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# Trametinib, a novel MEK kinase inhibitor, suppresses lipopolysaccharide-induced tumor necrosis factor (TNF)-α production and endotoxin shock

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ABSTRACT

Lipopolysaccharide (LPS), one of the most prominent pathogen-associated molecular patterns (PAMPs), activates macrophages, causing release of toxic cytokines (i.e. tumor necrosis factor (TNF)-α) that may provoke inflammation and endotoxin shock. Here, we tested the potential role of trametinib, a novel and highly potent MAPK/ERK kinase (MEK) inhibitor, against LPS-induced TNF-α response in monocytes, and analyzed the underlying mechanisms. We showed that trametinib, at nM concentrations, dramatically inhibited LPS-induced TNF-α mRNA expression and protein secretion in transformed (RAW 264.7 cells) and primary murine macrophages. In *ex-vivo* cultured human peripheral blood mononuclear cells (PBMCs), this MEK inhibitor similarly suppressed TNF-α production by LPS. For the mechanism study, we found that trametinib blocked LPS-induced MEK-ERK activation in above monocytes, which accounted for the defective TNF-α response. Macrophages or PBMCs treated with a traditional MEK inhibitor PD98059 or infected with MEK1/2-shRNA lentivirus exhibited a similar defect as trametinib, and nullified the activity of trametinib. On the other hand, introducing a constitutively-active (CA) ERK1 restored TNF-α production by LPS in the presence of trametinib. *In vivo*, mice administrated with trametinib produced low levels of TNF-α after LPS stimulation, and these mice were protected from LPS-induced endotoxin shock. Together, these results show that trametinib inhibits LPS-induced TNF-α expression and endotoxin shock probably through blocking MEK-ERK signaling.



## 1. Introduction

Inflammation is a complex interaction between pathogen-associated molecular patterns (PAMPs) and different type of immune cells [1,2]. It could happen in any tissues or organs in response to traumatic, infectious, toxic, or autoimmune injuries [3]. The appropriate inflammatory response is a self-defensive mechanism to protect affected cells/tissues from injurious stimuli, and to initiate the healing process [2,4,5]. Severe and acute inflammations, on the other hand, could cause tissue damages, sepsis, cancer and endotoxin shock if not properly treated [2,4,5].

Lipopolysaccharide (LPS), the endotoxin from the Gram-negative bacteria walls, is one of the most prominent PAMPs [3,6,7]. LPS, with the help from LPS-binding protein (LBP) and CD-14, recognizes toll-like receptor 4 (TLR4) on the plasma surface of macrophages [3,7,8]. The LPS-TLR4 binding will recruit several adaptor proteins, such as MyD-88 (Myeloid differentiation primary response gene 88) and TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN), causing subsequent activation of multiple intracellular signaling pathways [6]: mainly mitogen activated protein kinase (MAPK) cascades and nuclear factor-κB (NF-κB) cascades, which will induce translation, expression and secretion of tumor necrosis factor-α (TNF-α) and many other inflammatory factors and cytokines [6]. For example, studies have shown that activation of MAPK/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) signaling by LPS plays a major role in TNF-α response [9–11]. Thus, MEK-ERK pathway represents a valuable therapeutic target for preventing LPS-mediated inflammations.

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Trametinib, also named GSK1120212 or JTP-74057, is a highly selective and potent kinase inhibitor of MEK1/2. Its half-maximum inhibitory concentration (IC-50) for MEK kinases is less than 1 nM [12–14]. It showed no activity on ERK kinases [12,15,16]. In the current study, we show that trametinib dramatically inhibits LPS-induced TNF-α production in macrophages and in *ex-vivo* cultured human PBMCs. *In vivo*, trametinib protected mice from LPS-induced endotoxin shock. We suggest that to block MEK-ERK cascade activation by trametinib is causally linked to the defect in LPS-induced TNF-α response.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Trametinib was purchased from Selleck China (Shanghai, China). LPS and D-galactosamine were obtained from Sigma (St. Louis, MO). PD98059 was obtained from Calbiochem (Darmstadt, Germany).

### 2.2. Antibodies

Anti-ERK1/2, MEK1/2, tubulin, rabbit and mouse horseradish peroxidase (HRP)-conjugated IgG antibodies were purchased from Santa Cruz (Santa Cruz, CA). Antibodies against phospho (p)-ERK1/2 (Thr-202/Tyr-204) and p-MEK1/2 (Ser-217/221) were purchased form Cell Signaling Tech (Denver MA).

### 2.3. RAW 264.7 mouse macrophage culture

RAW 264.7 cells, purchased from the Cell Bank of Shanghai Institute of Biological Science of CAS (Shanghai, China), were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL streptomycin, and 2 mM glutamine at 37 °C in a 5% $CO_2$ humidified incubator.

### 2.4. Bone marrow–derived macrophages (BMDMs) culture

As reported [17], the bone marrow was flushed from femurs of C57/BL6 mice (2 month old) with 7 mL of RPMI (Sigma) supplemented with 10% FBS. Cell pellets were resuspended in ACK hypotonic buffer to remove red blood cells, and were subsequently washed with RPMI plus 10% FBS and cultured in RPMI supplemented with 10% FBS and 30% L-929 cell conditioned media. After Six-seven days, adherent macrophages were trypsinized, counted, re-plated for experimental use. Prior to stimulation, BMDMs were starved overnight in RPMI medium.

### 2.5. Ex-vivo culture of peripheral blood mononuclear cells (PBMCs)

PBMCs of healthy participants (male, 20–30 years old) were isolated by centrifugation over lymphocyte separation medium (Biotyme, Shanghai, China). After three washes in phosphate-buffered saline (PBS), the PBMCs were counted and cultured in DMEM supplemented with 15% FBS, 2 μg/mL of phytohemagglutinin (PHA), 10 ng/mL of phorbol 12-myristate-13-acetate, nonessential amino acids, 5 mM β-mercaptoethanol, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. PBMCs were serum starved for experimental uses. The study was approved by the institutional review board of authors' institution, and written informed consent was obtained from each participant. All investigations were conducted according to the principles expressed in the Declaration of Helsinki.

### 2.6. Cell survival assay

Cells were plated at a density of $5 \times 10^4$ cells/well in 96-well plates in 100 μl medium. After treatment, cell viability was evaluated using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. Trypan blue exclusion assay was performed to analyze PBMCs' survival.

### 2.7. TNF-α protein assay

For analysis of TNF-α secretion in conditional medium, macrophages and PBMCs were plated onto 96-well tissue culture plates. After the applied treatment/s, TNF-α content in the supernatant was measured with a TNF-α ELISA kit (R&D, Shanghai, China), according to the manufacturer's instructions. The concentrations of TNF-α in each sample were calculated from a standard curve prepared using known concentrations of recombinant TNF-α (R&D).

### 2.8. Western blots

Total cell lysates (40–60 g protein per sample) were denatured in sample buffer, samples were subjected to SDS–PAGE on 10% gel, and separated proteins were transferred onto PVDF membrane by Western blots. Membranes were blocked with blocking buffer for 1 h at room temperature and, as desired, probed with primary antibody against indicated kinase or the loading control overnight at 4 °C followed by peroxidase-conjugated appropriate secondary antibody and ECL detection.

### 2.9. RNA and Quantitative-PCR (Q-PCR)

RNA was isolated from the cells using Qiagen RNeasy mini kit (Shanghai, China) using manufacturer's protocol. Reverse transcription (RT) was performed using 1 μg of RNA with the Promega cDNA synthesis kit. Q-realtime-PCR was performed with ABI fast 7500 (Applied Biosystems, CA) and genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Following primers were used: TNF-α-forward: 5′-ATGAGCACTGAAAGCATGATC-3′; reverse: 5′-CAGATGACCTAGTAACGGACT-3′ [18]. Glyceraldehyde-3phosphate dehydrogenase (GAPDH)-forward: 5′-CAATGACCCCTTCATTGACC-3′; reverse: 5′-GACAAGCTTCCCGTTCTCAG-3′ [18]. The GAPDH gene was chosen as the reference gene for normalization, and the $2^{-\Delta\Delta Ct}$ method [19] was applied to quantify TNF-α mRNA change within samples.

### 2.10. MEK1/2 shRNA and stable cell selection

The MEK1/2 short hairpin RNA (shRNA)-containing lentiviral particles or the scramble shRNA control lentiviral particles were obtained from Sigma, the lentiviral particles (10 μl/mL medium each) were added to macrophages for 48 h, and stable colonies expressing targeted shRNA was selected by puromycin (1 μg/mL). The culture medium (containing puromycin) was replaced every 24 h for a total of 6 days. The expression of MEK1/2 and equal loading (tubulin) was tested by Western blotting in the resistant colonies.

### 2.11. Constitutively-active (CA) ERK1 plasmid and transfection

A constitutively-active (CA) ERK1 (T217D/Y221D)-GFP plasmid was designed, synthesized verified by Kaiji Biotech (Shanghai) based on protocol from previous studies [20]. Lipofectamine 2000 was introduced to transfect CA-ERK1 plasmid or the empty vector into *RAW 264.7 cells*. After transfection, more than 50% of RAW

264.7 cells were GFP positive, indicating a fine transfection efficiency.

### 2.12. Data analysis

Data were expressed as the mean ± S.D. Statistical differences were analyzed by one-way *ANOVA* followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 18). Values of $p < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Trametinib inhibits LPS-induced MEK-ERK activation and TNF-α production in RAW 264.7 mouse macrophages

We first tested the potential effect of trametinib on LPS-induced TNF-α production in RAW 264.7 mouse microphages. ELISA results in Fig. 1A demonstrated that LPS at the concentration of 0.01–1 μg/mL induced dramatic TNF-α production in RAW 264.7 cells. Significantly, pretreatment of trametinib (10 nM) dramatically decreased TNF-α content in the supernatant of RAW 264.7 cells treated with the same LPS regimen. Quantitative real-time PCR assay results in Fig. 1B showed that LPS-induced TNF-α mRNA expression was also inhibited by trametinib pretreatment. The inhibitory activity of trametinib on LPS-induced TNF-α production was dose-dependent (Fig. 1C). Since trametinib is a novel and potent MEK inhibitor [12–14,21], its effect on LPS-induced MEK-ERK activation was examined. Western blot results showed that LPS-induced phosphorylation of MEK1/2-ERK1/2 was largely inhibited by trametinib (1–100 nM) (Fig. 1D). Trametinib is being tested in both pre-clinical and clinical studies as a cytotoxic anticancer drugs, we thus examined whether trametinib could affect RAW 264.7 cell viability. CCK-8 assay results showed that trametinib at applied concentrations had no significant effect on RAW 264.7 cell survival alone or in combination with LPS (Fig. 1E). Together, these results show that trametinib inhibits LPS-induced MEK-ERK activation and TNF-α production in RAW 264.7 cells.

### 3.2. Trametinib inhibits LPS-induced MEK-ERK activation and TNF-α production in mouse bone marrow–derived macrophages and human peripheral blood mononuclear cells

To further examine the role of trametinib on LPS-induced TNF-α production, we repeated above experiments in primary monocytes. First, the activity of this MEK inhibitor on mouse bone marrow–derived macrophages (BMDMs) was tested. Results showed that LPS-induced TNF-α production (Fig. 2A) and expression (Fig. 2B) in BMDMs were dramatically inhibited by trametinib pretreatment. The MEK inhibitor also blocked LPS-induced MEK-ERK activation in BMDMs (Fig. 2C). Survival of BMDMs was again not affected by trametinib (Fig. 2D). Similarly in *ex-vivo* cultured human peripheral blood mononuclear cells (PBMCs), trametinib dose-dependently inhibited TNF-α production by LPS (Fig. 2E). MEK-ERK activation by LPS in human PBMCs was also blocked by trametinib (Fig. 2F). The MEK inhibitor was again safe to human PBMCs (Fig. 2G). Together, we show that trametinib inhibits LPS-induced TNF-α production in mouse BMDMs and human PBMCs.

### 3.3. To block MEK-ERK activation by trametinib is causally linked to the defect in LPS-induced TNF-α production

Next, we studied the link between trametinib-mediated MEK-ERK inhibition and the defect in LPS-induced TNF-α production. RAW 264.7 cells added with PD98059, a well-known MEK inhibitor, or infected with MEK1/2-shRNA containing lentivirus, exhibited a

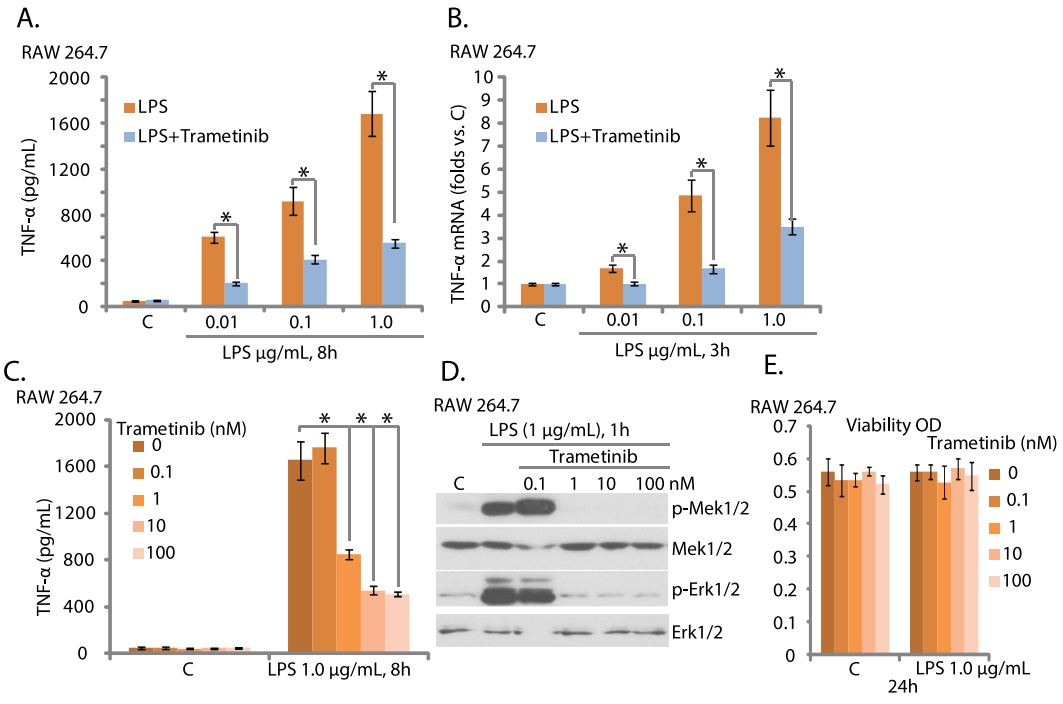


**Fig. 1.** Effect of trametinib on LPS-induced TNF-α production in RAW 264.7 cells. Serum starved RAW 264.7 mouse macrophages were pre-treated with indicated concentrations of trametinib for 1 h, followed by LPS (at applied concentrations) stimulation for indicated time, TNF-α content in conditional medium was tested by ELISA (**A** and **C**), TNF-α mRNA level was quantitatively analyzed by Q-PCR (**B**), phosphorylated (p-) and regular MEK1/2 and ERK1/2 were tested by Western blots (**D**), and cell viability was tested by CCK-8 assay (**E**). The data were expressed as mean ± S.D. of one representative experiment (Same for all figures). Experiments in this and all following figure were repeated three times, and similar results were obtained. "C" stands for untreated control group. *$p < 0.05$ (ANOVA, same for all figures).

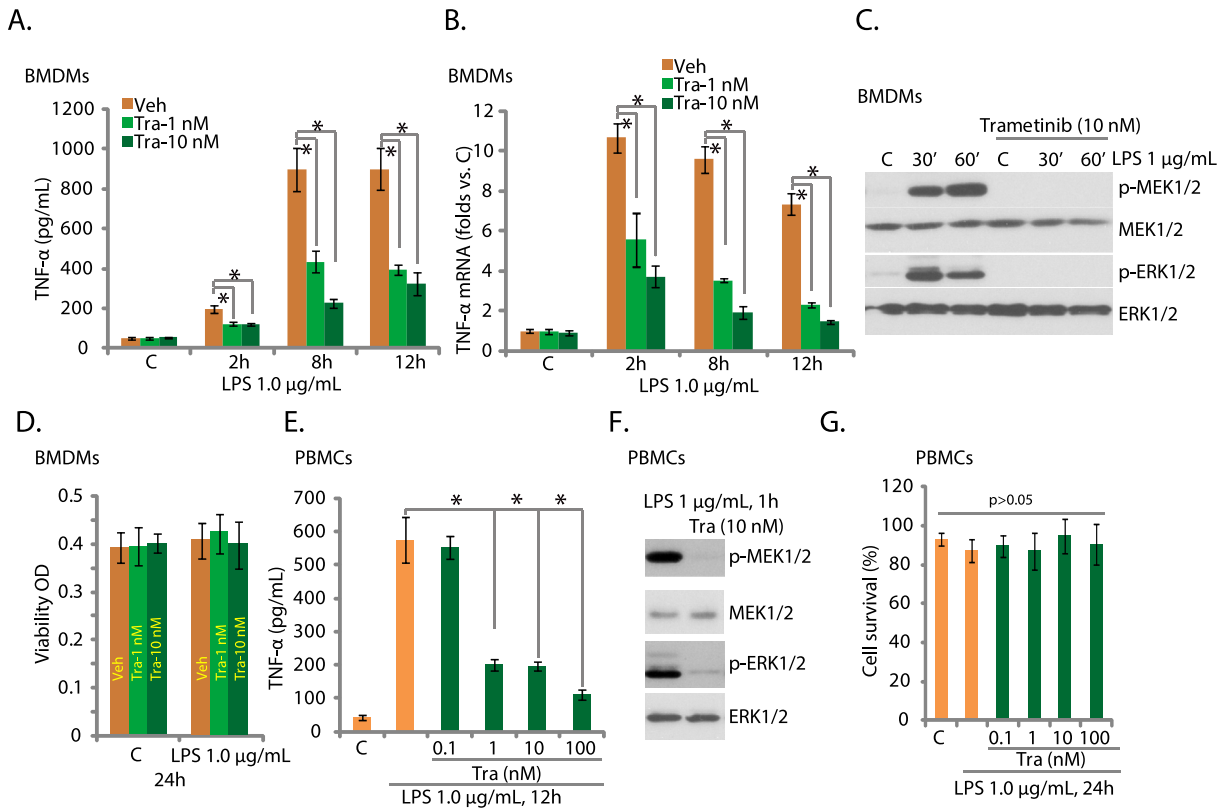


**Fig. 2.** Effect of trametinib on LPS-induced MEK-ERK activation and TNF-α production in primary monocytes. Serum starved mouse bone marrow–derived macrophages (BMDMs) or human peripheral blood mononuclear cells (PBMCs) were pre-treated with applied concentration of trametinib for 1 h, followed by LPS (1.0 μg/mL) stimulation for indicated time, TNFα content in conditional medium and its mRNA level were tested by ELISA (**A** and **E**) and real-time Q-PCR assay (**B**, for BMDMs) respectively, p- and regular MEK1/2 and ERK1/2 were tested by Western blots (**C** and **F**), cell survival was tested by CCK-8 assay (**D**, for BMDMs) or trypan blue staining assay (**G**, for PBMCs). The data were expressed as mean ± S.D. "C" stands for untreated control group. "Tra" stands for trametinib. *$p < 0.05$.

similar phonotype as cells treated with trametinib, showing defective TNF-α induction by LPS (Fig. 3A). Further, the activity of trametinib was depleted when MEK1/2 was silenced (Fig. 3A). Western blot results in Fig. 3B confirmed MEK1/2 knockdown by targeted-shRNA, the latter also prevented MEK1/2-ERK1/2 phosphorylation by LPS. On the other hand, introducing an constitutively-active (CA) ERK1 to RAW 264.7 cells restored TNF-α production by LPS even in the presence of trametinib (Fig. 3C). Western blot results in Fig. 3D confirmed CA-ERK1 expression. Note that CA-ERK1 alone also induced TNF-α production in RAW 264.7 cells (Fig. 3C). Similarly in BMDMs, MEK inhibition by PD98059 or MEK1/2-shRNA lentivirus (Fig. 3F) mimicked trametinib's actions and inhibited TNF-α production by LPS (Fig. 3E), which again almost nullified the activity of trametinib (Fig. 3E). In *ex-vivo* cultured PBMCs, the activity of trametinib was abolished when PD98059 was present, the latter also suppressed LPS-induced TNF-α production (Fig. 3G). Together, these results indicate that MEK-ERK inactivation by trametinib is causally linked to the defect in LPS-induced TNF-α production in monocytes.

### *3.4. Trametinib inhibits LPS-induced TNF-α production and endotoxin shock in mice*

To determine whether trametinib has a role in the response to severe inflammatory response, mice were inoculated intraperitoneally (IP) with LPS and D-galactosamine, the latter is a hepatotoxic transcriptional inhibitor which sensitizes the animals to the cytotoxic effects of TNF-α [11]. The results revealed that trametinib-treated mice were resistant to the induction of endotoxin shock (Fig. 4A). Thus, while the fast majority of mice died within 24 h following injection of LPS plus D-galactosamine, most mice survived following trametinib co-administration (Fig. 4A). The surviving trametinib-administrated mice were followed for a period of 4 days without any evidence of late occurring side effects. The death of LPS/D-galactosamine-treated mice was mainly due the action of TNF-α [11]. We therefore examined TNF-α level in above mice. ELISA results analyzing mice tail vein in Fig. 4B confirmed that trametinib administration significantly inhibited LPS/D-galactosamine-induced TNF-α production. Together, These results confirm that trametinib administration inhibits LPS-induced endotoxin shock and TNF-α production in mice.

## 4. Discussion

Macrophages are the main innate immune cells that initiate inflammation and immune responses when facing various stimuli [5,22,23]. LPS-activated macrophages release various pro-inflammatory factors and cytokines, which may cause extensive tissue damages and pathological changes [5,6,22,23]. Among all the inflammatory factors, TNF-α plays a predominant role in promoting above inflammatory responses and damages [11,24]. Thus, the results showing that trametinib dramatically inhibited LPS-induced TNF-α production and endotoxin shock should have a major translational value for managing those severe inflammatory diseases associated with TNF-α over-production.

Of the MAPK cascades (p38, JNK and MEK-ERK), it was early suggested that TNF-α induction in macrophages by LPS relyed on p38 and JNK pathways [6]. Later on, it was shown that MEK-ERK

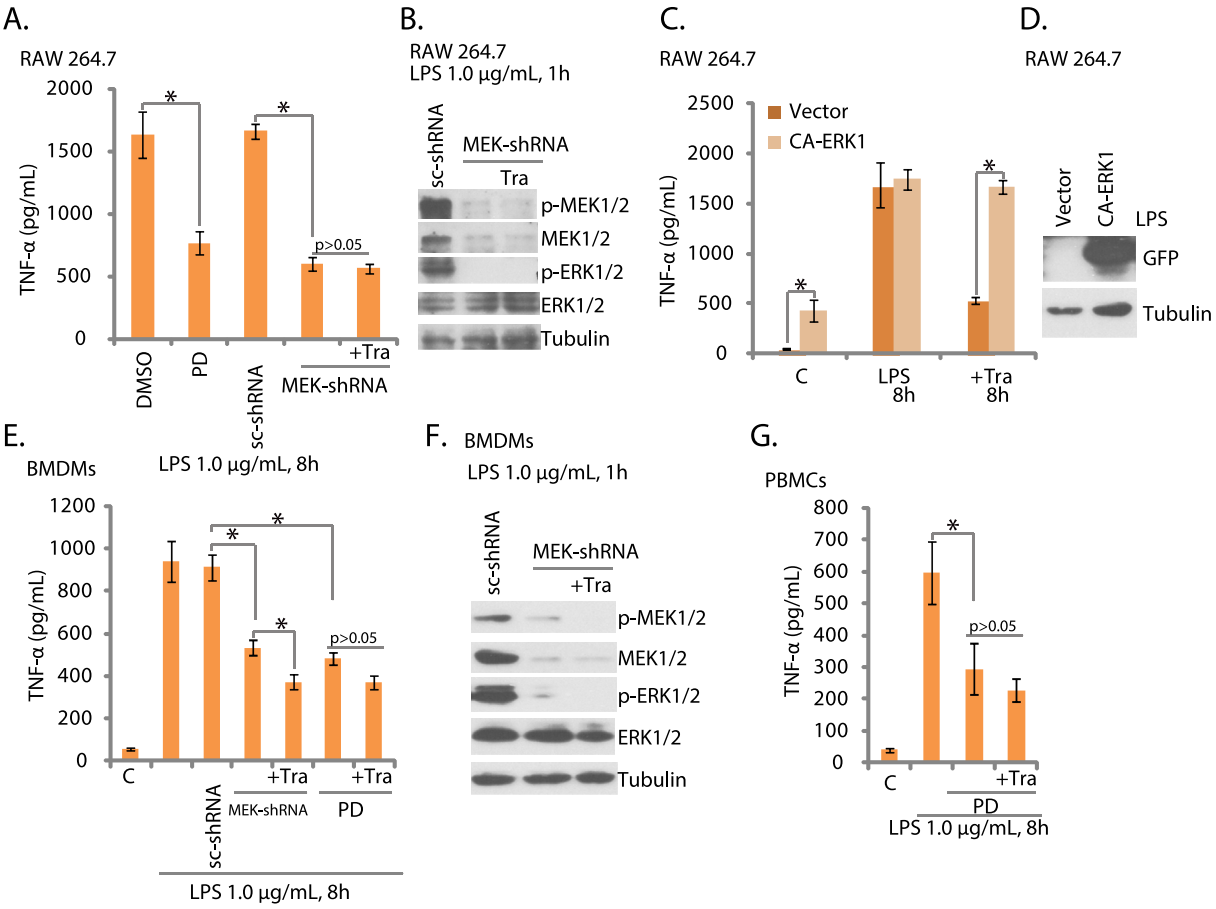


**Fig. 3.** The link between MEK-ERK in-activation and trametinib-caused defect in TNF-α production by LPS. Parental RAW 264.7 cells, scramble shRNA ("sc-shRNA")- or MEK1/2 shRNA ("MEK-shRNA")-infected stable RAW 264.7 cells or BMDMs, pre-treated with PD98059 (PD, 1 μM, 1 h) or trametinib (10 nM, 1 h), were stimulated with LPS (1.0 μg/mL) for indicated time, TNF-α content in the conditional medium was tested by ELISA (**A** and **E**), p- and regular MEK1/2-ERK1/2 as well as tubulin (equal loading) were tested by Western blots (**B** and **F**). RAW 264.7 cells, transfected with empty vector or constitutively-active ERK1 (CA-ERK1), were treated with LPS (1.0 μg/mL) or plus trametinib pre-treatment (10 nM, 1 h), TNF-α production was analyzed (**C**), CA-ERK1-GFP and tubulin expression was shown (**D**). *Ex vivo* cultured PBMCs were pre-treated with PD98059 (PD, 1 μM, 1 h) or plus trametinib (10 nM, 1 h), followed by LPS (1.0 μg/mL) stimulation for 8 h, TNF-α production was analyzed (**G**). "C" stands for untreated control group. "Tra" stands for trametinib. The data were expressed as mean ± S.D. *p < 0.05.

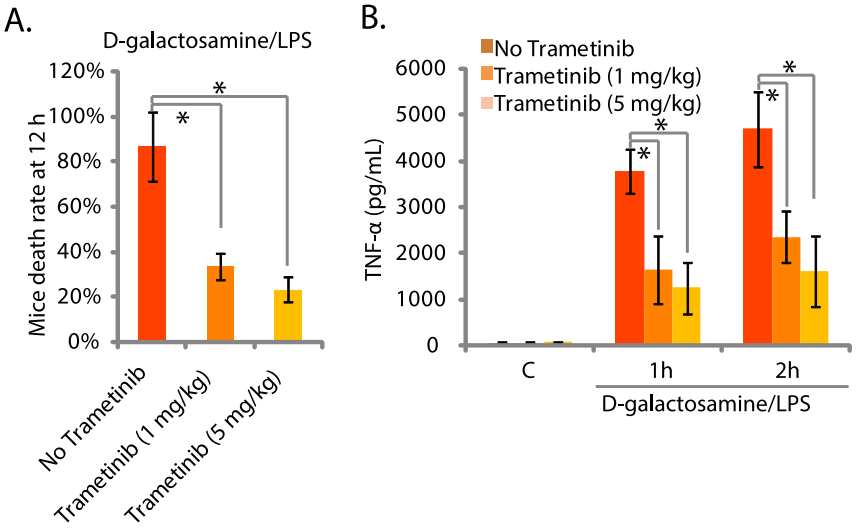


**Fig. 4.** Trametinib administration on LPS/D-galactosamine-induced endotoxin shock and TNF-α production in mice. Eight-to twelve-week-old mice (10 mice per group) were injected i.p. with D-galactosamine (1 mg/g body weight) and LPS (0.05 mg/g body weight), or with oral administration of indicated dosage of trametinib (1–5 mg/kg), mice death rate was recorded 24 h after LPS administration (**A**), tail vein serum samples were collected 1 h or 2 h after LPS stimulation, TNF-α content was determined by ELISA (**B**). The data were expressed as mean ± S.D. *p < 0.05.

activation by LPS was also required in TNF-α induction at both translational and post-translational levels [9–11,25]. The MEK-ERK kinase pathway is involved in the activation of AP-1 and C/EBPβ, both were transcription factors mediating TNF-α transcription [26,27]. Pharmacological or genetic inhibition of MEK-ERK signaling reduced LPS-mediated TNF-α expression and secretion [9–11,25]. Mice knockout of Tpl2, a potential upstream kinase of ERK signaling, were defective in LPS-induced TNF-α response, and those transgenic mice were resistance to LPS/D-galactosamine-induced endotoxin shock [11]. ERK signaling was also shown to be important for TNF-α mRNA stability and its nucleocytoplasmic transport [11]. Activation of MEK-ERK signaling alone was shown to induce TNF-α transcription in PBMCs and RAW 264.7 cells [9–11,25], here we found that CA-ERK1 alone also promoted TNF-α production in RAW 264.7 cells.

In light of these information, we show that in-activation of MEK-ERK signaling by trametinib counted for the defective TNF-α response-induced by LPS in monocytes. Evidences include that macrophages or PBMCs treated with the traditional MEK inhibitor PD98059 or with MEK1/2 shRNA lentivirus exhibited a similar defect as trametinib, and nullified the activity of trametinib on LPS-induced TNF-α response. On the other hand, introducing a CA-ERK1 restored TNF-α production by LPS in the presence of trametinib in RAW 264.7 cells. Thus, MEK-ERK signaling represents an important therapeutic target for the development of drugs against severe and acute inflammation and the septic shock syndrome.

The one advantage using this novel MEK inhibitor is its superior efficiency [15,21]. *In vitro*, trametinib at nM was able to achieve its dramatic inhibitory activity against LPS, while other well-known inhibitors (i.e. PD98059) were utilized at μM levels [11]. The another advantage of using this novel MEK inhibitor is its oral availability, making it an ideal candidate drug for anti-inflammatory injuries. Here we found that oral administration of low doses (1–5 mg/kg body weight) of trametinib could significantly protect mice from endotoxin shock. The safety of using trametinib has also been tested in clinical studies. For example, Phase 1 clinical studies have already shown that trametinib has an acceptable safety profile when administrated in human [12,13]. Further, Phase 2 and 3 monotherapy studies and novel combination studies with trametinib are ongoing [28]. In summary, our investigations indicate that trametinib has potential inhibitory effect on LPS-induced expression and release of TNF-α in macrophages by blocking the activation of MEK-ERK signaling pathways. These observations therefore suggest that trametinib, the potent and oral available MEK inhibitor, has potentials to prevent a variety of severe acute inflammation diseases.

## Conflict of interests

The authors have no conflict of interests.

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