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Original Article

# Glutamine Inhibits TNF-α-induced Cytosolic Phospholipase $A_2$ Activation via Upregulation of MAPK Phosphatase-1

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Tumor necrosis factor alpha (TNF-α) is a principal regulator of inflammation and immunity. The proinflammatory properties of TNF-α can be attributed to its ability to activate the enzyme cytosolic phospholipase $A_2$ ($cPLA_2$), which generates potent inflammatory lipid mediators, eicosanoids. L-glutamine (Gln) plays physiologically important roles in various metabolic processes. We have reported that Gln has a potent anti-inflammatory activity via rapid upregulation of mitogen-activated protein kinases (MAPKs) phosphatase (MKP)-1, which preferentially dephosphorylates the key pro-inflammatory enzymes, p38 MAPK and cytosolic phospholipase $A_2$ ($cPLA_2$). In this study, we have investigated whether Gln could inhibit TNF-α-induced $cPLA_2$ activation. Gln inhibited TNF-α-induced increases in $cPLA_2$ phosphorylation in the lungs and blood levels of the $cPLA_2$ metabolites, leukotrine B4 (LTB4) (lipoxygenase metabolite) and prostaglandin E2 (PGE2) (cyclooxygenase metabolite). TNF-α increased p38 and $cPLA_2$ phosphorylation and blood levels of LTB4 and PGE2, which were blocked by the p38 inhibitor SB202190. Gln inhibited TNF-α-induced p38 and $cPLA_2$ phosphorylation and production of the $cPLA_2$ metabolites. Such inhibitory activity of Gln was no longer observed in MKP-1 small interfering RNA-pretreated animals. Our data indicate that Gln inhibited TNF-α-induced $cPLA_2$ phosphorylation through MKP-1 induction/p38 inhibition, and suggest that the utility of Gln in inflammatory diseases in which TNF-α plays a major role in their pathogenesis.

**Key Words:** Glutamine, TNF-α, $cPLA_2$, p38 MAPK, MKP-1

## INTRODUCTION

TNF-α is produced mainly by monocytes and macrophages, and is a key cytokine regulating inflammation and immunity (Taylor et al., 2004). Many studies have showed that TNF-α is a principal therapeutic target for many inflammatory diseases (Kalliolias and Ivashkiv, 2016). TNF-α activates the pro-inflammatory enzyme, cytosolic phospholipase $A_2$ ($cPLA_2$) through increasing $cPLA_2$ phosphorylation and causing the translocation of $cPLA_2$ from perinuclear regions to the plasma membrane (Hoeck et al., 1993; Hirabayashi and Shimizu, 2000; Sapirstein and Bonventre, 2000), which can explain the proinflammatory properties of TNF-α. TNF-α-uses a serial pathway involving ROS/mitogen-activated protein kinase (MAPK)s/NF-κB/p300 (Lee et al., 2013; Lin et al., 2016) for $cPLA_2$ activation. $cPLA_2$ is involved in the production of potent lipid inflammatory mediator, eicosanoids such as platelet-activating factor (PAF), leukotrienes (LTs) (5-lipoxygenase products),

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and prostaglandins (PGs) and thromboxane (cyclooxygenase products) via releasing arachidonic acid (for an overview, see Dennis et al., 2011). Studies using $cPLA_2$ knock-out animals which display reduction in inflammatory and allergic responses indicate the key role of $cPLA_2$ in inflammatory, cardiovascular and neurological diseases (Leslie, 2015).

L-glutamine (Gln), a non-essential amino acid, is the most abundant amino acid in human body and used as an energy fuel in most cells (Fox et al., 1996; Encarnacion et al., 1998). Gln is an important molecule in the synthesis of peptides, nucleotide bases, neurotransmitters, and glutathione (Albrecht et al., 2010; Amores-Sanchez and Medina, 1999). Critically ill patients undergo Gln's alteration, which leads to muscle proteolysis activation, insulin resistance, and increased liver gluconeogenesis (Griffiths et al., 1997). Gln supplementation has been shown to decreases infectious complications and shortens hospitalization (Griffiths, 2003). We have reported that Gln exerts beneficial effects against several experimental inflammatory diseases (Kim et al., 2006; Ko et al., 2008; Ayush et al., 2013; Im et al., 2018). Such effect of Gln was due to its ability to induce MAPK phosphatase-1 (MKP-1) protein (Ko et al., 2009), which dephosphorylates p38 and JNK (Franklin and Kraft, 1997; Hammer et al., 2006). As a result, MKP-1 inactivates $cPLA_2$ by dephosphorylating p38, as $cPLA_2$ is one of p38 substrate (Su and Karin, 1996). Although we have reported MKP-1 upregulation as the anti-inflammatory mechanism of Gln in many experimental inflammatory diseases, it is unknown whether the same mechanism will be operated in Gln inhibition of the pro-inflammatory property of TNF. Therefore, in this study, we have investigated whether Gln could inhibit TNF-α-induced $cPLA_2$ phosphoylation via MKP-1 induction and p38 inhibition.

## MATERIALS AND METHODS

### Animals

Specific pathogen-free female C57BL/6 mice were obtained from Orient Bio (Seongnam, Gyounggi, Korea) and housed in clean, pathogen-free rooms in an environment with controlled temperature (23℃), humidity (55%), and a 12 hr light/dark cycle. All mice were used at 6~7 weeks of age. All experiments were conducted in accordance with the guidelines of the Chonnam National University Institutional Animal Care and Use Committee (Approval No. CNU IACUC-YB-2018-05). We included 4 mice/group/time point /experiment.

### Reagents

L-Gln (biotechnology performance certified, G-8540) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Gln was dissolved in sterilized distilled water to reach 4%, the saturated concentration at room temperature, Gln (750 mg /kg) was administered to animals via intraperitoneally (i.p.). Control mice received vehicle only. Recombinant mouse TNF-α was purchased from R&D System (Minneapolis, MN, USA). The p38 MAPK inhibitor SB202190 was obtained from Calbiochem (San Diego, CA, USA). SB202190 (5 mg /kg i.p.) was injected twice 48 hr and 24 hr (Lee et al., 2012; Kim et al., 2021) before TNF-α (25 μg/kg) injection (Jia et al., 2013). Antibodies against phospho-p38, phospho-$cPLA_2$, and MKP-1 were purchased from Cell Signaling Technology (Danvers, MA, USA).

### Cell culture

Murine alveolar macrophage cells, MH-S (ATCC CRL-2019), were maintained in RPMI 1640 containing 2 mM of Gln (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% antibiotics (Invitrogen, Carlsbad, CA). The cells were maintained at 37℃ in a humidified atmosphere at 5% $CO_2$. Cell passages, between 4~20, used throughout this study.

### Measurement of LTB4 and PGE2

Serum levels of LTB4 and PGE2 were quantified using competitive enzyme-linked immunosorbent assay (ELISA) according to the protocol of the manufacturers from Cayman Chemical Company (Ann Arbor, MI, USA) and R&D System (Minneapolis, MN, USA), respectively.

### Western blot analysis

Mice were sacrificed by cervical dislocation and the lungs were collected, frozen immediately in liquid nitrogen and were stored at -70℃ until analysis. The lung specimens

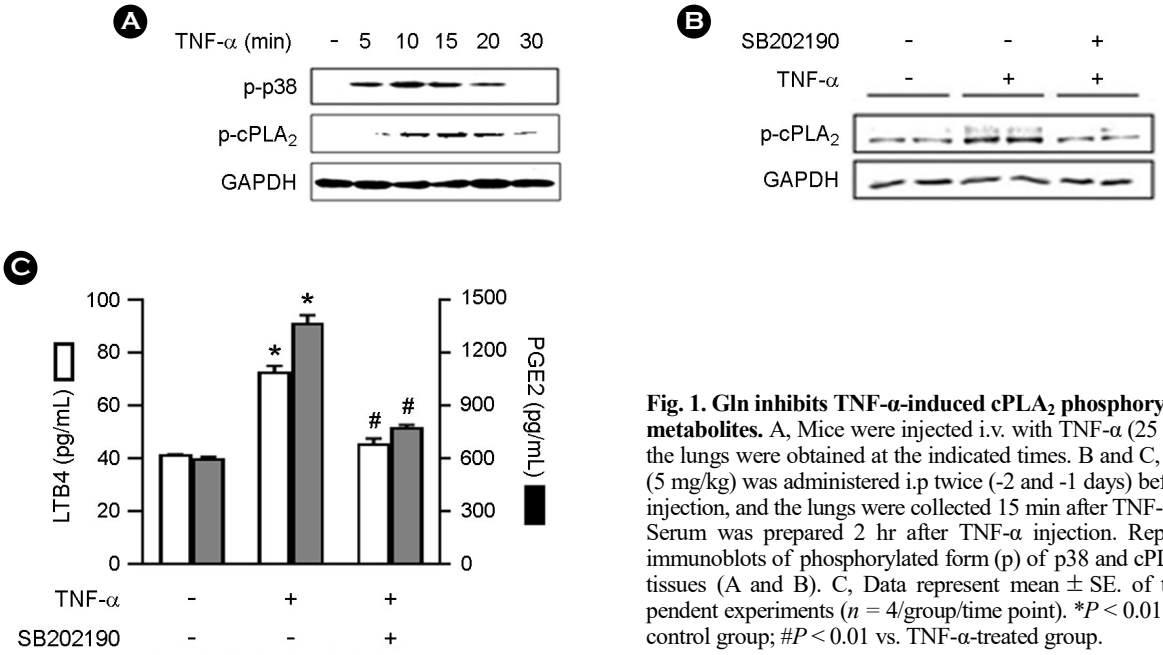


**Fig. 1. Gln inhibits TNF-α-induced $cPLA_2$ phosphorylation and metabolites.** A, Mice were injected i.v. with TNF-α (25 μg/kg) and the lungs were obtained at the indicated times. B and C, SB202190 (5 mg/kg) was administered i.p twice (-2 and -1 days) before TNF-α injection, and the lungs were collected 15 min after TNF-α injection. Serum was prepared 2 hr after TNF-α injection. Representative immunoblots of phosphorylated form (p) of p38 and $cPLA_2$ in lung tissues (A and B). C, Data represent mean ± SE. of three independent experiments ($n$ = 4/group/time point). *$P$ < 0.01 vs. normal control group; #$P$ < 0.01 vs. TNF-α-treated group.

and whole cell extracts were homogenized in PhosphoSafe Extraction Reagent (Novagen Merck, Darmstadt, Germany) with phenylmethylsulfonyl fluoride protease inhibitor (Sigma -Aldrich, St. Louis, MO, USA). Western blot analysis was performed as described previously (Jeong and Im, 2019).

**Small interfering RNA interference**

Small interfering RNA (siRNA) strands for MKP-1 and controls were obtained from Santa Cruz Biotechnology (mRNA accession # NM: 013642, Santa Cruz, CA, USA). The target sequences are as follow; Duplex 1 sense strand: GGUUCAACGAGGCUAUUGA, Duplex 2 sense strand: CGAGGCUAUUGACUUCAUA, Duplex 3 sense strand: GCAAGACAUUUGCUGAACU. *In vivo* delivery of siRNA was performed using *In vivo*-jet polyethyleneimine (PEI; Polyplus-transfection, Illkirch, France), according to the instructions of the manufacturer. In brief, MKP-1 siRNA and PEI dissolved in 5% glucose were mixed in a volume of 200 μL for i.v. injection at room temperature for 20 min, and the mixture was administered 24 hr before Gln administration. The mixture containing control siRNA and PEI dissolved in 5% glucose without siRNA were used as controls. The mixture almost completely inhibits the target molecule expression (Ko et al., 2009; Ayush et al., 2013).

**Statistical analysis**

Data are expressed as means ± SE. Statistical significance was determined via one-way analysis of variance (StatView; Abacus Concepts Inc., Berkeley, CA, USA). A value of $P < 0.05$ was regarded as statistically significant. All experiments were conducted at least twice. Reproducible results were obtained and representative data are, therefore, provided in the figures.

## RESULTS

**p38 MAPK plays a key role in TNF-α-mediated phosphorylation of $cPLA_2$**

We first examined how p38 MAPK regulates TNF-α-induced $cPLA_2$ phosphorylation in the lungs. Administration of TNF-α resulted in phosphorylations of p38 MAPK and $cPLA_2$ at 5~20 min (Fig. 1A). The p38 inhibitor SB202190 inhibited TNF-α-induced $cPLA_2$ phosphorylation (Fig. 1B) and increases in blood levels of the two $cPLA_2$ metabolites, LTB4 and PGE2 (Fig. 1C), indicating that p38 is required for $cPLA_2$ activation in response to TNF-α.

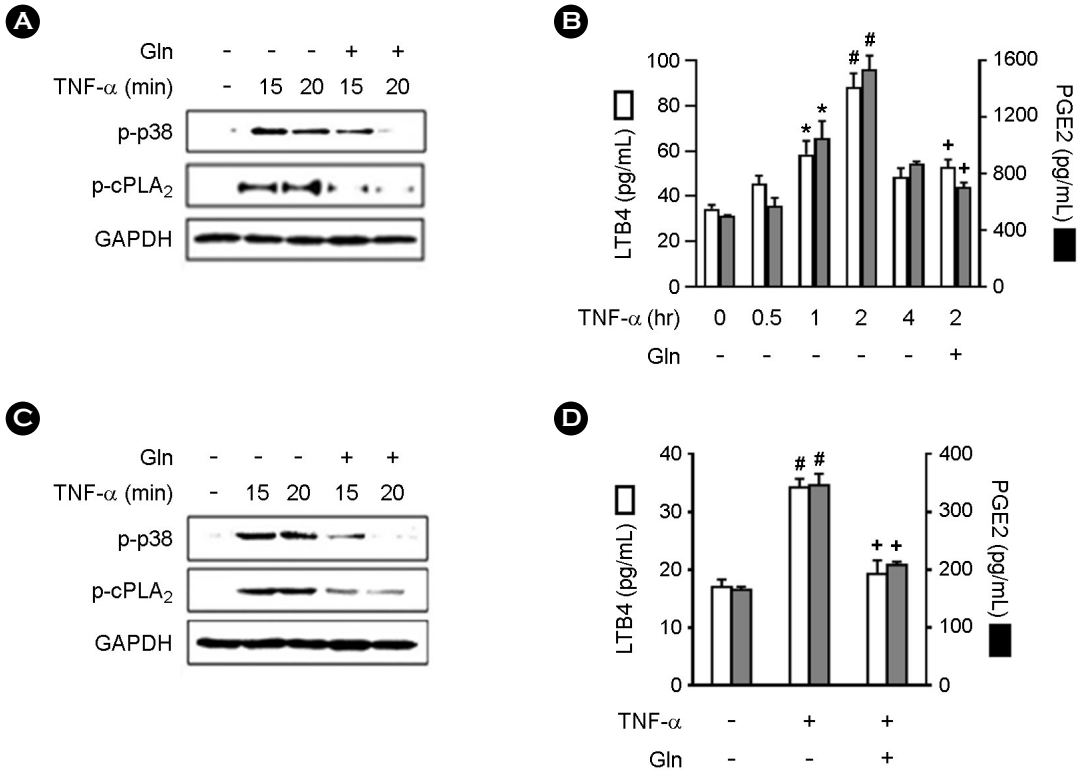


**Fig. 2. Gln inhibits TNF-α-induced phosphorylation of p38 and cPLA$_2$ phosphorylation as well as the production of LTB4 and PGE2.** A and B, Gln (750 mg/kg, i.p.) was given 10 min after TNF-α injection. A, The lungs were obtained at the indicated times. B, Serum was prepared 2 hr after TNF-α injection. C and D, MH-S cells ($2 \times 10^6$) were treated with Gln (40 mM) 10 min after TNF-α treatment. Cell lysate was prepared at the indicated times. Representative immunoblots of p-p38 and p-cPLA$_2$ using lung tissues (A) and cell lysates (C). GAPDH served as a loading control. B and D, Data represent mean ± SE. of three independent experiments ($n$ = 4/group/time point). *$P$ < 0.05 vs. normal control group; #$P$ < 0.01 vs. normal control group; +$P$ < 0.01 vs. TNF-α-treated group.

### Gln inhibits TNF-α-mediated phosphorylation of p38 and cPLA$_2$ and production of cPLA$_2$ metabolites

Gln nearly completely inhibited TNF-α-induced phosphorylation of p38 and cPLA$_2$ at 15 and 20 min when Gln was given at 10 min post TNF-α injection (Fig. 2A). The blood levels of the LTB4 and PGE2 in TNF-α-injected mice were also inhibited by Gln (Fig. 2B). We also examined the Gln's effect using the murine alveolar macrophage cell line MH-S. Addition of TNF-α increased the phosphorylation of p38 and cPLA$_2$, which were dephosphorylated by adding Gln 10 min after TNF-α stimulation (Fig, 2C). Gln also inhibited TNF-α-induced increases in LTB4 and PGE2 production (Fig. 2D), as seen in *in vivo* study.

### MKP-1 induction/p38 inhibition is involved in Gln inhibition of TNF-α-mediated phosphorylation of cPLA$_2$

MKP-1 appeared from 15 min in response to TNF-α in the lungs (Fig. 3A). Gln administration at 10 min post-TNF-α resulted in not only early appearance but also potentiation of MKP-1 upregulation (Fig. 3B).

We examined the involvement of MKP-1 in Gln inhibition of TNF-α-induced cPLA$_2$ phosphorylation using MKP-1 siRNA. Gln again upregulated MKP-1 and inhibited p38 and cPLA$_2$ phosphorylation, and the effects of Gln were no longer observed in MKP-1 siRNA-, but not control siRNA-, treated mice (Fig. 3C). Furthermore, administration of MKP-1 siRNA, but not control siRNA, abolished the Gln-induced inhibition of LTB4 and PGE2 production (Fig. 3D). These data indicate that Gln deactivates TNF-α-induced cPLA$_2$ activation through MKP-1 upregulation.

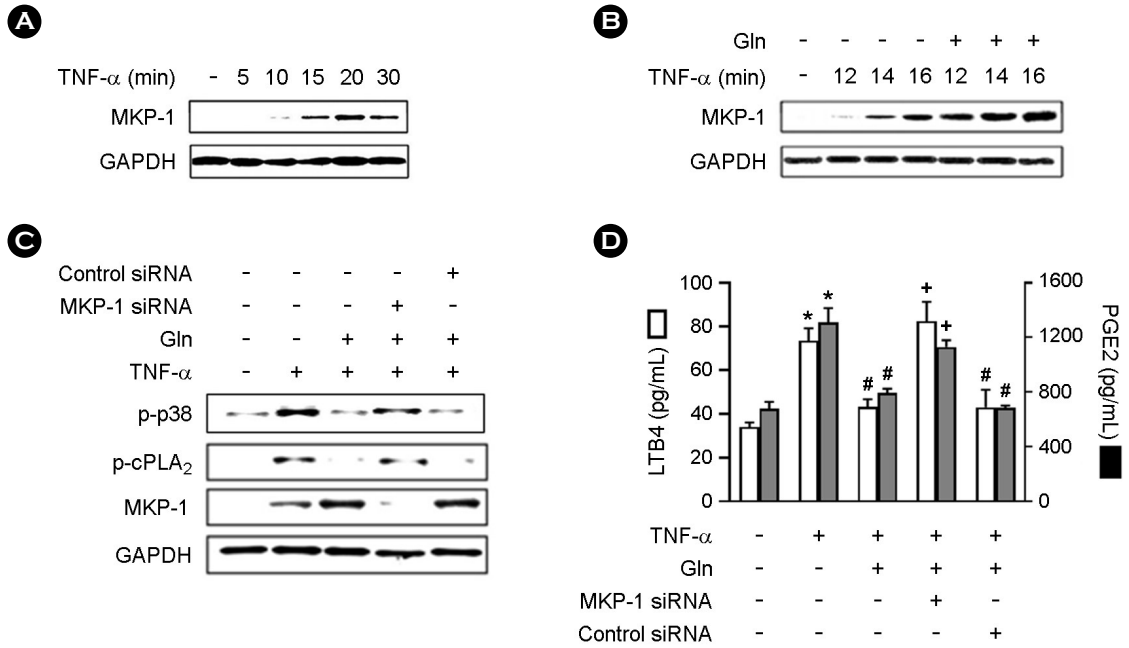


**Fig. 3. Gln potentiated TNF-α-induced MKP-1 induction and MKP-1 siRNA abrogates Gln inhibitions of TNF-α-induced $cPLA_2$ phosphorylation and production of LTB4 and PGE2.** A-D, Gln was given 10 min after TNF-α injection. Lungs were removed 20 min (C) and serum was prepared at 2 hr (D) after TNF-α injection. Representative immunoblots of MKP-1 (A-C) and p-p38, p-$cPLA_2$ (C) in lung tissues. D, Data represent mean ± SE. of three independent experiments ($n$ = 4/group/time point). *$P$ < 0.01 vs. normal control group; #$P$ < 0.01 vs. TNF-α-treated group; +$P$ < 0.01 vs. TNF-α + Gln-treated group.

## DISSCUSSION

Our previous reports have shown that Gln acts as a MKP-1 inducer, which deactivates not only p38 and JNK MAPKs, but also $cPLA_2$ by dephosphorylating them in lung tissues in many disease models (Kim et al., 2006; Ko et al., 2009). Therefore, we here demonstrated that Gln, in addition to the beneficial effects against inflammatory disease, also exerts an anti-inflammatory action against a proinflammatory cytokine itself through the same mechanism. In this study, our observation that 1) Gln administration resulted in earlier and stronger upregulation of MKP-1 and dephosphorylation of p38 and $cPLA_2$ in TNF-α-injected mice, and 2) MKP-1 siRNA abolished such effect of Gln, indicated that such effect of Gln was attributed to the early upregulation of MKP-1. Regarding the administration time and concentration of Gln *in vivo*, we have reported that Gln induced MKP-1 upregulation within 5 min after administration and the optimum concentration was 750 mg/kg (Ko et al., 2009, Ayush et al., 2013; Im et al., 2018).

MKPs are subfamilies within a larger group of dual-specificity protein phosphatases which dephosphorylate MAPK. MKP-1 has been reported as an ERK-specific phosphatase (Sun et al., 1993; Misra-Press et al., 1995), but dephosphorylate and inactivate both p38 and JNK MAPKs later (Franklin and Kraft, 1997; Chi et al., 2006; Hammer et al., 2006; Zhao et al., 2006). Given that both p38 and $cPLA_2$ are key enzymes involved in inflammation, MKP-1 is regarded as a negative regulator of inflammatory responses. Some stress stimuli can induce MKP-1 through transcriptional (Li et al., 2001; Wang et al., 2007) and post-transcriptional mechanisms (Brondello et al., 1999; Lin and Yang, 2006). These include oxidative stress and heat shock (Keyse and Emslie, 1992), anti-cancer drugs (Chattopadhyay et al., 2006; Wang et al., 2006) and UV light (Franklin et al., 1998). As a post-transcriptional mechanism, Brondello et al. (Brondello et al., 1999) reported that ERK MAPK phosphorylates MKP-1 on two carboxyl-terminal serine residues - serine 359 and serine 364, resulting in the stabilization of MKP-1 by preventing proteosomal degradation. We have reported

that Gln increase of ERK activity via activation of the $Ca^{2+}$/Ras/c-Raf/MEK (ERK cascade) pathway as a post-transcriptional mechanism (Ayush et al., 2016).

TNF-α is importantly involved in the pathogenesis of many important inflammatory diseases such as rheumatoid arthritis, Crohn's disease, psoriatic arthritis, juvenile idiopathic arthritis, psoriasis, and ankylosing spondylitis (Kalliolias and Ivashkiv, 2016). Although the action of TNF-α has not been fully elucidated in the context, the ability of TNF-α to activate $cPLA_2$ can explain its proinflammatory properties because $cPLA_2$ is involved in the generation of the potent pro-inflammatory lipid mediator, eicosanoids. These molecules are importantly associated with the pathogenesis of rheumatoid arthritis (Feuerherm et al., 2019), Crohn's disease (Rosengarten et al., 2016), psoriasis (Omland et al., 2017), and other autoimmune diseases (Marusic et al., 2008; Yang et al., 2014).

The approved anti-TNF agents have been widely used in the treatment of TNF-associated diseases (Monaco et al., 2015). However, it has been reported that the clinical use of TNF has been turned out to have several limitations, such as 1) low rates of disease remission, 2) increase in common and opportunistic infections, i.e., reactivation of latent tuberculosis, and 3) induction of autoantibodies, lupus-like symptoms, and increased risk for specific malignancies, such as lymphomas (Smith and Kauffman, 2009; Deepak et al., 2013; Feldmann and Maini, 2015).

In summary, we found that Gln successfully inhibited TNF-α-induced $cPLA_2$ phosphorylation. Given that supportive nutritional Gln therapy is safe (Wischmeyer et al., 2001; Novak et al., 2002), Gln may provide a therapeutic regimen to many inflammatory diseases in which TNF-α plays an important role in their pathogenesis.

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**CONFLICT OF INTEREST**

No potential conflict of interest relevant to this article was reported.

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