Mechanism of glutamine inhibition of cytosolic phospholipase A$_2$ (cPLA$_2$): evidence of physical interaction between glutamine-induced mitogen-activated protein kinase phosphatase-1 and cPLA$_2$

C.-H. Lee,* H.-K. Kim,* J.-S. Jeong,*
Y.-D. Lee,* Z. Wu Jin,[†] S.-Y. Im[‡]
and H.-K. Lee*

Department of Immunology and Institute for Medical Science, Chonbuk National University Medical School, Jeonju, Korea, [†]Department of Anatomy and Histology and Embryology, Yanbian University Medical College, YanJi City, China, and [‡]Department of Biological Sciences, College of Natural Sciences, Chonnam National University, Gwangju, Korea

Summary

Non-essential amino acid L-glutamine (Gln) possesses anti-inflammatory activity via deactivating cytosolic phospholipase A$_2$ (cPLA$_2$). We showed previously that Gln deactivated cPLA$_2$ indirectly via dephosphorylating p38 mitogen-activated protein kinase (MAPK), the major kinase for cPLA$_2$ phosphorylation, through inducing MAPK phosphatase-1 (MKP-1). In this study, we investigated the precise mechanism underlying Gln deactivation of cPLA$_2$. In lipopolysaccharide (LPS)-treated mice, Gln injection resulted in dephosphorylation of phosphorylated cPLA$_2$ (p-cPLA$_2$), which coincided with rapid Gln induction of MKP-1. MKP-1 small interfering RNA (siRNA) abrogated the ability of Gln to induce MKP-1 as well as the dephosphorylation of cPLA$_2$. Co-immunoprecipitation and in-situ proximity ligation assay revealed a physical interaction between MKP-1 and p-cPLA$_2$. In a murine model of allergic asthma, we also demonstrated the physical interaction between MKP-1 and p-cPLA$_2$. Furthermore, Gln suppressed various allergic asthma phenotypes, such as neutrophil and eosinophil recruitments into the airway, airway levels of T helper type 2 (Th2) cytokines [interleukin (IL)-4, IL-5 and IL-13], airway hyperresponsiveness, mucin production and metabolites (leukotriene B$_4$ and platelet-activating factor) through inhibiting cPLA$_2$ in a MKP-1-dependent manner. These data suggest that MKP-1 uses cPLA$_2$, in addition to p38, as a substrate, which further potentiates the anti-inflammatory action of Gln.

Keywords: asthma, cPLA$_2$, glutamine, inflammation, MKP-1

Introduction

Phospholipase A$_2$ (PLA$_2$) is a family of proteins that consists of five subtypes: cytosolic PLA$_2$ (cPLA$_2$), secreted PLA$_2$, calcium-independent PLA$_2$, platelet-activating factor (PAF) acetylhydrolase and lysosomal PLAs [1,2]. Among PLA$_2$s, many research studies have focused on cPLA$_2$ due to its preference for hydrolyzing membrane glycerophospholipids at position *sn*-2 to form arachidonic acid (AA) [3]. AA is converted to potent inflammatory lipid mediator eicosanoids, including leukotrienes (LTs), lipoxins, thromboxanes and prostaglandins, through catalytic action of lipoxygenase or cyclooxygenase [4,5]. The involvement of cPLA$_2$ in lipid mediator production makes it a potentially important pharmacological target to control inflammation and cancer. Despite its impor-

tance, the mechanisms for cPLA$_2$ down-regulation have not yet been determined. Understanding the mechanisms of down-regulation of cPLA$_2$ will provide a novel approach to improve the management of inflammatory diseases.

Non-essential amino acid L-glutamine (Gln), the most abundant amino acid in plasma [6], is an energy substrate for most cells [7,8]. Consequently, Gln is important in multiple ways in the nitrogen- and carbon-skeleton exchange of different tissues [9]. Human clinical trials with several patient populations have demonstrated that Gln treatment can decrease infectious complications, shorten hospitalization and decrease hospital costs [10]. Additionally, extensive research studies using animal models of endotoxin shock, including severe injury models, have demonstrated that Gln supplementation can

improve survival, enhance immune and gut barrier function, decrease bacteraemia and inhibit gut mucosal atrophy [11,12].

To understand the molecular mechanism of Gln anti-inflammatory activity, we have shown that Gln can protect animals effectively from endotoxin shock as well as inhibit bronchial allergic asthma by inhibiting $cPLA_2$ phosphorylation [13,14]. To decipher the mechanism of Gln inhibition of $cPLA_2$, we demonstrated recently that Gln deactivated p38 and Janus kinase (JNK) mitogen-activated protein kinases (MAPKs) by rapidly inducing MAPK phosphatase (MKP)-1 protein [15], which preferentially dephosphorylated p38 and JNK [16,17], suggesting that Gln inhibited $cPLA_2$ phosphorylation indirectly by blocking the kinase activity of p38, a major upstream pathway for $cPLA_2$ phosphorylation [18].

In this study, we further investigated the precise mechanism of how Gln inhibits $cPLA_2$ phosphorylation. We found a physical interaction between Gln-induced MKP-1 and phosphorylated $cPLA_2$ (p-$cPLA_2$). Our data suggest that MKP-1 can directly dephosphorylate $cPLA_2$, thus further delineating the mechanism underlying Gln deactivation of $cPLA_2$.

Materials and methods

Animals

Specific pathogen-free female BALB/c mice were purchased from Samtako Bio Korea (Osan, Korea) and kept in our animal facility for at least 1 week before use. Mice used at the start of each experiment were at 7–8 weeks of age. All experimental animals used in this study protocol were approved by the Institutional Animal Care and Use Committee of Chonbuk National University Medical School.

Cell culture

MH-S murine alveolar macrophage cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% antibiotics (Invitrogen) at $37°C$ in a 5% CO_2 atmosphere.

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* (O127:B8) and L-Gln (biotechnology performance certified, G-8540) were purchased from Sigma-Aldrich (St Louis, MO, USA). LPS (2·5 mg/mouse) was injected intravenously (i.v.) via the lateral tail vein. Gln was dissolved in sterilized distilled water to 3%. A volume of 0·5 ml (750 mg/kg) [12–15] was administered intraperitoneally (i.p.) 10 min before or 10 min after LPS injection.

Specific $cPLA_2$ inhibitor pyrrophenone (Cayman Chemical Company, Ann Arbor, MI, USA) was adminis-

tered i.p. at 20 mg/kg 30 min before LPS injection. Triptolide (1 μM) (Calbiochem, Darmstadt, Germany), a MKP-1 inhibitor, was added to the culture 30 min before LPS stimulation. Antibodies against $cPLA_2$ and p-$cPLA_2$ were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-MKP-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunoblotting analysis

Mice were killed by cervical dislocation. Lungs were collected immediately thereafter, washed briefly with cold phosphate-buffered saline (PBS) and dried with blotting paper. Isolated lung tissues were frozen in liquid nitrogen and stored at $-70°C$ until analysis. Small lung specimens were homogenized in PhosphoSafe Extraction Reagent (Novagen, Madison, WI, USA) and subjected to immunoblotting analysis, as described previously [15].

Immunoprecipitation

Lungs and cells were lysed in non-denaturing lysis buffer consisting of 20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM ethylendiamine tetra-acetic acid (EDTA), protease inhibitor cocktail and phosphatase inhibitor. Equal amounts (100 μg) of cell or tissue extracts were incubated with primary antibodies for 4 h at $4°C$ in the same total volume of lysis buffer. Protein A/G-conjugated agarose beads (Santa Cruz Biotechnology) were added and incubated overnight. The beads were washed four times with lysis buffer and boiled to separate proteins. The beads were removed by centrifugation. The supernatant was used for Western blot.

RNA interference

Small interfering RNA (siRNA) targeting mouse MKP-1 and non-targeting siRNA controls were purchased from Santa Cruz Biotechnology. *In-vivo* siRNA transfection was performed using polyethyleneimine (PEI) purchased from Polyplus-tansfection (BP 90018 ; F-67401 Illkirch Cedex, France) according to the manufacturer's instruction. siRNA and PEI dissolved in 5% glucose was mixed in a volume of 200 μl for i.v. or 30 μl for intratracheal (i.t.) injection. The mixture was incubated for 15 min at room temperature and injected 24 h before LPS treatment [12].

In-situ proximity ligation assay (PLA)

Interaction of p-$cPLA_2$ and MKP-1 was detected *in situ* by PLA technology. Duolink II fluorescence compound was purchased from Olink Bioscience (Uppsala, Sweden). The assay was performed according to the manufacturer's protocol. Briefly, 1×10^4 of MH-S cells were cultured in a covered glass-bottomed dish overnight and treated with LPS (0·1 μg/ml). Cells were washed with PBS for 50 min, fixed with 4% paraformaldehyde for 15 min and treated with cold 100% methanol for 10 min for membrane

permeabilization. Samples were blocked with blocking solution for 30 min and incubated with primary antibodies (anti-p-cPLA$_2$ at 1 : 500 or anti-MKP-1 at 1 : 500) at 4°C for 2 h. After washing twice for 5 min, samples were incubated with PLA probes at 37°C in a humidity chamber for 30 min and washed twice for 5 min. Oligonucleotides conjugated with PLA probes were ligated for 30 min and washed twice for 2 min. Ligated oligonucleotides were amplified for 60 min in a preheated humidity chamber and washed. Covered glass-bottomed dishes were mounted with mounting medium and covered with a coverslip. Samples were analysed by confocal microscopy.

Immunization and challenge

Mice were immunized i.p. with 20 μg OVA (grade V; Sigma-Aldrich) plus 1·0 mg aluminium hydroxide adjuvant (Imject® Alum; Pierce, Rockford, IL, USA) on day 0 and ovalbumin (OVA) alone without alum on day 14. The immunized mice were exposed to aerosolized OVA on days 28 and 35. Aerosolization of OVA was performed using a chamber adapted for mice. Animals were exposed to 1% OVA using a model NE-U12 ultrasonic nebulizer at output of 0·8 ml/min (Omron, Tokyo, Japan) for 30 min at first challenge and 10 min at second challenge [19]. Gln (750 mg/kg) was administered i.p. immediately after the cessation of the second challenge. Pyrrophenone (20 mg/kg) was administered i.p. 30 min before the second airway challenge.

Bronchoalveolar lavage (BAL)

Mice were anaesthetized and the trachea was cannulated while gently massaging the thorax. Lungs were lavaged with 800 μl of saline. An equal volume of BAL fluid sample was centrifuged. The supernatant was collected and stored at −70° until cytokine assay. The pellet was resuspended in saline and cytospin preparations (5 min, 202 g) of BAL cells were stained with Diff-Quik (International Reagents, Kobe, Japan). Different cell types were enumerated based on morphology and staining profile.

Determination of airway hyperresponsiveness (AHR)

AHR was determined as changes in airway function after challenge by aerosolized methacholine using published procedures [14,19]. After methacholine challenge, airway resistance (RL) data were collected continuously. Maximum RL values representing percentage of change from baseline after aerosol saline challenge were used to express changes in airway function.

Histological analysis

Lungs were fixed with 10% formalin. Tissues were embedded in paraffin. To detect mucin, sections (4 μm) were stained with periodic acid–Schiff and counterstained with haematoxylin.

Enzyme-linked immunosorbent assay (ELISA)

Levels of T helper type 2 (Th2) cytokines interleukin (IL)-4, IL-5 and IL-13 in BAL fluids were measured using ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The lower limits of detection for the cytokines were as follows: IL-4 (>2 pg/ml), IL-5 (>7 pg/ml) and IL-13 (>1·5 pg/ml). Levels of lipid mediators, leukotriene B$_4$ (LTB$_4$) and PAF in the blood and BAL fluids were also measured using ELISA according to the manufacturer's instructions. The lower limits of detection for lipid mediators were: LTB$_4$ (>13 pg/ml; Cayman Chemical Company) and PAF (>0·78 ng/ml; MyBiosource, San Diego, CA, USA).

Statistical analysis

Data were expressed as mean ± standard deviation (s.d.). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Statistically significant difference was considered when P-values were less than 0·05.

Results

Gln dephosphorylates p-cPLA$_2$ in a MKP-1-dependent manner

In the course of our studies on Gln deactivation of cPLA$_2$, we have found consistently that Gln could deactivate cPLA$_2$ by dephosphorylating p-cPLA$_2$ [13,14,20]. In this study, we confirmed this finding in the lungs of LPS-injected mice. Time kinetics of cPLA$_2$ phosphorylation and MKP-1 induction are shown in Fig. 1a. When Gln was administered 10 min after LPS injection, Gln rapidly dephosphorylated the p-cPLA$_2$ within 5 min, concurrent with cPLA$_2$ phosphorylation (Fig. 1b). Gln administration also resulted in an early induction of MKP-1 protein (Fig. 1b), consistent with our previous report [15]. MKP-1 siRNA potentiated cPLA$_2$ phosphorylation and suppressed MKP-1 protein induction (Fig. 1c). In mice treated with MKP-1 siRNA, Gln could no longer dephosphorylate cPLA$_2$ (Fig. 1d). To verify the MKP-1/cPLA$_2$ linkage, cPLA$_2$ phosphorylation and MKP-1 expression in response to LPS were evaluated in MH-S murine alveolar macrophage cells. The time kinetics of cPLA$_2$ phosphorylation and MKP-1 induction are shown in Fig. 1e. Addition of Gln resulted in decreased phosphorylation of cPLA$_2$ and early induction of MKP-1 (Fig. 1f). Inhibition of MKP-1 using MKP-1 inhibitor triptolide enhanced the magnitude of cPLA$_2$ phosphorylation but reduced MKP-1 expression (Fig. 1g). Gln-mediated inhibition of cPLA$_2$ phosphorylation was reversed by triptolide (Fig. 1h). Taken together, the observation that Gln deactivated cPLA$_2$ by dephosphorylating it only when cPLA$_2$ was phosphorylated in
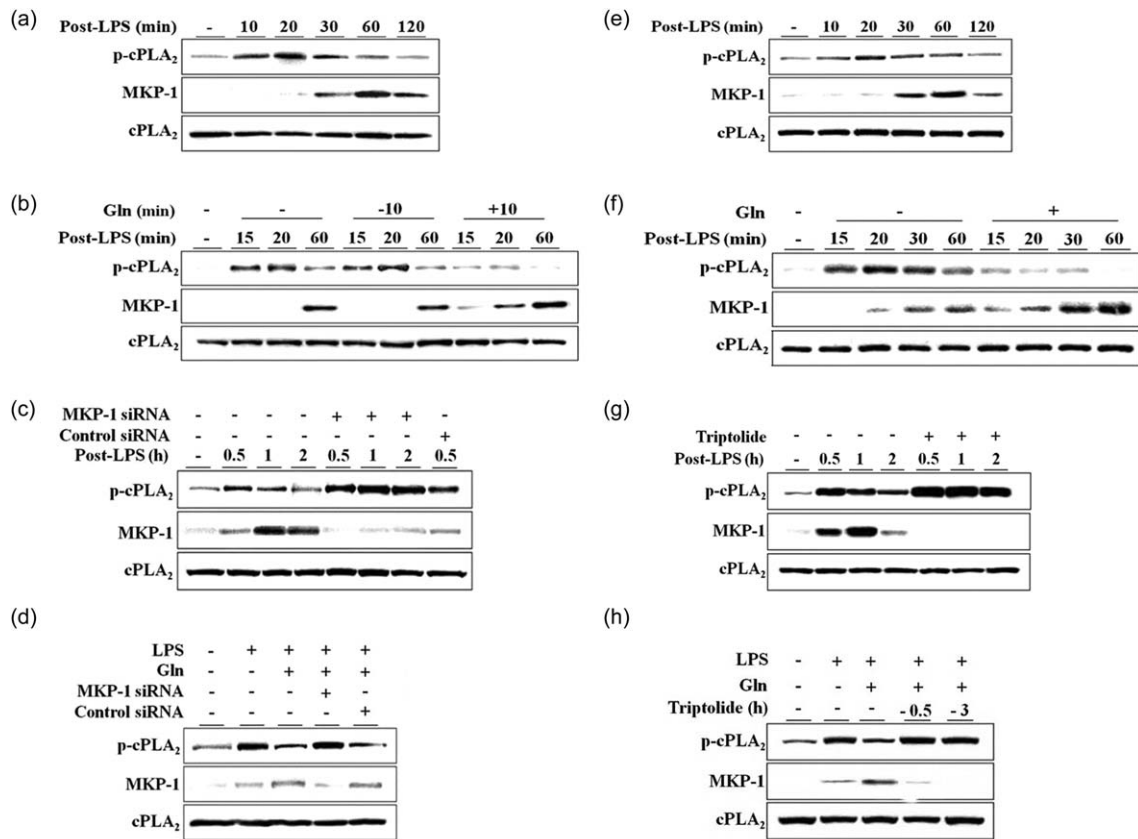
Fig. 1. L-glutamine (Gln) dephosphorylates phosphorylated cPLA$_2$ in a mitogen-activated protein kinase phosphatase-1 (MKP-1)-dependent manner. (a) Lungs were removed at the indicated time-points after lipopolysaccharide (LPS) injection [50 μg/mouse, intravenously (i.v.)]; (b) Gln [750 mg/kg, intraperitoneally (i.p.)] was injected 10 min before or 10 min after LPS and mice were killed at the indicated time-points. (c) MKP-1 small interfering RNA (siRNA) or control siRNA (0·4 nM each) was injected i.v. 24 h before LPS; (d) siRNA and Gln were injected 24 h before and 10 min after LPS, respectively; (e) LPS (0·1 μg/ml) was added to the culture medium and cells were harvested at the indicated time-points; (f,h) Gln (20 mM) was added 10 min after LPS; (g,h) triptolide (1 μM) was added 3 h before LPS. (a–d) A representative of three to five independent experiments with three mice/time-point/experiment is shown. (e–g) A representative of two or three independent experiments with three dished/time-point/experiment is shown.

response to LPS in a MKP-1-dependent manner suggested that Gln could dephosphorylate cPLA$_2$ directly through the induction of MKP-1.

Physical interaction between MKP-1 and p-cPLA$_2$

How could Gln dephosphorylate p-cPLA$_2$ within 5 min? cPLA$_2$ phosphorylation is known to depend upon the kinase activity of p38. In fact, pretreatment of p38 inhibitor SB202190 inhibited cPLA$_2$ phosphorylation significantly [20]. Injection of Gln resulted in a rapid dephosphorylation of p38 through MKP-1 induction [15]. However, the extent to which Gln-mediated cPLA$_2$ dephosphorylation is p38-dependent remains to be elucidated. Phosphatase activity of MKP-1 could be another possible mechanism underlying Gln dephosphorylation of cPLA$_2$ based on the finding that the activity of Gln was exclusively MKP-1-dependent. To test this hypothesis, we investigated whether or not there was a physical interaction between

MKP-1 and cPLA$_2$. Lung lysates from LPS-injected mice were co-immunoprecipitated using anti-MKP-1 and anti-p-cPLA$_2$ antibodies targeting the Ser[505] residue. MKP-1 was detected in immunoprecipitates using anti-p-cPLA$_2$ (Fig. 2a). A reciprocal immunoprecipitation and Western blotting experiment using anti-MKP-1 antibody also detected p-cPLA$_2$ (Fig. 2b). A co-immunoprecipitation assay using LPS-treated MH-S cells showed similar results (Fig. 2c,d). However, immunoprecipitates were not detected using anti-MKP-1 or anti-cPLA$_2$ antibodies (Fig. 2e,f). These data support the theory that there was a physical interaction between MKP-1 and p-cPLA$_2$, but not cPLA$_2$ itself. *In-situ* PLA was performed to confirm further the physical interaction between MKP-1 and p-cPLA$_2$. After MH-S cells were treated with LPS for 50 min, cells were probed with anti-p-cPLA$_2$ and anti-MKP-1 antibodies and conjugated with oligonucleotide PLA probes. When the complementary PLA probes were in close proximity (<40 nm), oligonucleotide strands could be hybridized
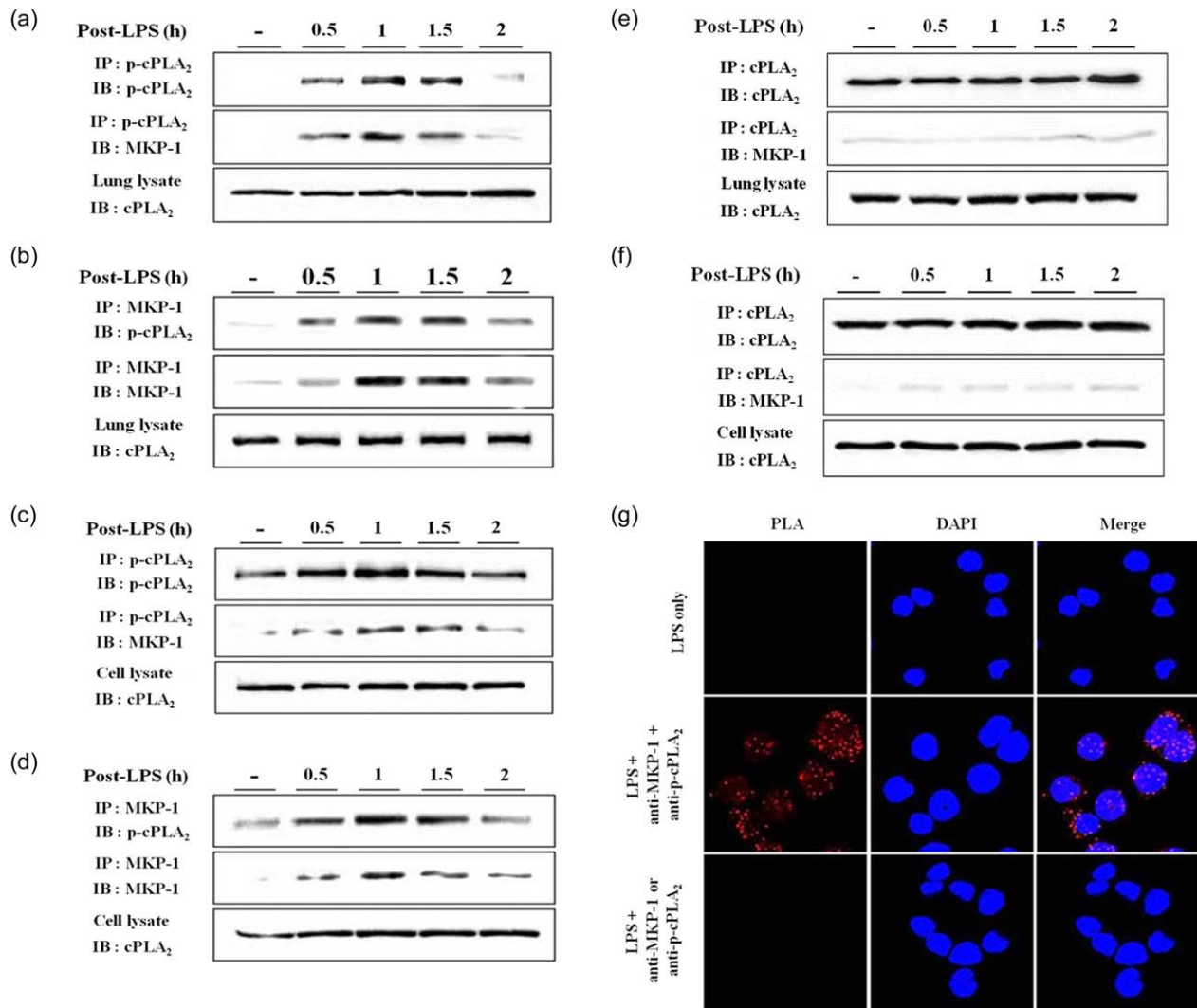
Fig. 2. Physical interaction between mitogen-activated protein kinase phosphatase-1 (MKP-1) and phosphorylated cytosolic phospholipase A2 (cPLA$_2$). (a–d) Lung and cell lysates were immunoprecipitated using anti-p-cPLA$_2$ and anti-MKP-1 antibodies. (e,f) Lung and cell lysates were immunoprecipitated using anti-cPLA$_2$ antibody. (g) Cells were probed with anti-phospho-cPLA$_2$ or anti-MKP-1 antibody and conjugated with oligonucleotide PLA probe: nucleus was counterstained with 4',6-diamidino-2-phenylindole (DAPI). A representative of two or three independent experiments with three mice/time-point/experiment is shown in (a–f). A representative of four independent experiments is shown in (g).

and amplified via the rolling circle method. As a result of this process, red dots were visualized. When viewed with a fluorescence microscope, the red fluorescence was observed mainly around nucleus (Fig. 2g). However, application of either anti-p-cPLA$_2$ or anti-MKP-1 failed to produce red fluorescence, confirming that there was physical interaction between MKP-1 and p-cPLA$_2$.

Gln inhibits the production of cPLA$_2$ products

We assessed whether Gln inhibition of cPLA$_2$ phosphorylation could lead to the inhibition of cPLA$_2$ products LTB$_4$ and PAF. Serum levels of LTB$_4$ (Fig. 3a) and PAF (Fig. 3b) were increased progressively after LPS injection.

Gln administration resulted in the inhibition of both LTB$_4$ (Fig. 3c) and PAF (Fig. 3d). The suppressive activity of Gln was comparable to those of pyrrophenone, the specific cPLA$_2$ inhibitor.

Physical interaction between MKP-1 and p-cPLA$_2$ in asthmatic lungs

We have reported previously a beneficial effect of Gln on allergic asthma in mice [14,20]. In this study, we assessed whether cPLA$_2$/MKP-1 linkage was involved in the beneficial effect of Gln. Phosphorylation of cPLA$_2$ was prominent at 10–30 min after the second airway challenge. Expression of MKP-1 was at 30–60 min after the cessation
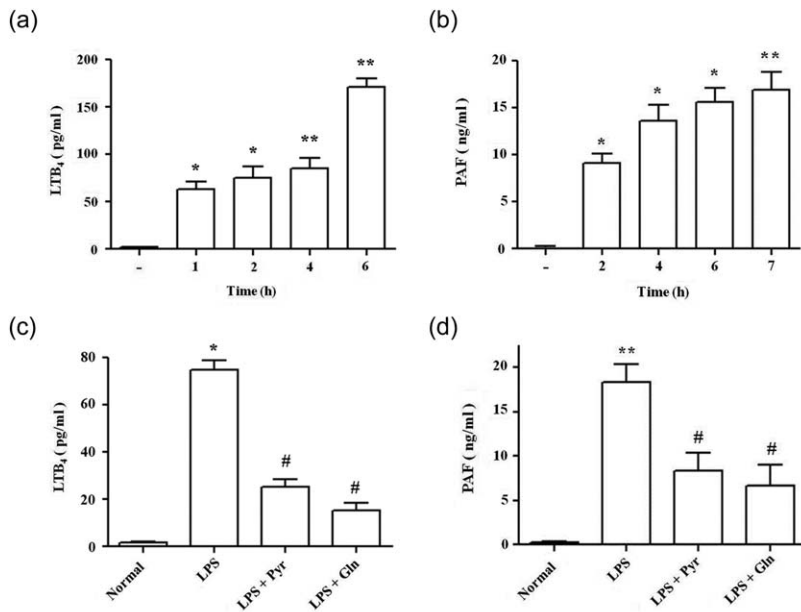
Fig. 3. L-glutamine (Gln) and pyrrophenone suppress serum levels of leukotriene B4 (LTB_4) and platelet activating factor (PAF). Serum levels of LTB_4 (a) and PAF (b) were determined at the indicated time-points. Gln (750 mg/kg, i.p.) and pyrrophenone [20 mg/kg, intraperitoneally (i.p.)] were administered 10 min after and 30 min before LPS injection, respectively. LTB_4 (c) and PAF (d) were determined at 1 and 4 h after LPS. Data representing the mean $\pm$ standard deviation (s.d.) of three independent experiments with three to five mice/group/experiment are shown. $*P < 0.05$ compared to saline control; $**P < 0.01$ compared to saline control; $\#P < 0.05$ compared to the LPS group.

of the second OVA airway challenge (Fig. 4a). MKP-1 siRNA abrogated MKP-1 induction and potentiated $cPLA_2$ phosphorylation in asthmatic lungs (Fig. 4b). Administration of Gln immediately after the cessation of the second airway challenge resulted in the dephosphorylation of $cPLA_2$ and induced early strong induction of MKP-1 protein (Fig. 4c). MKP-1 siRNA reversed Gln inhibition of $cPLA_2$ phosphorylation as well as MKP-1 induction (Fig. 4d). We assessed the interaction between MKP-1 and p-$cPLA_2$ in asthmatic lungs. MKP-1 was detected in immunoprecipitates using anti-p-$cPLA_2$ antibody (Fig. 4e). Immunoprecipitates using anti-MKP-1 antibody showed p-$cPLA_2$ (Fig. 4f). Taken together, these data indicated that MKP-1-deactivated $cPLA_2$, through their physical interaction, also occurred in asthmatic lungs.

Gln suppresses allergic pulmonary inflammation in a MKP-1-dependent manner

We evaluated the role of MKP-1 in the suppression of allergic asthmatic reactions by Gln using MKP-1 siRNA. Gln and pyrrophenone suppressed the recruitment of neutrophils (Fig. 5a) and eosinophils (Fig. 5b), but not macrophages (Fig. 5c), BAL fluid levels of Th2 cytokines (Fig. 5d), AHR (Fig. 5e) or mucus secretion (Fig. 5f). MKP-1 siRNA abrogated all the suppressive effects of Gln. We also assessed LTB_4 and PAF in the airways. BAL fluid levels of LTB_4 (Fig. 6a) and PAF (Fig. 6b) were increased progressively up to 3 h after challenge, but declined thereafter. Gln and pyrrophenone suppressed the BAL levels of both LTB_4 and PAF. The suppressive effect of Gln was reversed by MKP-1 siRNA (Fig. 6c). These data indicate that Gln ameliorated allergic asthma by inhibiting $cPLA_2$ after MKP-1 induction.

Discussion

Our data demonstrate a physical interaction between Gln-induced MKP-1 and p-$cPLA_2$, suggesting that Gln could deactivate $cPLA_2$ directly via early induction of MKP-1. Our findings not only provided the underlying mechanism of Gln's anti-inflammatory activity, but also delineated a possible mechanism underlying the down-regulation of $cPLA_2$. Gln deactivates p38 MAPK by rapidly inducing MKP-1 protein [15], which dephosphorylates p38 and JNK preferentially [16,17]. Both MKP-1 induction and MKP-1 mediated p38 dephosphorylation within 5 min following Gln administration, suggesting that Gln dephosphorylated $cPLA_2$ indirectly through MKP-1-mediated p38 dephosphorylation. However, Gln dephosphorylated p-$cPLA_2$ rapidly within 5 min, as in the case of p38 in LPS-injected mice [13,14,20], suggesting that phosphatase is involved. Gln injection resulted in a rapid induction of MKP-1 within 5 min, which coincided with Gln-induced dephosphorylation of $cPLA_2$ (Fig. 1b). MKP-1 siRNA abrogated the ability of Gln to induce dephosphorylation of $cPLA_2$, suggesting that MKP-1 was involved in the dephosphorylation of $cPLA_2$ by Gln. Our time-kinetic study revealed that Gln failed to deactivate $cPLA_2$, consistent with our previous report [13]. As shown in this and other studies [15,20], Gln dephosphorylation of $cPLA_2$ is an exclusively MKP-1-dependent phenomenon. MKP-1 has been reported to be a labile protein that is normally degraded via the ubiquitin/proteasome pathway. Its phosphorylation reduces its ubiquitination and degradation [21,22] and phosphorylation of MKP-1 is mediated by transient activation of extracellular-regulated kinase (ERK), which enhances MKP-1 stabilization [23]. Therefore, in the absence of

Fig. 4. L-glutamine (Gln) deactivates phosphorylated (p)-cytosolic phospholipase A2 (cPLA$_2$) through mitogen-activated protein kinase phosphatase-1 (MKP-1) in asthmatic lungs. After a second ovalbumin (OVA) airway challenge, lungs were removed at the indicated time-points (a–c). Small interfering RNAs (siRNAs) (0·2 nM) were administered intratracheally (i.t.) 24 h before the second challenge (b,d). Gln (750 mg/kg) was administered intraperitoneally (i.p.) immediately after the cessation of the second challenge (c,d). Lungs were removed at 10 min post-challenge (d). Lungs were removed at the indicated time-points. Lung lysates were immunoprecipitated using anti-p-cPLA$_2$ or anti-MKP-1 antibodies (e,f). A representative of three to five independent experiments with three to five mice/time-point/experiment is shown.
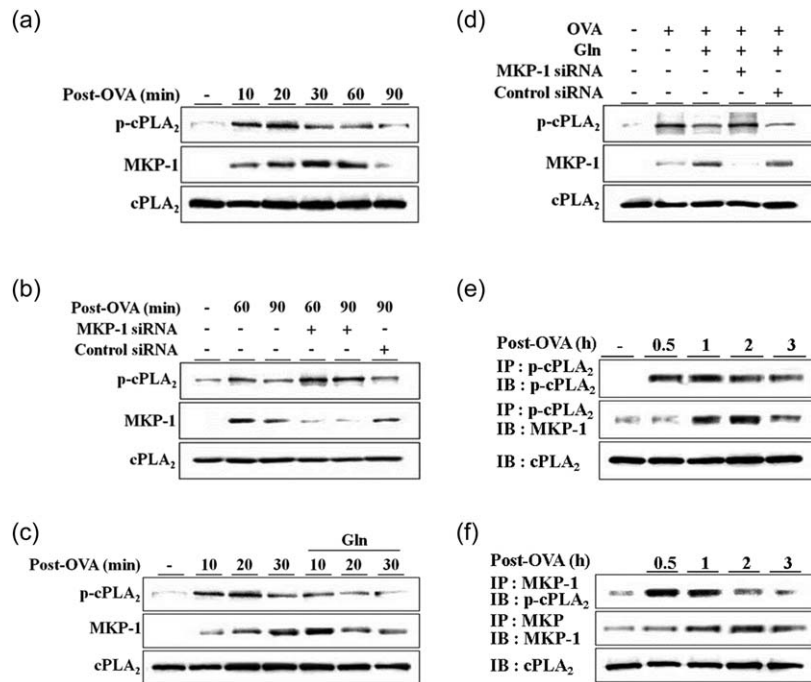
ERK activation by an exogenous stimulus, Gln cannot up-regulate MKP-1 induction. This is the probable reason why Gln failed to deactivate cPLA$_2$ when it was administered before LPS injection.

If MKP-1 could dephosphorylate cPLA$_2$, these two molecules should interact physically with each other. Co-immunoprecipitation and *in-situ* PLA experiments revealed a physical interaction between MKP-1 and p-cPLA$_2$, identifying cPLA$_2$ as a novel MKP-1-interacting protein. *In-situ* PLA revealed red fluorescence in nucleus and the immediate vicinity. MKP-1 is a nuclear protein [24]. Activated cPLA$_2$ translocates from the cytosol to cell membranes, such as those of endoplasmic reticulum, Golgi apparatus and perinuclear region [5]. Thus, MKP-1 may interact with cPLA$_2$ during its translocation to the perinuclear membrane. These data suggest that Gln could deactivate cPLA$_2$ directly by MKP-1-dependent cPLA$_2$ dephosphorylation. Taken together, these data suggest that two mechanisms are involved in the Gln deactivation of cPLA$_2$. The first mechanism is by indirect cPLA$_2$ dephosphorylation through MKP-1-mediated p38 inactivation; the other mechanism is by direct physical interaction between Gln-induced MKP-1 and p-cPLA$_2$.

In this study, we used anti-p-cPLA$_2$ antibody to recognize phosphorylated Ser[505]. When MKP-1 was induced by Gln, the antibody could no longer detect p-cPLA$_2$, indicating that MKP-1 could bind to at least the Ser[505] residue of cPLA$_2$. Further studies are required to address the possible involvement of phosphorylation site besides Ser[505]. MKP-1, an archetypal member of the MKP family, plays a pivotal role in the negative control of p38 and JNK. p38 has a role in the production of inflammatory molecules through both transcription-dependent mechanism and post-transcriptional regulation [25–29]. MKP-1 functions as a critical negative regulator of inflammation. In a septic shock model, MKP-1-deficient mice produced greater amounts of proinflammatory cytokines and chemokines, with increased incidence and severity of multi-organ failure as well as higher mortality [30–32]. These studies identified MKP-1 as an important target for the treatment of inflammatory diseases. Thus, developing novel ligands to up-regulate MKP-1 would be a therapeutically attractive anti-inflammatory strategy.

It is important to discuss the mechanism underlying Gln suppression of neutrophilic infiltration into the airway and Th2-dependent asthmatic phenotypes. Many investigators have reported an association between cPLA$_2$ metabolites and Th2 response in asthmatic reactions [33–36]. Importantly, LTs play a key role in dendritic cell influx into airways in asthma [37–39] which, in turn, results in inhibition of recruitment of T cells and eosinophils into asthmatic airways and late AHR. Regarding neutrophil infiltration, we reported previously that airway neutrophilia in the asthmatic lungs is mediated by LTB4 rather than CXC chemokines such as keratinocyte-derived chemokine and macrophage inflammatory protein (MIP)-2 [20], which are principally chemotactic for neutrophils. Taken together, cPLA$_2$ metabolites play an important role in induction of airway neutrophilia and Th2-dependent asthmatic phenotypes. As a result, Gln can suppress these parameters. In contrast to neutrophils and eosinophils, macrophage infiltration was not suppressed by Gln. This may be due to the fact that
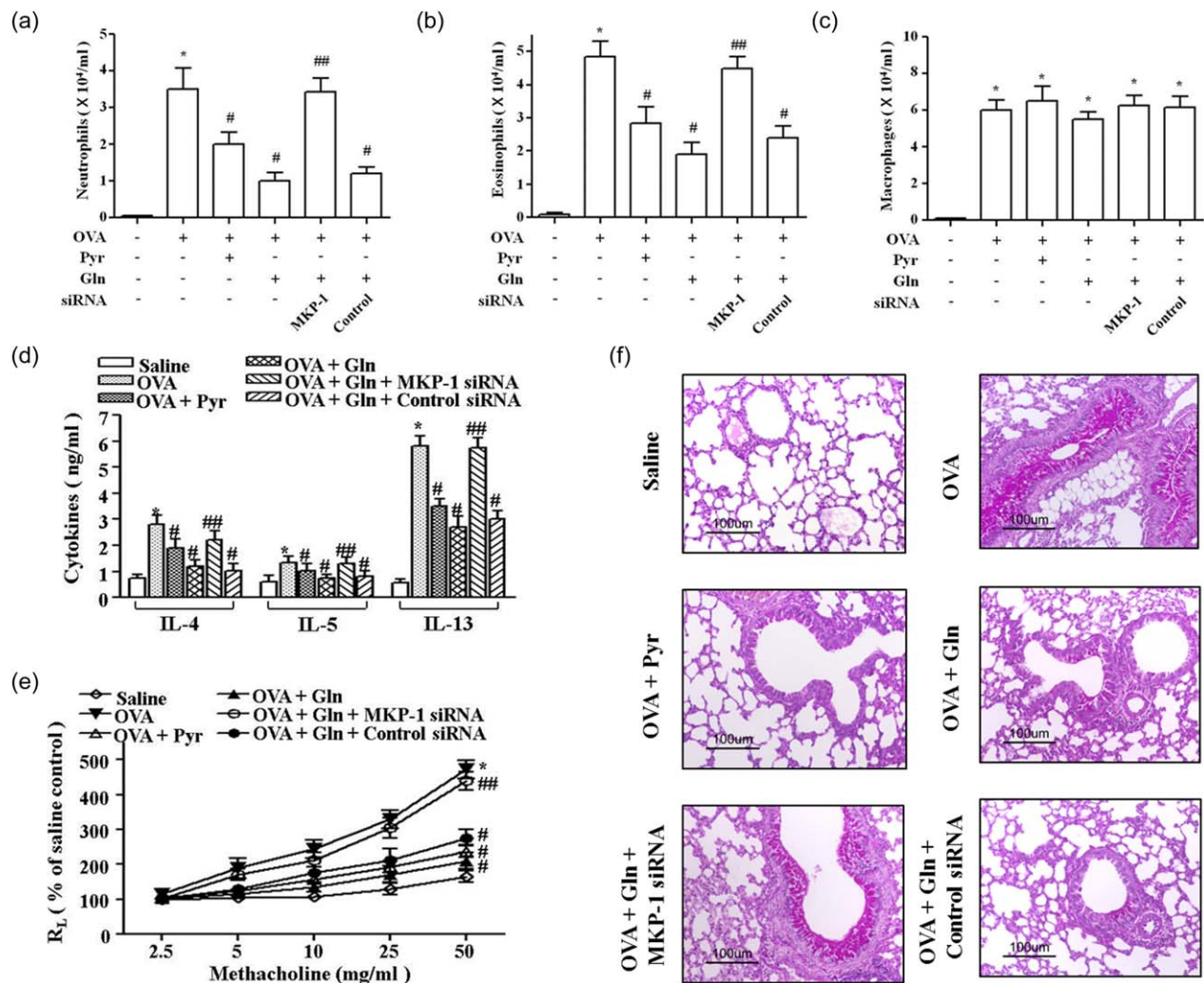
Fig. 5. Mitogen-activated protein kinase phosphatase-1 (MKP-1) Small interfering RNA (siRNA) abrogates the suppressive effect of L-glutamine (Gln) in allergic pulmonary inflammation. Small interfering RNAs (siRNA) and Gln were administered as described in Fig. 4. Pyrrophenone (Pyr; 20 mg/kg) was administered intraperitoneally (i.p.) 30 min before the second challenge. Bronchoalveolar lavage (BAL) fluids were collected 12 h (a), 48 h (b) and (c) and 18 h (d) after the second ovalbumin (OVA) challenge. Airway hyperresponsiveness (AHR) was performed at 48 h (e). Lungs were removed at 48 h post-challenge for mucin production (f). Data representing the mean ± standard deviation (s.d.) of three independent experiments with three to five mice/group/experiment are shown. $*P < 0.05$ compared to saline control; $**P < 0.01$ compared to saline control; $\#P < 0.05$ compared to the OVA group; $\#\#P < 0.05$ compared to the Gln group.

macrophage migration is not influenced by $cPLA_2$ metabolites; therefore, the monocytes/macrophages chemokines, C-C chemokines, such as monocyte chemotactic protein-1 and MIP-1, are not inhibited by Gln. In fact, the C-C chemokines are not suppressed by Gln in asthmatic lungs (data not shown). $cPLA_2$ has high selectivity in liberating arachidonic acid that is metabolized subsequently by a panel of downstream enzymes for eicosanoid production. Absence of $cPLA_2$ in mice resulted in their resistance to a variety of inflammatory diseases [40–44], indicating the potential contribution of $cPLA_2$ to inflammation. Therefore, $cPLA_2$ has attracted considerable attention as a potential therapeutic target for the control of inflamma-

tion and cancer. Inhibition of $cPLA_2$ as a pharmacological strategy has been proposed for the treatment of asthma [45], inflammatory skin diseases [46,47] and arthritis [48,49]. Despite promising preclinical data, the use of $cPLA_2$ inhibitors as a therapeutic approach in inflammatory diseases has not yet been confirmed in clinical trials, due probably to toxicity [50] or poor bioavailability [51]. In this regard, our findings showing that Gln exerted a potent anti-inflammatory action by deactivating both p38 and $cPLA_2$, probably the most potent enzymes involved in inflammatory reactions, might make Gln a useful therapeutic agent for many inflammatory diseases that involve $cPLA_2$ in their pathogenesis.
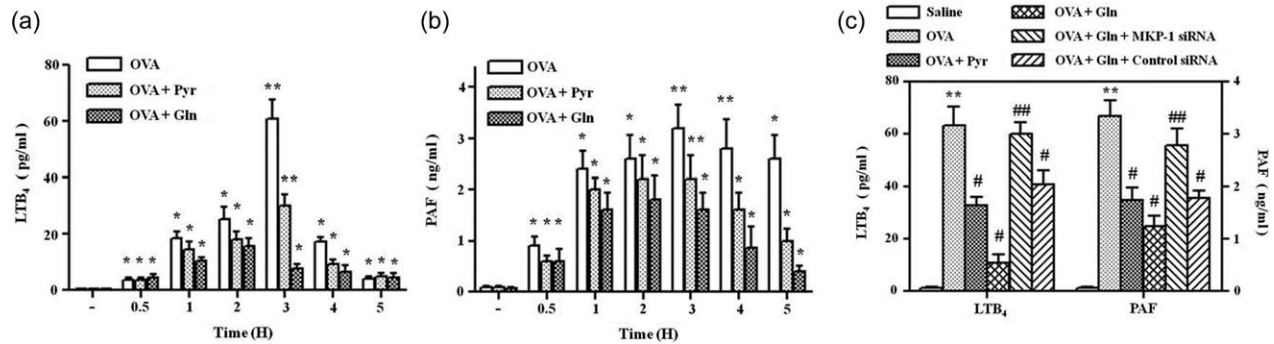
Fig. 6. L-glutamine (Gln) and pyrrophenone suppress bronchoalveolar lavage (BAL) levels of leukotriene B4 (LTB$_4$) and platelet activating factor (PAF). BAL fluid levels of LTB$_4$ (a) and PAF (b) were determined at the indicated time-points. Gln [750 mg/kg, intraperitoneally (i.p.)] and pyrrophenone (Pyr; 20 mg/kg, i.p.) were administered immediately after and 30 min before the second airway challenge, respectively. (c) LTB$_4$ and PAF were measured at 3 h. Data representing the mean ± standard deviation (s.d.) of three independent experiments with three to five mice/group/experiment are shown. *$P < 0.05$ compared to saline control; **$P < 0.01$ compared to saline control; #$P < 0.05$ compared to ovalbumin (OVA) challenge group.

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Disclosures

The authors declare that there are no conflicts of interest.

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