represents a critical transcription factor involved in the inflammation process. LPS stimulates a profound activation of NF-κB (Fig. 7, lanes 1 and 2). Addition of unlabeled NF-κB to the reaction mixture showed the specificity of the detected signals. Triptolide generated a significant reduction of LPS-induced activation of NF-κB in murine peritoneal macrophage.

Triptolide inhibits TNF-α, IL-1ß, IL-6 and IFN-γ expression at the level of transcription. Recent studies have revealed that inflammatory cytokines play a critical role in underlying inflammatory processes and related studies may provide effective therapeutic strategies (20). We investigated the effects of triptolide on gene expression of pro-inflammation cytokines at the mRNA level *in vitro*. The down-regulation of the mRNA for the pro-inflammatory cytokines TNF-α, IL-1ß, and IL-6 was observed after stimulation with triptolide. These results are shown in Figs. 8 and 9. For each of the cytokines (TNF-α, IL-1ß, IL-6), the maximal suppressive dose of triptolide was 40 ng/ml, at which the mRNA levels of those cytokines decreased by 21.8%, 21.6% and 29.7%, respectively. These results demonstrated that triptolide was very efficient in the reduction of those cytokines *in vitro*. Furthermore, IFN-γ mRNA levels were proved to be decreased by triptolide. The greatest inhibition rate was 31.0% at 30 ng/ml. As shown in Fig. 9, triptolide had an obvious general inhibitory effect on IFN-γ mRNA abundance, though this effect was not dose-dependent.

Simulation of triptolide inhibition on superoxide anion production and iNOS, TNF-α, IL-1ß gene expression. Using mathematical modeling, our proposed hypothetical expression

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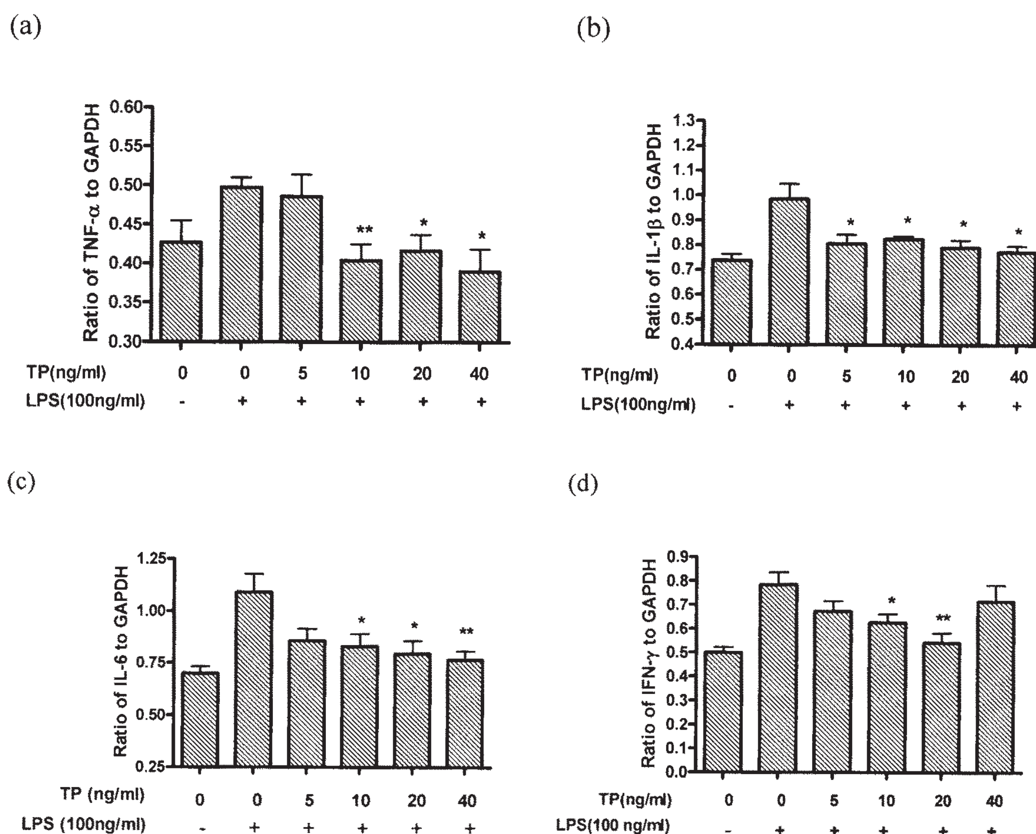

Figure 9. Abundance of (a) TNF-α, (b) IL1-ß, (c) IL-6, (d) INF-γ and GAPDH mRNA in macrophages stimulated with LPS and triptolide at indicated dose for 24 h. Total RNA was prepared and analyzed by RT-PCR. GAPDH expression was used to confirm equal loading of RNA in each lane. The difference between the positive control group (LPS treatment only) and treated group were determined by Student's t-test. Data are expressed as mean ± SE of three experiments with quadruple cultures. Asterisks denote a response that is significantly different from the positive control (*P<0.05, **P<0.01). Only the representative gels are shown.

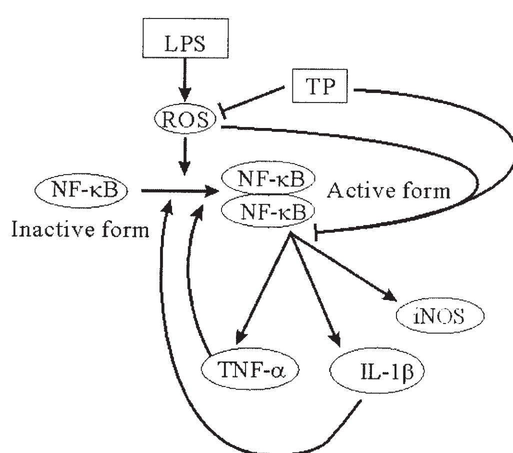

Figure 10. A possible triptolide-ROS-NF-κB pathway.

patterns can be tested *in silico*. Fig. 11 shows the simulated levels of superoxide anion production and iNOS, TNF-α, IL-1ß gene expression in response to different doses of triptolide. In general, this mathematical model showed expression patterns for the cytokines and iNOS that were consistent with experimental data, proving the viability of the triptolide-ROS-NF-κB pathway (Fig. 10). At high doses of triptolide, however, the simulated expression levels of TNF-α and IL-1ß are lower than those observed, indicating that other regulatory mechanisms may be involved in this triptolide-mediated pathway.

Discussion

Macrophages are the main components of the innate immune system. They are involved in both host defense and various other pathological settings characterized by excessive inflammation. Accordingly, they are key targets for immunomodulatory drugs (21). In order to clarify the mechanism underlying triptolide-induced innate immunity suppression, we examined the effects of triptolide on cellular oxidative stress status, NF-κB activation and the gene expression patterns of multiple key pro-inflammatory cytokines in murine primary macrophages. We chose concentrations of triptolide that did not affect cell viability, as indicated by the trypan blue exclusion and LDH releasing assays. Therefore, the immunosuppression effects of triptolide could not be attributed to cell death or variations in membrane permeability.

Our experimental results showed that triptolide suppressed cellular phagocytic activity in a LPS-stimulated macrophage activation model. Phagocytosis plays a pivotal role in autoimmune reactions by removing pathogens and tissue debris during inflammatory reactions. In some instances the over-

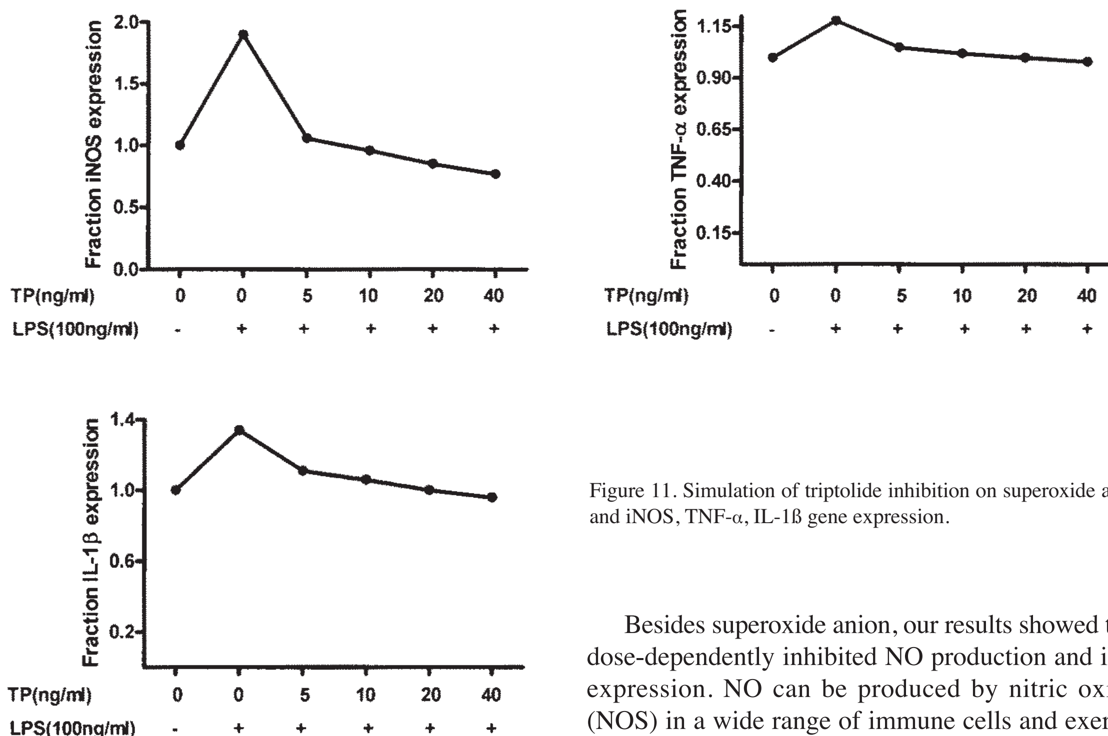

Figure 11. Simulation of triptolide inhibition on superoxide anion production and iNOS, TNF-α, IL-1ß gene expression.

reaction of the phagocytosis leads to massive recruitment of peripheral macrophages and can amplify the inflammatory response, which is potentially harmful. Physiologically, it is important for the body to control the adverse amplified phagocytosis and direct macrophages toward a restorative inflammatory response. The LPS-stimulated macrophage activation model used in our experiment mimics *in vitro* the excessive immuno-inflammation of the body. Thus, our description of triptolide's down-regulatory effects on LPS-simulated phagocytosis in murine macrophages might be used to help alleviate trauma stress caused by inflammation and infection.

We also demonstrated that triptolide could suppress the generation of reactive oxygen species (ROS) such as superoxide anion and NO. Superoxide anion and NO are short-lived free radicals with important physiological functions. They are second messengers and mediators of tissue damage during inflammation. Superoxide anion is produced by activated macrophages, leading to a rapid and impressive consumption of oxygen by a membrane oxidase, NADPH oxidase, which reduces the oxygen to superoxide anion, creating the well-known 'respiratory burst' (22). Superoxide anion radicals released by macrophages during respiratory burst are observed to have dual effects: they can attenuate the infection process by killing foreign organisms, but are themselves potentially injurious to the surrounding tissues (23). For example, superoxide anions are known to attack or degrade hyaluronic acid, which, in turn, results in a decrease in the viscosity of rheumatoid synovial fluid, leading to the development of rheumatoid arthritis. The decline of superoxide anion production as a result of triptolide treatment indicated the regulatory role of triptolide on ROS and the spread of tissue damage during the development of inflammation and infection.

Besides superoxide anion, our results showed that triptolide dose-dependently inhibited NO production and iNOS mRNA expression. NO can be produced by nitric oxide synthase (NOS) in a wide range of immune cells and exerts regulatory effects on these cells. There are three NOS isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. Usually, nNOS and eNOS are constitutively expressed and regulated primarily at the post-transcriptional level, while iNOS is regulated predominantly at the transcriptional level. It is known that, once induced, iNOS can synthesize large amounts of NO, thereby significantly increasing its concentration. Many studies have shown that NOS inhibition reduces inflammation development in chronic inflammation (24). Triptolide's obvious transcriptional level inhibition of NO production may allow it to act as an immunosuppressor on the innate immune system. Our observations suggested that triptolide also plays a role in the regulation of oxidative stress disbalance, which, if left unchecked, would otherwise lead to tissue damage and immune system dysfunction.

Furthermore, our results presented the first evidence that triptolide modulates the induction of TNF-α, IL-1ß, IL-6 and IFN-γ cytokines at the transcription level. These cytokines can originate from macrophages and be secreted at a very early stage of inflammation. They play a crucial role in the initiation of inflammatory cascade reactions and subsequent peripheral tissue destruction. TNF-α, the primary mediator in rheumatoid arthritis, is secreted very early in the inflammatory process and joins in many immune reactions. IL-1ß has been observed to have a wide range of biological activities on many different target cells, including B cells, T cells and monocytes (25). IL-6 is a mediator of host response to tissue injury. Finally, IFN-γ is a pleiotropic cytokine endowed with potent immunomodulatory effects whose expression has long been considered to be restricted to T and NK cells. Only recently has it become evident that IFN-γ production can also occur in many other cell types, including monocytes and macrophages (26). Its release may act as an auto-stimulatory signal for further cytokine production (27). Moreover, IFN-γ has also been found to have an effect on T cell growth and differentiation. It is a growth-promoting factor for T-lymphocytes and can potentiate the response of these cells

ns or growth factors (28). Triptolide's suppressive n the gene expression of these pro-inflammatory cytokines implicated it as an anti-inflammatory factor in immune reactions.

NF-κB, a transcription factor sensitive to oxidative stress, is known to regulate iNOS gene expression in macrophages (29,30). Binding sites for NF-κB have been identified in the promoter regions of those cytokines assessed above (31-33). In this study, we demonstrate the inhibitory potential of triptolide through its attenuation of NF-κB activation. There is a large set of genes with NF-κB binding regions in their cis-acting elements. These genes encode defensive and signaling proteins, including cell surface molecules involved in immune function, such as the immunoglobulin κ light chain, class I and II major histocompatibility complexes (MHC) and various cytokines (IL-1, IL-2, IL-6, IL-8, IFN-γ, G-CSF, GM-CSF and TNF). Because of its role in inhibiting the activation of NF-κB, triptolide should be considered an inflammation suppressor.

NF-κB has been proven to be activated by TNF-α and IL-1ß. In order to validate these experimental results and gain further insight into the mechanism of triptolide-mediated regulation of NF-κB, we combined mathematical modeling with computer simulation to create a novel approach to the study of triptolide-induced immunosuppression. By optimizing various parameters and running Matlab simulations, we generated different graphs to predict the expression of superoxide anion, iNOS and the pro-inflammatory cytokines TNF-α and IL-1ß when macrophages were exposed to increasing doses of triptolide. Our mathematical predictions agreed with the data from our PCR quantization of iNOS mRNA levels. Moreover, the model predicted the same trend for the decrease of mRNA levels for TNF-α and IL-1ß (upon increasing doses of triptolide) as found experimentally, though there were slight differences between the predicted and experimental values. It is entirely possible that the expression of TNF-α and IL-1ß is not regulated by NF-κB alone; additional factors and/or interactions may be involved and await discovery.

Based on the above analysis, however, we proposed a hypothetical model of a triptolide-ROS-NF-κB pathway (Fig. 10). In this pathway, in addition to inhibiting NF-κB, triptolide also suppresses multiple other aspects of the macrophage immune response, including free radical generation (superoxide anion, NO) and the transcriptional level production of some key pro-inflammatory cytokines (TNF-α, IL-1ß, IL-6 and IFN-γ).

In conclusion, the immunosuppression mechanism of triptolide is complex and still elusive. A variety of cells are involved in this process in which macrophages play a leading role. Considering the complexity of experimental research into the mechanisms of triptolide, a combination of data from conventional experimentation with mathematical computer simulation is a useful, time-saving approach to research. The present study is the first report presenting both the experimental results of various triptolide-induced cytokine gene expression patterns as well as the accompanying mathematical simulations. Our results cast new light on the anti-inflammatory and immunosuppressive effects of triptolide. Still, much more effort is required to fully explore the mechanisms of triptolide involved in immune regulation.

Acknowledgements

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