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Perifosine inhibits lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α production via regulation multiple signaling pathways: New implication for Kawasaki disease (KD) treatment

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ABSTRACT

Kawasaki disease (KD) is a multisystem vasculitis of unknown etiology, with coronary artery aneurysms occurring in majority of untreated cases. Tumor necrosis factor (TNF)-α is the pleiotropic inflammatory cytokine elevated during the acute phase of KD, which induces damage to vascular endothelial cells to cause systemic vasculitis. We here investigated the potential role of perifosine, a novel Akt inhibitor, on TNFα expression in LPS-stimulated macrophages and in *ex-vivo* cultured peripheral blood mononuclear cells (PBMCs) of acute KD patients. Here, we found that perifosine inhibited LPS-induced TNFα expression and production in mouse macrophages (RAW 264.7 cells and bone marrow-derived macrophages (BMDMs)). Meanwhile, perifosine administration down-regulated TNFα production in PBMCs isolated from acute KD patients. For the mechanism study, we found that perifosine significantly inhibited Akt and ERK/mitogen-activated protein kinases (MAPK) signaling, while activating AMP-activated protein kinase (AMPK) signaling in both patients' PBMCs and LPS-stimulated macrophages. Interestingly, although perifosine is generally known as an Akt inhibitor, our data suggested that ERK inhibition and AMPK activation, but not Akt inactivation were possibly involved in perifosine-mediated inhibition against TNFα production in monocytes. In conclusion, our data suggested that perifosine significantly inhibited TNFα production via regulation multiple signaling pathways. The results of this study should have significant translational relevance in managing this devastating disease.

1. Introduction

Kawasaki disease (KD) is a common acute febrile disease in children [1]. The dominant pathological changes of this autoimmune disease is systemic vasculitis, which will lead to serious cardiovascular complications, especially coronary artery lesions [2]. The abnormal activation of immunocompetent cells such as monocytes/macrophages and lymphocytes is the major characteristic of KD. These cells synthesize and secrete various inflammatory cytokines and chemokines including tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL-6), which will activate the endothelial cells to cause vasculitis [3].

Despite appropriate therapy with i.v. γ-globulin, coronary artery aneurysms continue to develop in 5% of affected children, making it the leading cause of acquired heart disease in children in the developed counties [4]. The role of TNFα in the vascular inflammation of KD is now well-established, TNFα expression is increased in the peripheral blood of KD patients during the acute phase [5–8]. Because TNFα is a potent mediator of inflammation and causes damage to vascular endothelial cells, it is becoming an important factor in the pathogenesis of both the immune activation and endothelial cell damage in KD [5–8]. TNFα blocking agents have been used as salvage therapy in isolated cases to treat KD patients [9]. In the current study, we aimed to understand the role of perifosine, a novel Akt inhibitor, on TNFα production in *ex-vivo* cultured peripheral blood mononuclear cells (PBMCs) of acute KD patients.

As the first oral available alkylphospholipid, perifosine interacts with the cell membrane and affects Akt and other signaling pathways [10–12]. In clinical phase I trials, perifosine administration leads to a profound cytotoxicity against a number of human tumor cells with few side effects [13,14]. Perifosine blocks Akt activation by disrupting the recruitment of Akt to the plasma membrane [10,15,16]. Interestingly, recent studies have confirmed other actions by perifosine. For example, perifosine promotes AMP-activated kinase (AMPK) activation [17,18]. Meanwhile, studies also shown that perifosine in-activates extracellular signal-regulated kinases (ERK) mitogen-activated protein kinase (MAPK) signaling in certain cells [16,19,20].

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Here we found that perifosine significantly inhibits TNFα expression and production in lipopolysaccharide (LPS)-stimulated macrophages (Raw 264.7 and primary cells) and *ex-vivo* cultured cute KD patients' PBMCs. This inhibitory efficiency is associated with perifosine's effect on multiple signaling pathways in monocytes.

2. Materials and methods

2.1. Chemical and regents

Perifosine and A769662 were purchased from Cayman Chemical (Ann Arbor, MI). LPS was obtained from Sigma (St. Louis, MO). PD 98059, LY 294002 and AICAR(5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside) were obtained from Calbiochem (Darmstadt, Germany).

2.2. Antibodies

Anti-AMPKα1, acetyl-CoA carboxylase (ACC), Akt1, S6, ERK1/2, P38, JNK1/2, rabbit and mouse horseradish peroxidase (HRP)-conjugated IgG antibodies were purchased from Santa Cruz (Santa Cruz, CA). Antibodies against phospho(p)-AMPKα (Thr 172), p-ACC (Ser 79), p-Akt (Ser 473), p-S6 (Ser 235/236), p-ERK1/2 (Thr202/Tyr204), p-JNK1/2 (Thr183/Tyr185) and p-P38 (Thr180/Tyr182) were purchased form Cell Signaling Tech (Denver MA).

2.3. RAW 264.7 cell culture

RAW 264.7 mouse macrophage cells (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml streptomycin, and 2 mM glutamine at 37 °C in a 5% CO_2 humidified incubator.

2.4. Bone marrow-derived macrophages (BMDM) culture

The bone marrow was flushed from femurs of mice (2 month old) with 7 ml of DMEM supplemented with 10% FBS. Cell pellets were resuspended in ACK hypotonic buffer to remove red blood cells, and were subsequently washed with DMEM with 10% FBS and cultured at the concentration of 10^7 cells/ml in DMEM supplemented with 20% FBS and 30% L 929 cell conditioned media. Six–seven days later, adherent macrophages were trypsinized, counted, and replated to be used experimentally. Prior to stimulation, BMDM were cultured overnight in DMEM supplemented with only 0.5% FBS.

2.5. Ex-vivo culture of peripheral blood mononuclear cells (PBMCs) isolated from acute KD patients

PBMCs of 3 acute KD patients (each patient PBMCs were used for one set of experiment) 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 10% FBS, 2 μg of phytohemagglutinin (PHA) per ml, 10 ng of phorbol 12-myristate-13-acetate per ml, nonessential amino acids, 5 mM β-mercaptoethanol, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The study was approved by the institutional review board of author's institution, and written informed consent was obtained from each acute KD patients. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

2.6. Cell viability assay

Cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5diphenyltetrazolium bromide (''MTT'') assay. Briefly, after the treatment, cells were collected and seeded in 96-well plates at a density of 2×10^5 cells/cm^2. After indicated treatment, MTT tetrazolium salt (0.5 mg/ml, Sigma, St. Louis, MO) was added to culture well. Afterwards, DMSO (150 μl/well) was added to dissolve formazan crystals, the absorbance of each well was observed by a plate reader at a test wavelength of 490 nm with a reference wavelength of 630 nm.

2.7. TNFα protein analysis

For analysis of TNFα, macrophages and PBMCs were plated onto either 24-well or 96-well tissue culture plates at a density 1.5×10^6 cells/ml. After the indicated treatment, TNFα protein content in culture supernatant was measured with an ELISA kit (R&D, Shanghai, China), according to the manufacturer's instructions. The concentrations of TNFα in each sample were calculated from a standard curve prepared using known concentrations of recombinant TNFα (R&D).

2.8. Western-blots

Cells with the indicated treatment were harvested in a lysis buffer (Jingmei, Shanghai, China). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Beijing, China). Aliquots of 30–40 μg of lysates were electrophoresed on 10% SDS–PAGE gel and transferred to PVDF membranes. The blots were then incubated with primary antibodies at 4 °C overnight. Appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) were then added. Antigen–antibody complex was detected by using enhanced chemiluminescence (ECL) reagent. All Western-blots in this study were subjected to different exposures: from 10 s to 10 min, the best exposures were selected for data presentation.

2.9. Total RNA isolation and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared by RNA-TRIZOL extraction (Gibco). Concentration and purity of the extracted RNA were measured spectrophotometrically at A260 and A280. Real time-reverse transcription-polymerase chain reaction (RT-PCR) was performed by using TOYOBO ReverTra Ace RT-PCR kit according to the manufacturer's instructions. Primers were 5′-ATGAGCACTGAAAGCATGATC-3′, 5′-CAGATGACCTAGTAACGGACT-3′ for TNFα. A typical reaction (50 μl) contained 1/50 of reverse transcription-generated cDNA and 200 nM of primer in 1× SYBR Green RealTime Master Mix (Toyobo, Shanghai, China) buffer. The PCR reactions were carried out on a Bio-Rad IQ5 multicolor detection system by using 2 μg of synthesized cDNA under the following conditions: 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. All real-time PCRs were performed at least in triplicate. The TNFα mRNA expression level was expressed as percentage changes vs. control group.

2.10. AMPK knockdown in RAW 264.7 cells

To decrease the levels of endogenous AMPK, RAW 264.7 cells were pretreated for 48 h with 25 μM phosphothiorated antisense (AS) oligonucleotide (5′-CGCCCGTCGTCGTGCTTCTGC-3′) directly against both the α_1- and α_2-subunits of AMPK [21]. A missense (MS) oligonucleotide (5′-CTCCCGGCTTGCTGCCGT-3′) was used in control cultures. Transient transfections were performed on 6-well

plates using FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions.

2.11. Data analysis

The data were expressed as means ± S.D. Western blot band intensity was quantified by densitometric analyses using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software. Data were collected using a minimum of three experiments and used to calculate the mean ± S.D. Statistical differences were analyzed by one-way *ANOVA* followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 15). Values of $p < 0.05$ were considered statistically significant. The significance of any differences between two groups was tested using paired-samples *t* test when appropriated.

3. Results

3.1. Perifosine inhibits LPS-induced TNFα expression and production in RAW 264.7 mouse macrophages

In the current study, we aimed to understand the effect of perifosine on LPS-induced TNFα production in macrophages. First, we wanted to know whether perifosine, the cytotoxic drug for cancer treatment, affected the cell viability of RAW 264.7 macrophages. As shown in Fig. 1A, perifosine didn't show significant inhibitory role on RAW 264.7 cell viability until at a relative high dose (10 μM). ELISA results in Fig. 1B showed that perifosine (1 μM) significantly inhibited LPS-induced TNFα production in RAW 264.7 mouse macrophages, and the inhibitory effect appeared to be dose-dependent (Fig. 1C). The effect of perifosine on TNFα mRNA expression was also examined. As shown in Fig. 1D, perifosine supplementation significantly decreased LPS-induced TNFα mRNA expression in RAW 264.7 mouse macrophages. Similarly the perifosine's effect on TNFα mRNA expression was dose-dependent (Fig. 1E). Based on these information, we demonstrated that perifosine attenuated LPS-induced TNFα mRNA expression and production in RAW 264.7 mouse macrophages.

3.2. Perifosine inhibits TNFα production in ex-vivo cultured peripheral blood mononuclear cells of acute Kawasaki disease patients

As discussed early, studies have indentified increased TNFα production in peripheral blood mononuclear cells (PBMCs) of acute KD patients, which contributed to vasculitis. Next, we wanted to know if perifosine could affect TNFα production in PBMCs of KD patients. As expected, ELISA results in Fig. 2B demonstrated a high basal TNFα production in *ex-vivo* cultured PBMCs from KD patients. Importantly, perifosine dose-dependently inhibited TNFα production in the PBMCs (Fig. 2B). Again, patient's PBMCs cell viability was also not affected by low dose of perifosine treatment (<10 μM) (Fig. 2A). We then tested the effect of perifosine on LPS-induced TNFα expression and production in primary cultured mouse bone marrow derived macrophages (BMDMs), and results clearly showed that perifosine dramatically suppressed LPS-induced TNFα mRNA expression (Fig. 2C) and protein secretion (Fig. 2D) in BMDMs.

3.3. Perifosine inhibits Akt/mTOR and ERK/MAPK activation in monocytes

Activation of MAPK signaling including JNK, ERK and P38 cascades is required for LPS-induced TNFα production in monocytes [22–24]. We tested these signaling cascades in LPS-treated RAW 264.7 mouse macrophages. Western blot results in Fig. 3A showed that LPS induced significant JNK, ERK and P38 MAPK cascades as well as Akt and its downstream mTOR activation RAW 264.7 mouse macrophages. mTOR activation was reflected by S6 phosphorylation at ser 235/236 (Fig. 3A). As expected, perifosine inhibited LPS-induced Akt and downstream mTOR activation

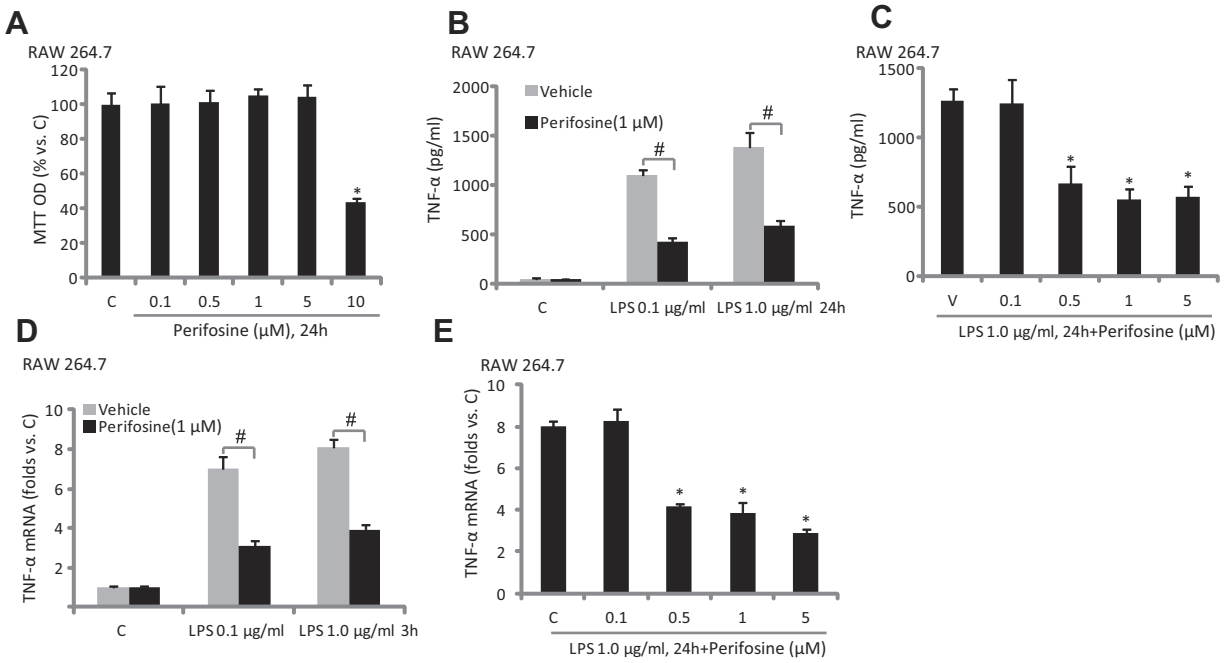

Fig. 1. Perifosine inhibits LPS-induced TNFα expression and production in RAW 264.7 mouse macrophages. RAW 264.7 mouse macrophages were treated with indicated concentration of perifosine for 24 h, cell viability was analyzed by MTT assay (A). RAW 264.7 mouse macrophages were stimulated with LPS (0.1 or 1.0 μg/ml), co-supplemented with indicated concentration of perifosine, TNFα in culture supernatant was measured with an ELISA kit 24 h after stimulation (B and C). TNFα mRNA level was analyzed by real-time PCR 3 h after stimulation (D and E). The results presented are representative of three independent experiments. The values were expressed as the means ± SD. $^{*}p < 0.05$ compared with "C" group (A, C and E). $^{\#}p < 0.05$ (B and D).

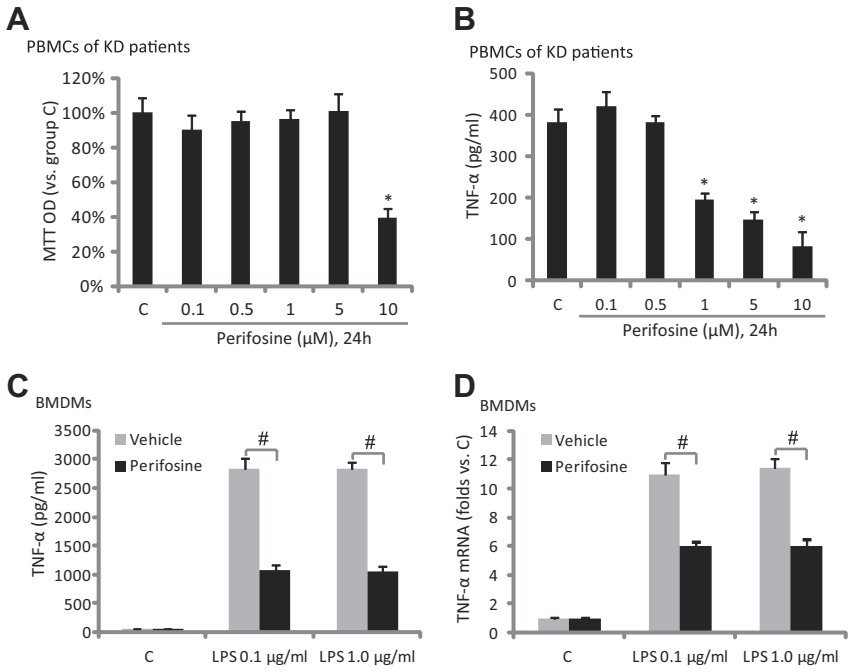

Fig. 2. Perifosine inhibits TNFα production in *ex-vivo* cultured peripheral blood mononuclear cells of acute Kawasaki disease patients. *Ex-vivo* cultured peripheral blood mononuclear cells (PBMCs) of acute Kawasaki disease (KD) patients were either left untreated ("C") or treated with different concentration of perifosine for 24 h, cell viability was tested through MTT assay (A), TNFα in culture supernatant was measured with an ELISA kit (B). Primary cultured BMDMs were stimulated with LPS (0.1 or 1.0 μg/ml), co-supplemented with perifosine (5 μM), TNFα protein level in culture supernatant was measured with an ELISA kit 24 h after stimulation (C), while TNFα mRNA expression level was examined by real-time PCR 3 h after stimulation (D). The results presented are representative of three independent experiments. The values were expressed as the means ± SD.$^{*}p < 0.05$ compared with "C" group (A and B). $^{\#}p < 0.05$ (C and D).

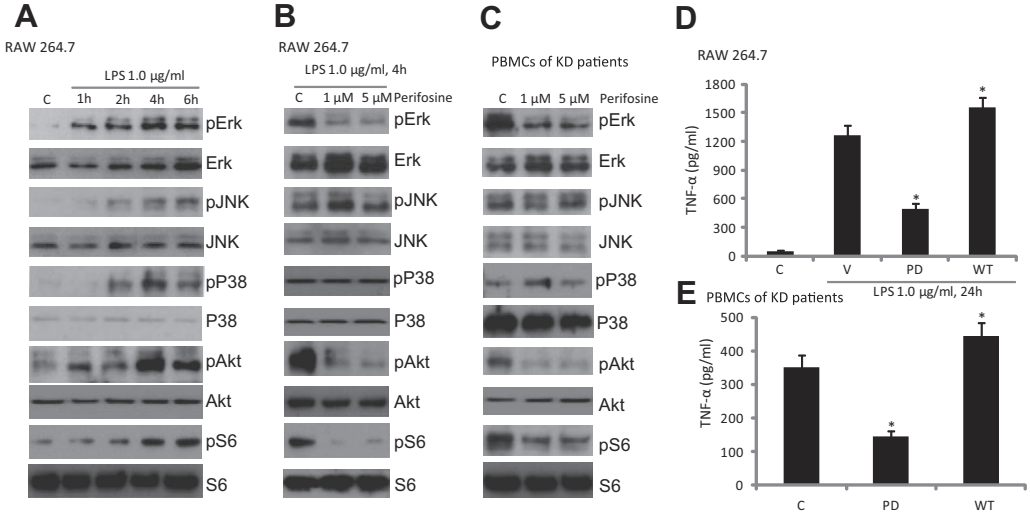

Fig. 3. Perifosine inhibits Akt/mTOR and ERK/MAPK activation in monocytes. RAW 264.7 mouse macrophages were treated with LPS for indicated time points, phospho- and total-ERK, JNK1/2, P38, Akt and S6 were tested by Western blots (A). RAW 264.7 mouse macrophages were treated LPS (1.0 μg/ml) in the presence or absence of perifosine (1 and 5 μM) for 4 h, phospho- and total-ERK, JNK1/2, P38, Akt and S6 were tested by Western blots (B). *Ex-vivo* cultured PBMCs of acute KD patients were incubated with perifosine (1 and 5 μM) for 4 h, phospho- and total- above signal molecular were tested (C). RAW 264.7 mouse macrophages were pre-treated with ERK inhibitor PD 98059 (PD, 10 μM) or Akt inhibitor LY 294002 (LY, 10 μM), followed by LPS (1.0 μg/ml) stimulation for 24 h, TNFα in culture supernatant was measured with an ELISA kit (D). *Ex-vivo* cultured PBMCs of acute KD patients were treated with PD 98059 (PD, 10 μM) or LY 294002 (LY, 10 μM) for 24 h, TNFα protein content in culture supernatant was examined (E). The results presented are representative of three independent experiments. The values were expressed as the means ± SD. $^{*}p < 0.05$ compared with "V" or "C" group (D and E).

(Fig. 3B). Meanwhile, perifosine suppressed ERK, but not P38 and JNK activation in RAW 264.7 mouse macrophages (Fig. 3B). Importantly, in *ex-vivo* cultured KD patients' PBMCs, we observed a high basal level of MAPK (JNK, ERK and P38), Akt and mTOR activation, and perifosine administration significantly inhibited Akt/mTOR and ERK activation, leaving JNK and P38 activation unaffected. Interestingly, as shown in Fig. 3D, ERK inhibitor PD 98059 inhibited LPS-induced TNFα production in RAW 264.7 mouse macrophages, while Akt blocker LY 294002 enhanced it (Fig. 3D). Further, PD 98059 decreased TNFα secretion from PBMCs of KD patients, and LY 294002 again increased it (Fig. 3E). These results indicated that ERK inhibition, but not Akt/mTOR inactivation may be associated with perifosine-mediated TNFα inhibition in monocytes.

3.4. Perifosine-induced inhibition of TNFα was associated with AMPK activation

Recent studies have demonstrated that perifosine, the Akt inhibitor, also activated AMPK signaling in multiple cancer cells [17,18]. Meanwhile, activation of AMPK appears to be anti-inflammatory [21,25]. As such, we tested the potential role of AMPK in TNFα expression and production, and mainly focused on the effect of perifosine on AMPK activation in monocytes. Western blot results in Fig. 4A showed that LPS inhibited basal AMPK activation in RAW 264.7 mouse macrophages. Significantly, perifosine co-administration not only reversed AMPK inhibition by LPS, it significantly enhanced AMPK activation (Fig. 4B). More importantly, AMPK silencing by targeted anti-sense suppressed perifosine's effect on TNFα production (Fig. 4C). Note that AMPK silencing had almost no effect on LPS-induced TNFα production. These results suggested that perifosine-induced inhibition on TNFα was associated with AMPK activation. The fact that AMPK activators (AICAR and A769662) also inhibited LPS-induced TNFα production in RAW 264.7 cells (Fig. 4D) and BMDMs (Fig. 4E) further confirmed this hypothesis. As shown in Fig. 4F, we observed a relative low basal level of AMPK activation in *ex-vivo* cultured PBMCs of acute KD patients, and perifosine administration significantly promoted AMPK activation. Further, AMPK activators (AICAR and A769662) decreased TNFα content in the culture medium of PBMCs of KD patients (Fig. 4G).

4. Discussion

In the current study, we found that perifosine inhibited LPS-induced TNFα expression and production in mouse macrophages (RAW 264.7 cells and primary BMDMs). Meanwhile, perifosine down regulated TNFα production in *ex-vivo* cultured PBMCs of acute KD patients. For the mechanism study, we found that perifosine significantly in-activated Akt/mTOR and ERK signals, while activating AMPK in LPS-stimulated macrophages and KD patients' PBMCs. Interestingly, although perifosine is generally known as an Akt blocker, our RNA silencing and inhibitor data suggested that ERK inhibition and AMPK activation, but not Akt inactivation were related to perifosine's inhibitory effect on TNFα expression and/or production.

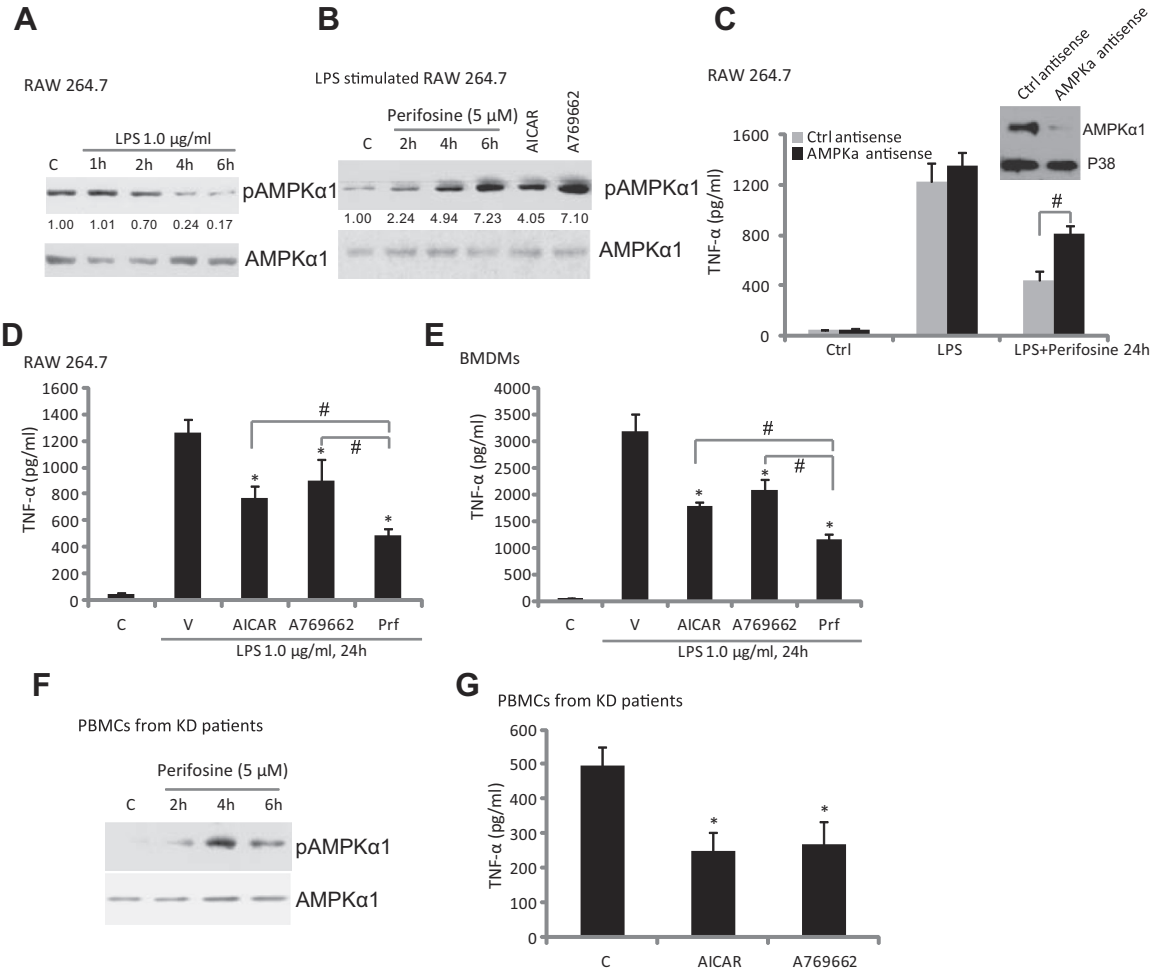

Fig. 4. Perifosine-induced inhibition of TNFα was associated with AMPK activation. RAW 264.7 mouse macrophages were treated with LPS (1.0 μg/ml) for indicated time points, phospho- and total-AMPKα1 were shown (A). RAW 264.7 mouse macrophages were treated with LPS (1.0 μg/ml) in the presence or absence of perifosine (5 μM) for indicated time points, or treated with AMPK activators AICAR (1 mM, 3 h) or A769662 (10 μM, 3 h), phospho- and total-AMPKα1 were tested by Western blot (B). Control ("Ctrl") or AMPKα1/2 antisense transfected RAW 264.7 mouse macrophages were treated with LPS (1.0 μg/ml) or plus perifosine (5 μM) for 24 h, TNFα protein content in culture supernatant was measured (C). RAW 264.7 (D) and BMDMs (E) were co-stimulated with LPS and AICAR (1 mM), A769662 (10 μM) or perifosine (5 μM) for 24 h, TNFα in culture supernatant was measured by ELISA (D and E). *Ex-vivo* cultured PBMCs of acute KD patients were treated with perifosine (5 μM) for indicated time points, phospho- and total-AMPKα1 were tested (F). *Ex-vivo* cultured PBMCs of acute KD patients were cultured in AICAR (1 mM) or A769662 (10 μM) containing medium for 24 h, TNFα protein content in culture supernatant was measured by ELISA kit (G). The results presented are representative of more than three independent experiments. The values were expressed as the means ± SD. $^{*}p < 0.05$ compared with "V" or "C" group (D, E and G). $^{\#}p < 0.05$ (C–E).

Regulation of pro-inflammatory gene (i.e. TNFα) expression in a biological system is a balance between positive and negative signal transduction pathways. LPS (the outer membrane component of Gram-negative bacteria) induces expression of many pro-inflammatory mediators in monocyte/macrophages, one of the key cell types involved in KD. Published studies have shown that LPS-induced TNFα expression in monocytes is mediated, in part, via the activation of the ERK/MAPK pathway [26,27]. ERK inhibition suppressed LPS-induced TNFα expression in macrophages [26,27]. In consistent with these studies, here we found that ERK inhibitor PD 98059 also inhibited TNFα expression in LPS-stimulated macrophages and KD patients' PBMCs. Further, we found that perifosine significantly inhibited ERK activation in above monocytes, indicating that ERK inhibition is involved in perifosine-medicated anti-TNFα efficiency. The fact that perifosine inhibits ERK signaling was consistent with previous observations in other cells [22–24], although the underlying mechanisms need further investigations.

On the other hand, recent studies have shown that activation of PI3K-Akt pathway inhibits the expression of inflammatory genes through negatively regulate NF-κB activation. Wortmannin, a specific inhibitor of Akt, enhanced LPS-induced inflammatory response in murine peritoneal macrophages [28]. Here we found that LY 294002, another well-known Akt inhibitor, also enhanced LPS-induced TNFα expression in macrophages. Hence, although the Akt inhibitor perifosine blocked Akt and its downstream mTOR activation, such effect might not be required for its anti-TNFα efficiency in the monocytes.

AMPK, the highly conserved heterotrimeric kinase, functions as a metabolic switch. It's main function is to coordinate the cellular enzymes involved in carbohydrate and fat metabolism to enable ATP conservation and synthesis [29]. AMPK is activated by conditions that increase the AMP:ATP ratio, i.e. exercise and metabolic stress [29]. Recent studies have studied the role of AMPK in modulating inflammatory responses, and have found that AMPK serves as an anti-inflammatory target [17,18]. AMPK activator AICAR down-regulates LPS-mediated induction of the pro-inflammatory cytokines by inhibiting nuclear factor κB (NF-κB) nuclear translocation [21]. Meanwhile, activation of AMPK by metformin attenuates the cytokine-induced expression of pro-inflammatory and adhesion molecule genes by inhibiting NF-κB activation [25]. Perifosine was shown to activate AMPK in other cells [17,18], in the current study, we observed a significant AMPK activation by perifosine in KD patients' PBMCs and LPS-stimulated macrophages. Activation of AMPK by perifosine appeared to be anti-inflammatory, as AMPK silencing almost reversed perifosine's inhibitory effect on TNFα production, while AMPK activators AICAR and A769662 inhibited TNFα production in patients' PBMCs and LPS-activated macrophages. The underlying mechanisms by which perifosine activates AMPK and AMPK activation negatively regulate TNFα production warrants further investigations.

We concluded that perifosine inhibits TNFα expression and production in monocytes probably via regulating AMPK and ERK signaling pathways. The results of this study should have significant translational relevance in managing this devastating disease.

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