

Glutamine Suppresses DNFB-Induced Contact Dermatitis by Deactivating p38 Mitogen-Activated Protein Kinase via Induction of MAPK Phosphatase-1

Otgonzaya Ayush¹, Chang-Hoon Lee¹, Hae-Kyoung Kim¹, Suh-Young Im², Baik-Hwan Cho³ and Hern-Ku Lee¹

L-glutamine (Gln) is a nonessential amino acid that is the most abundant amino acid in plasma. Gln has been reported to have an anti-inflammatory activity that involves deactivation of mitogen-activated protein kinases (MAPKs) in a MAPK phosphatase (MKP)-1-dependent manner. This study investigated the role of Gln in the inhibition of DNFB-induced allergic contact dermatitis (CD) in the ears of mice, and specifically the involvement of Gln in p38 MAPK inhibition. Topical application of Gln or the p38 inhibitor, SB202190, suppressed DNFB-induced CD. Gln application inhibited DNFB-induced p38 phosphorylation. Western blot analysis revealed that Gln application resulted in early phosphorylation and protein induction of MKP-1. MKP-1 small interfering RNA (siRNA), but not control siRNA, abrogated Gln-mediated early phosphorylation, protein induction of MKP-1, deactivation of p38, and Gln-mediated suppression of CD. The extracellular signal-regulated kinase (ERK) inhibitor, U0126, blocked Gln-induced MKP-1 phosphorylation and protein induction, as well as Gln suppression of CD. These results suggest that Gln suppresses DNFB-induced CD via deactivation of p38 MAPK through the early induction of MKP-1, the negative regulator of p38, in an ERK-dependent manner.

Journal of Investigative Dermatology (2013) **133**, 723–731; doi:10.1038/jid.2012.373; published online 18 October 2012

INTRODUCTION

The pathways of three mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, are activated by many inflammatory stimuli (Chen *et al.*, 2001). Among them, p38 principally functions in the production of inflammatory molecules (Kumar *et al.*, 2003; Saklatvala, 2004; Schieven, 2005). Therefore, the p38 pathway is crucial in the production of a variety of inflammatory response proteins, such as pro-inflammatory cytokines, chemokines, degradative enzymes, growth factors, and adhesion molecules (Kumar *et al.*, 2003; Saklatvala, 2004; Schieven, 2005).

p38 MAPK activation is associated with a set of skin inflammation events that include hapten-induced contact

dermatitis (CD), UV irradiation-induced dermatitis, and psoriatic skin lesion (Takanami-Ohnishi *et al.*, 2002; Hildesheim *et al.*, 2004; Johansen *et al.*, 2005; Funding *et al.*, 2009). Many investigators have tried to develop p38 inhibitors, and some have been tested in clinical trials. However, severe side effects halted their development (Zhang *et al.*, 2007).

The nonessential amino acid L-glutamine (Gln) is an abundant amino acid in plasma ($\sim 600 \mu\text{M l}^{-1}$) (Cynober, 2002) and is an energy substrate for most cells (Fox *et al.*, 1996; Encarnacion *et al.*, 1998). Consequently, Gln is important in multiple ways in the nitrogen- and carbon-skeleton exchange among different tissues (Kovacevic and McGivan, 1983). Human clinical trials with several patient populations have demonstrated that Gln treatment decreases infectious complications, shortens hospitalization, and decreases hospital costs (Griffiths *et al.*, 1997). In addition, extensive research in animal models of endotoxin shock, including severe injury models, has demonstrated that Gln supplementation improves survival, enhances immune and gut barrier function, decreases bacteremia, and inhibits gut mucosal atrophy (Newsholme *et al.*, 1985; Wischmeyer *et al.*, 2001a).

With regard to the molecular mechanism of Gln anti-inflammatory activity, we have shown that Gln effectively protects animals from endotoxin shock and inhibits bronchial allergic asthma by inhibiting cytoplasmic phospholipase A₂ (cPLA₂) activity (Kim *et al.*, 2006; Ko *et al.*, 2008), which specifically releases arachidonic acid from the sn-2 position of glycerophospholipids to provide the precursor to various

¹Department of Immunology and Institute for Medical Science, Chonbuk National University Medical School, Jeonju, Korea; ²Department of Biological Sciences, College of Natural Sciences, Chonnam National University, Gwangju, Korea and ³Department of Surgery and Research Institute of Clinical Medicine, Chonbuk National University Medical School, Jeonju, Korea

Correspondence: Hern-Ku Lee, Department of Immunology, Chonbuk National University Medical School, Chonju, Chonbuk 561-182, Korea. E-mail: leeh-k@chonbuk.ac.kr

Abbreviations: CD, contact dermatitis; cPLA₂, cytoplasmic phospholipase A₂; ERK, extracellular signal-regulated kinase; ESR, ear swelling response; Gln, L-glutamine; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase-1; PEI, polyethyleneimine; siRNA, small interfering RNA

Received 13 February 2012; revised 10 May 2012; accepted 8 June 2012; published online 18 October 2012

eicosanoids, such as leukotrienes, prostaglandins, and platelet-activating factor, which are implicated in a variety of inflammatory diseases (Kita *et al.*, 2006). As a mechanism of Gln inhibition of cPLA₂, we have recently demonstrated that Gln deactivates p38 and JNK MAPKs, which is a major upstream pathway for cPLA₂ phosphorylation, by a rapid induction of MAPK phosphatase (MKP)-1 protein, which preferentially dephosphorylates p38 and JNK (Ko *et al.*, 2009).

On the basis of these findings, it was of interest to investigate whether Gln could suppress dermatitis by inhibiting p38. In this study, we explored this possibility using a murine model of DNFB-induced CD. The results demonstrate that the topical application of Gln suppresses DNFB-induced CD by deactivating p38 MAPK via the early induction of MKP-1.

RESULTS

Gln suppresses DNFB-induced ear inflammation

Gln (4% in saline) was topically applied onto the ears of mice after DNFB challenge: once at 5 minutes, three times at 5, 10, and 15 minutes, or five times at 5, 10, 15, 20, and 25 minutes. Application of DNFB to the ears increased ear swelling response (ESR) by ~80% compared with ears of mice that were treated with vehicle. One-time application of Gln had little effect on ESR, but application of Gln three or five times significantly inhibited DNFB-induced ESR at 24 hours, as well as at 48 hours (Figure 1a). The inhibition was not evident in the Ala (five applications) control group. Hematoxylin and eosin staining of sections revealed the development of strong inflammatory reactions at the DNFB-challenged sites, which were characterized by marked spongiosis and extensive leukocyte infiltration in the swollen dermis (Figure 1b). The extent of infiltration of leukocytes and swelling were significantly lower in Gln-treated mice than that in Ala-treated mice. Gln, but not Ala, significantly reduced protein levels of the IFN- γ , IL-1 β , tumor necrosis factor- α , and IL-5 cytokines in the ears (Figure 1c). In addition to DNFB, Gln also suppressed picryl chloride, as well as oxazolone-induced ear inflammation (Figure 1d and e).

Gln inhibits p38 and JNK phosphorylation, and p38 inhibitor attenuates DNFB-induced CD

We assessed the kinetics of MAPK phosphorylation in the DNFB-challenged ears (Figure 2). The three MAPKs, p38, JNK, and ERK, were phosphorylated by DNFB. p38 and JNK reached their maximal activities within 30–40 minutes, which declined thereafter. ERK was also phosphorylated, but in contrast to p38 and JNK its phosphorylation was sustained at relatively high levels for up to 80 minutes (Figure 2). Application of vehicle only induced a weak phosphorylation. Application of Gln once had little effect on the phosphorylation of p38 and JNK, but application of Gln three or five times inhibited their phosphorylation, and the inhibition was nearly complete at 50–60 minutes. The inhibition was not evident in the Ala control group.

We examined the linkage between MAPKs and DNFB-induced CD; intraperitoneal administration of the p38 inhibitor, SB202190, significantly attenuated ESR (Figure 3a), ear

inflammation (Figure 3b), and cytokine expression (Figure 3c). JNK or ERK inhibitor did not show such inhibition. These data suggest that p38 MAPK has a key role in the DNFB-induced dermatitis, and that Gln suppresses CD through the inhibition of p38 phosphorylation.

Gln-induced inhibition of p38 was associated with the early induction of MKP-1

To gain insight into the molecular mechanism of Gln inhibition of p38 phosphorylation, we examined the effect of Gln on MKP-1 protein induction. Western blot analysis revealed that MKP-1 protein increased from 60 minutes and continued to rise over 120 minutes and persisted for another 2 hours in response to DNFB challenge (Figure 4a). The appearance of MKP-1 protein also correlated with the dephosphorylation of p38 seen in Figure 2. Gln application resulted in an early induction of MKP-1. Appearance of MKP-1 as early as 30 minutes was evident in the ears of mice that received more than three applications of Gln, but not Ala. This Gln-induced induction of MKP-1 also coincided with a Gln-induced dephosphorylation of p38 seen in Figure 2. To elucidate the role of MKP-1 in Gln-induced inhibition of p38 phosphorylation, we tried to block MKP-1 induction using MKP-1 small interfering RNA (siRNA). Mice were intravenously administered 0.1, 0.2, or 0.4 nmol of MKP-1 siRNA or control siRNA 24 hours before DNFB challenge. MKP-1 siRNA, but not control siRNA, significantly abrogated the DNFB-mediated induction of MKP-1 at 0.2 or 0.4 nmol (Figure 4b). In mice pretreated with 0.2 or 0.4 nmol of MKP-1 siRNA, Gln did not induce MKP-1 and did not inhibit p38 phosphorylation (Figure 4c). We examined the effect of Gln in the human keratinocyte cell line HaCaT. Treatment of the cells with DNFB resulted in phosphorylation of p38 as early as 5 minutes, and addition of Gln at 10 minutes not only dephosphorylated p38 at 30 minutes in a dose-dependent manner (Figure 4d) but also caused an early induction of MKP-1 and dephosphorylation of p38 (Figure 4e), as seen in an *in vivo* study. These data clearly indicate that Gln inhibits phosphorylation of p38 through the early induction of MKP-1.

MKP-1 siRNA attenuates Gln suppression of ear inflammation

By using MKP-1 siRNA, we examined whether the ability of Gln to induce MKP-1 protein was associated with the beneficial effect of Gln on CD. Pretreatment of MKP-1 siRNA, but not control siRNA, abrogated the Gln-mediated suppression of DNFB-induced ESR (Figure 5a), inflammation (Figure 5b), and cytokine expression (Figure 5c).

Gln-induced induction of MKP-1 is ERK dependent

MKP-1 is a labile protein that is normally degraded by the ubiquitin/proteasome pathway; its phosphorylation reduces its ubiquitination and degradation (Sun *et al.*, 1993; Kyriakis and Avruch, 2001). ERK is responsible for MKP-1 phosphorylation (Brondello *et al.*, 1999). We have previously shown that Gln-induced MKP-1 induction is ERK dependent by demonstrating that Gln-induced MKP-1 phosphorylation and protein induction were blocked by pretreatment of the ERK inhibitor (Ko *et al.*, 2009) in lipopolysaccharide-treated mice.

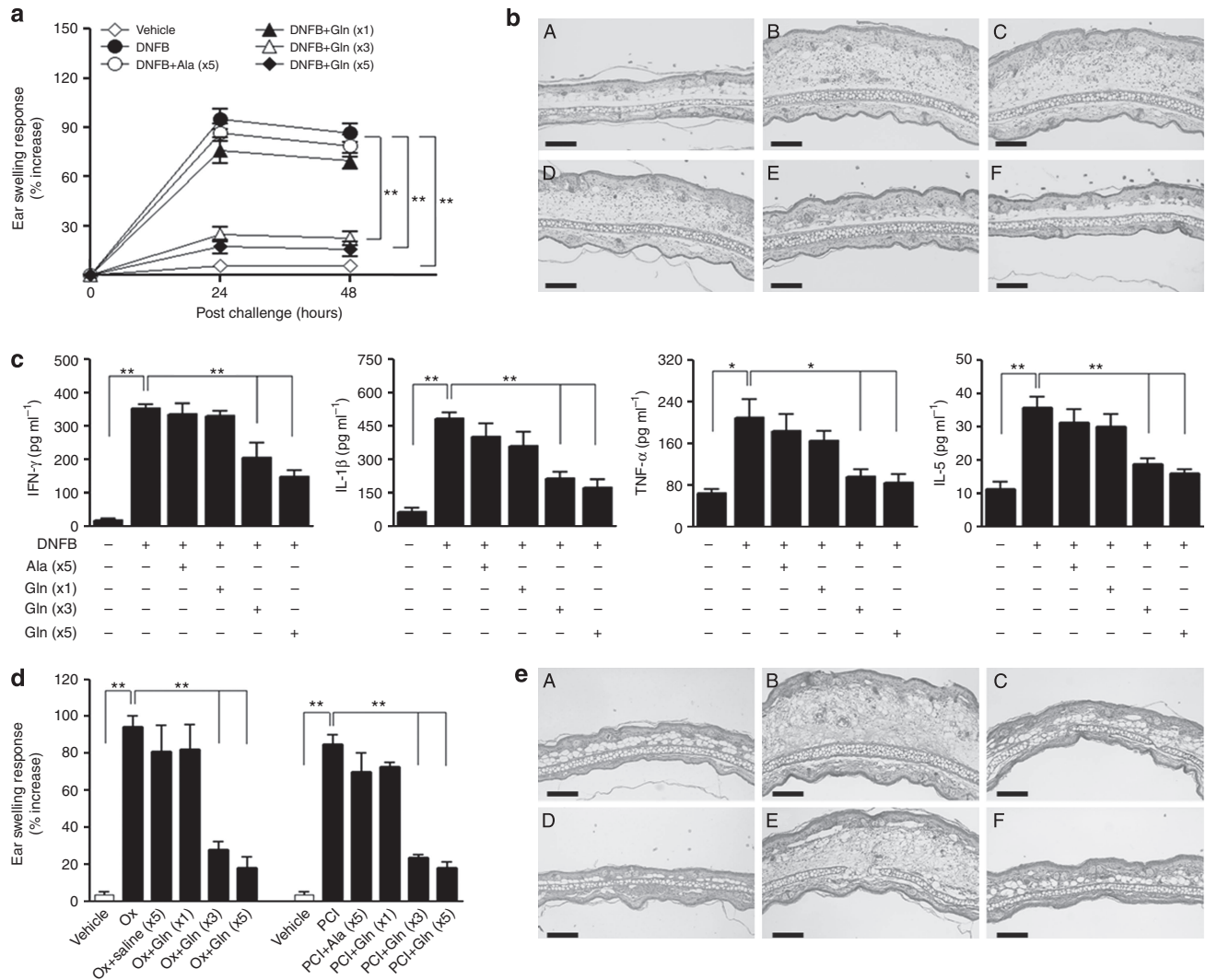


Figure 1. L-glutamine (Gln) suppresses DNFB-induced contact dermatitis. (a) Ear swelling responses (ESRs) were measured. (b) After measurement of ESR at 24 hours, ear sections from the (A) vehicle, (B) DNFB, (C) DNFB + Ala ($\times 5$), (D) DNFB + Gln ($\times 1$), (E) DNFB + Gln ($\times 3$), or (F) DNFB + Gln ($\times 5$)-treated group were stained with hematoxylin and eosin (H&E). (c) Cytokine levels were measured and calculated as picograms of protein per milligram of homogenized tissue. (d) ESR at 24 hours after challenge with oxazolone (Ox) or picryl chloride (PCI). (e) After measurement of ESR, ear sections from the (A) Ox vehicle, (B) Ox, (C) Ox + Gln ($\times 5$), (D) PCI vehicle, (E) PCI, or (F) PCI + Gln ($\times 5$)-treated group were stained with H&E. Data in **a**, **c**, and **d** represent the mean $\pm$ SD of three independent experiments with five mice per group. * $P < 0.05$; ** $P < 0.01$. TNF, tumor necrosis factor. Bar = 100 μ m.

Therefore, we currently assessed whether ERK could exert a similar role in skin inflammation.

We first verified the efficacy of U0126 to inhibit ERK phosphorylation (Figure 6a). Topical application of Gln three times resulted in MKP-1 phosphorylation (Figure 6b). U0126 abrogated not only Gln-induced MKP-1 phosphorylation, protein induction, and deactivation of p38 (Figure 6c), but also Gln-induced suppression of DNFB-induced ESR (Figure 6d) and inflammation (Figure 6e).

DISCUSSION

In this study, we demonstrate that Gln is capable of suppressing DNFB-induced CD by inhibiting p38 MAPK in a MKP-1-dependent manner. Application of DNFB induced the phosphorylation of p38 MAPK, and p38 inhibitor, SB202190,

suppressed all the DNFB-induced ESR, inflammatory cell infiltration, and expression of several proinflammatory cytokines, Th1 and Th2 cytokines in the ears we measured. To gain clinical relevance, Gln was topically applied after the challenge of DNFB, thus, Gln inhibits elicitation, but not induction, phase of contact allergy.

Many inflammatory response proteins, such as proinflammatory cytokines, chemokines, degradative enzymes, growth factors, and adhesion molecules, depend on p38 signaling for their production (Zhang *et al.*, 2007). Therefore, the abrogation of the p38 pathway results in diminished production of inflammatory mediators, which blocks inflammation (Kracht and Saklatvala, 2002; Kumar *et al.*, 2003; Saklatvala, 2004; Schieven, 2005; Zarubin and Han, 2005). Furthermore, the p38 pathway has a regulatory role in the proliferation and

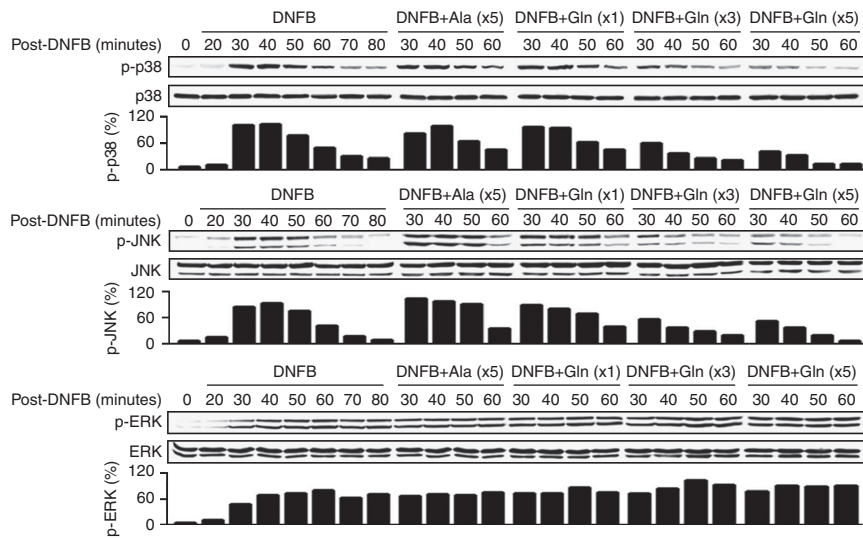


Figure 2. Gln inhibits p38 and c-Jun N-terminal kinase (JNK) phosphorylation. The ears were removed at the indicated time points after DNFB challenge. Protein levels and phosphorylation of mitogen-activated protein kinases were determined by western blotting and quantified by densitometric analysis. A representative of three to five independent experiments with 3–5 mice per time point per experiment is shown. ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated ERK; p-JNK, phosphorylated JNK.

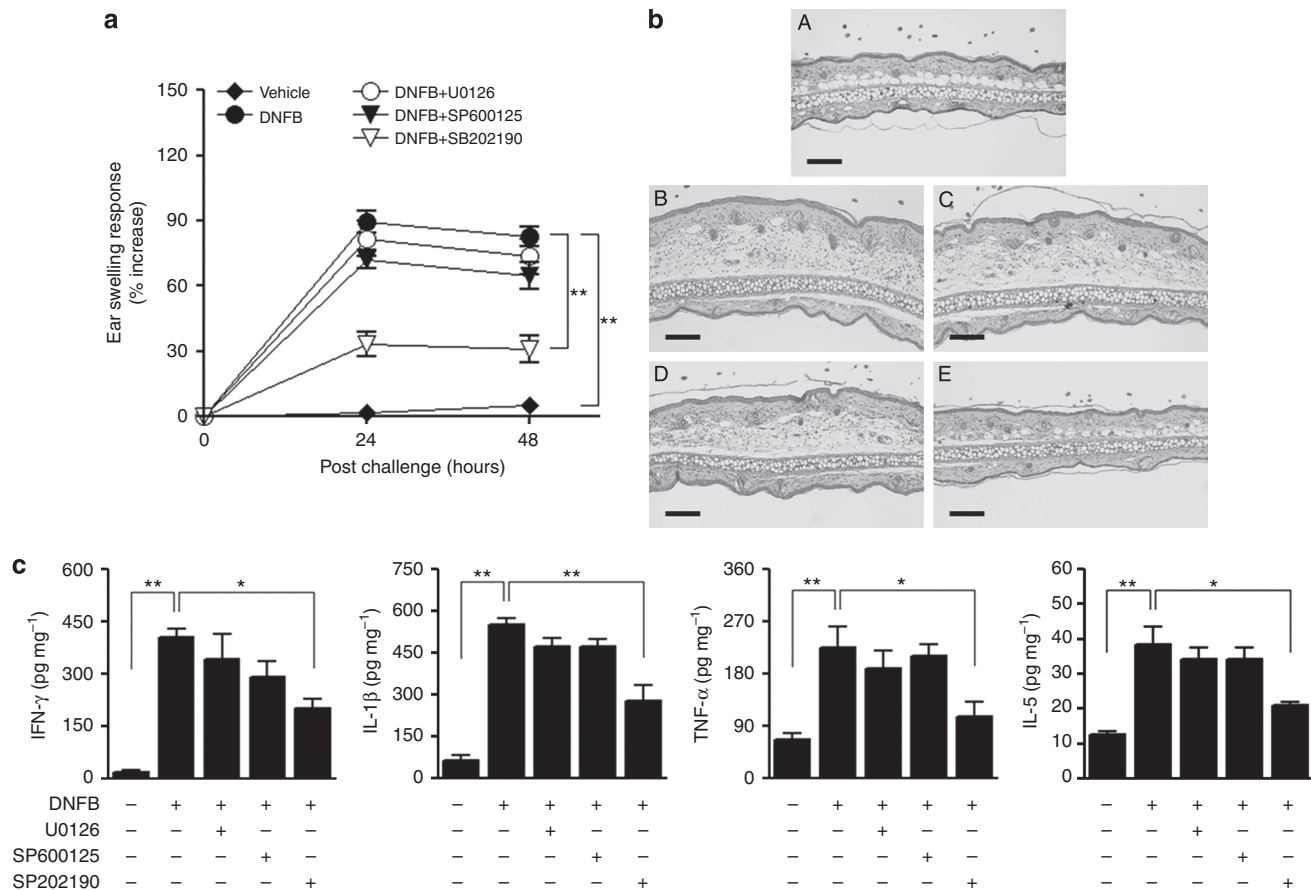


Figure 3. p38 inhibitor attenuates DNFB-induced contact dermatitis. (a) Mitogen-activated protein kinase inhibitors were injected intraperitoneally (i.p.) 24 hours before the challenge of DNFB. Ear swelling responses (ESRs) were measured at the indicated time points. (b) After measurement of ESR at 24 hours, the mice were killed, and ear sections from the (A) vehicle (DMSO), (B) DNFB, (C) DNFB + U0126, (D) DNFB + SP600125, or (E) DNFB + SB202190-treated group were stained with hematoxylin and eosin. (c) Ear biopsy homogenates were taken 24 hours after the challenge and cytokine levels were measured using ELISA and calculated as picograms of protein per milligram of homogenized tissue solution. Data in a and c represent the mean ± SD of three independent experiments with five mice per group. **P* < 0.05; ***P* < 0.01. TNF, tumor necrosis factor. Bar = 100 μm.

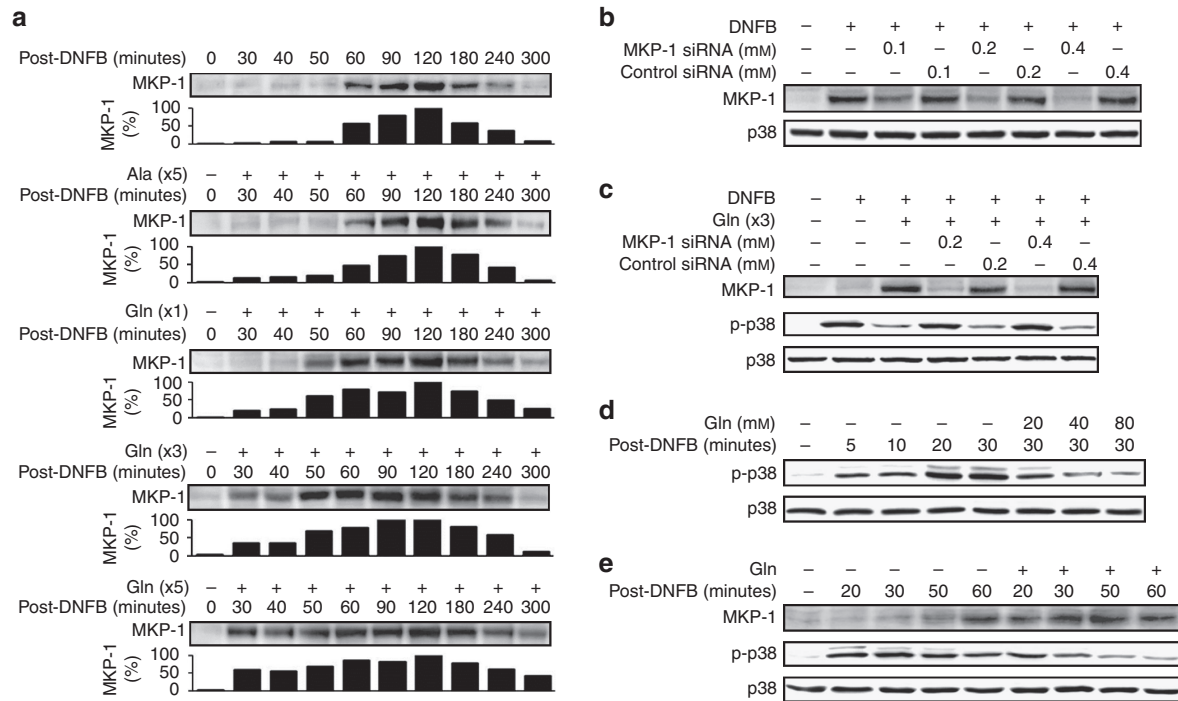


Figure 4. L-glutamine (Gln) deactivates p38 via mitogen-activated protein kinase phosphatase (MKP)-1. (a) Gln or Ala was applied onto the ears. (b) Mice received small interfering RNA (siRNA) 24 hours before challenge of DNFB. The ears were removed 90 minutes after challenge. (c) Mice were pretreated with siRNA, and Gln was applied three times ($\times 3$) after DNFB challenge. Mice were killed at 50 minutes. (d) HaCaT cells were stimulated with DNFB ($5 \mu\text{g ml}^{-1}$). Gln (20, 40, or 80 mM) was added to the HaCaT cells (2×10^6) 10 min after DNFB stimulation. (e) Gln (80 mM) was added at 10 minutes and the cells were harvested at the times indicated. A representative of three to five independent experiments with 3–5 mice per time point per experiment is shown.

differentiation of dendritic cells (Ardeshtna *et al.*, 2000; Arrighi *et al.*, 2001), T cells (Crawley *et al.*, 1997; Rincon *et al.*, 1998; Maneechotesuwan *et al.*, 2007), and B cells (Craxton *et al.*, 1998), all of which contribute to the development of inflammation. These findings support the view of the crucial function of the p38 pathway in the regulation of inflammation, including a set of skin inflammatory diseases, such as hapten-induced CD, UV irradiation-induced dermatitis, and psoriatic skin lesions (Takanami-Ohnishi *et al.*, 2002; Hildesheim *et al.*, 2004; Johansen *et al.*, 2005; Funding *et al.*, 2009). In this study, we found that p38 inhibitor exerted a suppressive activity, further supporting the notion that p38 has a key role in the pathogenesis of DNFB-induced CD.

Topical application of Gln resulted in significant suppression of DNFB-induced ear inflammation, as well as phosphorylation of p38. At present, Gln-induced inhibition of p38 was attributed to the early induction of the dual phosphatase MKP-1 protein. This conclusion arose from two observations. First, Gln application resulted in a rapid induction of MKP-1, which coincided with Gln-induced inhibition of p38. Second, MKP-1 siRNA abrogated the ability of Gln not only to induce MKP-1 induction and inhibition of p38, but also to suppress ear inflammation. These findings might explain our previous observation that Gln-mediated inhibition of cPLA₂ phosphorylation is due to its capability to inhibit the phosphorylation of MAPKs, which are the major upstream pathways for cPLA₂ phosphorylation (Kim *et al.*, 2006; Ko *et al.*, 2008). We have previously shown that Gln rapidly deactivates p38 by dephos-

phorylating them only when these MAPKs were phosphorylated in response to lipopolysaccharide, and this effect of Gln was mediated by rapid (~ 5 minutes) phosphorylation and protein induction of MKP-1 (Ko *et al.*, 2009). Therefore, a rapid and early induction of MKP-1, resulting in deactivation of p38 and cPLA₂, seems to be a common important mechanism of the anti-inflammatory activity of Gln. We found that, in addition to Gln, cysteine and valine were capable of inducing MKP-1 and inhibiting p38 phosphorylation, as well as suppressing ESR (data not shown). These findings indicate that other amino acids can act as modifiers in the phosphorylation-associated event. Further studies are required to address the precise mechanisms underlying the phenomenon.

MKP-1 was originally identified as an ERK-specific phosphatase (Sun *et al.*, 1993; Misra-Press *et al.*, 1995), but has since been also recognized as being able to dephosphorylate and inactivate both p38 and JNK MAPKs, its substrate specificity being dependent on cell type and context (Franklin and Kraft, 1997; Chi *et al.*, 2006; Hammer *et al.*, 2006; Zhao *et al.*, 2006). Among the MKPs, in addition to MKP-1, MKP-5 (Tanoue *et al.*, 1999) and MKP-7 (Masuda *et al.*, 2001) have also been reported to have specificity for p38 and JNK. Nevertheless, abrogation of the majority of activities of Gln by MKP-1 siRNA suggests that MKP-1 appears to be the critical MKP in this process.

MKP-1 has been reported to be a labile protein that is normally degraded via the ubiquitin/proteasome pathway. Its

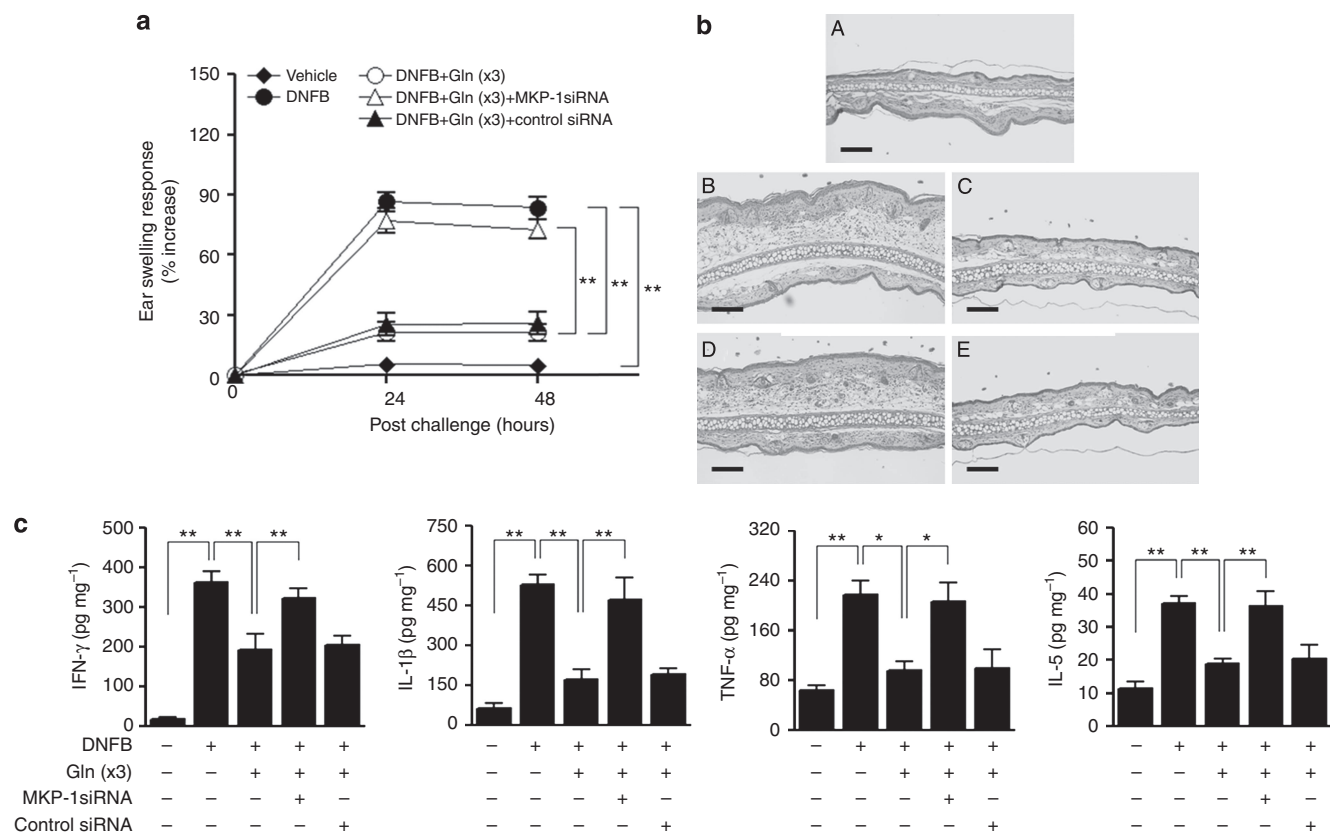


Figure 5. Mitogen-activated protein kinase phosphatase (MKP)-1 small interfering RNA (siRNA) abrogates the suppressive activity of L-glutamine (Gln) against DNFB-induced contact dermatitis. Mice were intravenously injected with 200 μ l of the polyethyleneimine mixture containing 0.4 nmol of siRNA 24 hours before challenge of DNFB. Gln was applied three times ($\times 3$) in the ear skin after DNFB challenge. (a) Measurement of ear swelling response (ESR), (b) hematoxylin and eosin (H&E) staining from mice that received (A) vehicle, (B) DNFB, (C) DNFB + Gln ($\times 3$), (D) DNFB + Gln ($\times 3$) + MKP-1 siRNA, or (E) DNFB + Gln ($\times 3$) + control siRNA, and (c) measurement of cytokine levels in the ear tissues were performed. Data in a and c represent the mean $\pm$ SD of three independent experiments with five mice per group. * $P < 0.05$; ** $P < 0.01$. TNF, tumor necrosis factor. Bar = 100 μ m.

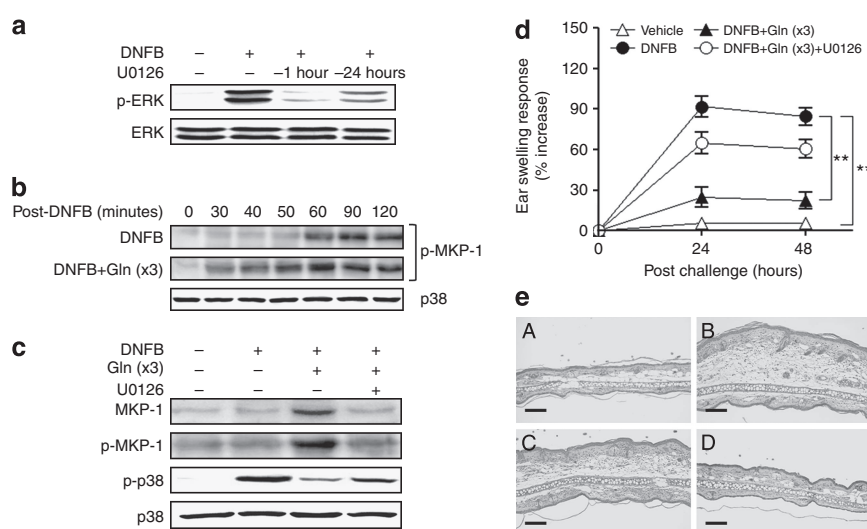


Figure 6. Extracellular signal-regulated kinase (ERK) inhibitor reverses the effects of Gln on mitogen-activated protein kinase phosphatase (MKP)-1 induction, p38 phosphorylation, and ear inflammation. ERK inhibitor U0126 was administered intraperitoneally 24 hours before DNFB challenge. Gln was applied three times ($\times 3$) in the ear skin after DNFB challenge. (a) ERK phosphorylation, (b) MKP-1 phosphorylation, (c) MKP-1 protein induction and p38 phosphorylation, and (d) measurement of ear swelling response were performed, as well as (e) histological examination of groups that received (A) vehicle, (B) DNFB, (C) DNFB + Gln ($\times 3$), or (D) DNFB + Gln ($\times 3$) + U0126. Data in a, b, and c are representative of three to five independent experiments with 3–5 mice per time point per experiment. Data in d represent the mean $\pm$ SD of three independent experiments with five mice per group. ** $P < 0.01$. Bar = 100 μ m.

phosphorylation reduces its ubiquitination and degradation (Brondello *et al.*, 1999; Orłowski *et al.*, 2002), and phosphorylation of MKP-1 is mediated by transient activation of ERK at the two extreme C-terminal Ser359 and Ser364, which enhances MKP-1 stabilization without altering its phosphatase activity (Brondello *et al.*, 1999). We currently demonstrated that (1) the kinetics of phosphorylation (Figure 6b) and protein induction of MKP-1 in response to DNFB challenge are very similar (Figure 4a), and (2) the ERK inhibitor, U0126, blocked not only Gln-induced MKP-1 phosphorylation and protein induction, but also the suppressive activity of Gln against DNFB-induced CD. These findings, together with our previous report showing similar results in lipopolysaccharide-induced septic shock model (Ko *et al.*, 2009), suggest that Gln induces the early induction of MKP-1 protein by preventing its degradation by inducing MKP-1 phosphorylation in an ERK-dependent manner.

As a result of its function to deactivate p38 MAPK, MKP-1 serves as an important negative regulator of the inflammatory responses. MKP-1 is induced not only by a number of growth factors and stresses in multiple cell types (Chen *et al.*, 2002; Zhao *et al.*, 2005; Chi *et al.*, 2006; Zhao *et al.*, 2006; Hu *et al.*, 2007), but also by multiple immunosuppressive agents, including glucocorticoids and anti-inflammatory cytokines, and this induction partially mediates the inhibitory effects of these agents on MAPK activation (Abraham *et al.*, 2006). Induction of MKP-1 by transforming growth factor- β and IL-10 also contributes to the suppressive effects of these potent anti-inflammatory cytokines on the expression of genes encoding proinflammatory mediators (Xiao *et al.*, 2002; Hammer *et al.*, 2005). Therefore, it appears that the induction of MKP-1 represents a common mechanism of immunomodulatory agents.

In summary, it seems clear that Gln exerts strong anti-inflammatory activity against DNFB-induced CD through the early induction of MKP-1, resulting in the inhibition of p38 MAPK, probably the most important enzyme in induction of inflammatory reactions. Given that even the parenteral administration of Gln as a component of supportive nutritional therapy is safe (Wischmeyer *et al.*, 2001b; Novak *et al.*, 2002; Noé, 2009), Gln targeting p38 could be very useful in a therapeutic regimen in a variety of skin inflammatory diseases.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice (7–8 weeks old, 16–18 g body weight) were purchased from the Orient Bio (Gapyeong, Gyeonggi, Korea) and kept in our animal facility for at least 1 week before use. All animal experiments in this study were conducted using a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

Chemicals and reagents

DNFB, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone), picryl chloride, L-Gln (Biotechnology performance certified, G-8540), and alanine (Ala) were obtained from Sigma-Aldrich (St Louis, MO). DNFB was dissolved in acetone:olive oil (3:1). Gln and Ala were dissolved in saline to reach the final concentration of

4%. SB202190 (p38 inhibitor), SP600125 (JNK inhibitor), and U0126 (MEK1/2 inhibitor) were from Calbiochem (San Diego, CA). SB202190 (5 mg kg⁻¹) (Kang *et al.*, 2008), SP600125 (12.5 mg kg⁻¹), and U0126 (12.5 mg kg⁻¹) (Ko *et al.*, 2009) were dissolved with DMSO and injected intraperitoneally 24 hours before DNFB challenge. The control group received vehicle.

Induction of allergic CD

Mice were sensitized on the shaved back of skin with 100 μ l of 0.3% DNFB in acetone:olive oil (3:1) (Takanami-Ohnishi *et al.*, 2002; Yuan *et al.*, 2010), 50 μ l of 0.5% oxazolone in acetone:olive oil (4:1) (Noordegraaf *et al.*, 2010), or 50 μ l of 5% picryl chloride in ethanol:acetone (3:1) (Mori *et al.*, 2008). Five days after sensitization, mice were challenged by painting the inner and outer surfaces of both sides of each ear with 20 μ l of 0.3% DNFB, 20 μ l of 0.3% oxazolone, or 20 μ l of 0.5% picryl chloride. Vehicle groups received acetone:olive oil or ethanol/acetone only.

Measurement of ESR

The thickness of the ears was measured using a No. 7326 thickness gauge (Mitutoyo, Tokyo, Japan). Percent ESR change was expressed as follows:

$$[(\text{value at post-DNFB application} - \text{value at pre-DNFB application}) \div \text{value at pre-DNFB application}] \times 100.$$

Histological analysis

Twenty-four hours after challenge, mice were anesthetized by inhalation of diethyl ether. Their ears were removed and immersed in 10% formaldehyde and then embedded in paraffin and cut into 5- μ m-thick sections using a SM2000R microtome (Leica, Jena, Germany). The ear sections were stained with hematoxylin and eosin.

Cell culture

The human keratinocyte cell line, HaCaT, was cultured in DMEM with high glucose (Thermo Scientific Hyclone No.: SH30243.02), supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Invitrogen, Grand Island, NY). The cells were maintained at 37 °C in a humidified atmosphere at 5% CO₂.

Cytokine ELISA of ear tissues

After DNFB challenge, ear samples were weighed (100 mg) and homogenized in 1 ml of PhosphoSafe Extraction Reagent (Novagen, Madison, WI) containing a protease inhibitor cocktail. Homogenates were then centrifuged at 12,000 g for 20 minutes at 4 °C to obtain the supernatant. IFN- γ , IL-1 β , tumor necrosis factor- α , and IL-5 levels were analyzed using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cytokine levels were calculated as picogram of protein per milligram of homogenized tissue.

siRNA interference

siRNA strands for mouse MKP-1 and controls were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). *In vivo* delivery of siRNA was performed using *in vivo*-jet polyethyleneimine (PEI) (Polyplus-transfection SA, 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 μ l for intravenous injection at room temperature for 20 minutes, and the mixture (0.1, 0.2, or 0.4 nmol)

was administered 24 hours before DNFB challenge. The mixture containing control siRNA and PEI dissolved in 5% glucose without siRNA was used as control. To confirm that the MKP-1 siRNA used really blocked the synthesis of its target, an immunoblotting analysis was performed.

Immunoblotting analysis

Mice were killed by cervical dislocation, and ear samples were frozen in liquid nitrogen and stored in -70°C until analysis. Ear samples were homogenized in the Phosphosafe Extraction Reagent (Novagen). Equal amounts of samples were separated by 10% SDS-PAGE under reducing conditions, and were then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were blocked by incubation for 1 hour at room temperature in 5% skimmed milk in Tris-buffered saline, followed by a further 2 hours of incubation with primary antibodies against mouse phospho-ERK, phospho-JNK, phospho-MKP-1, phospho-p38, ERK, JNK, MKP-1, and p38 (Santa Cruz Biotechnology). Blots were washed for 25 minutes with five changes of Tris-buffered saline-0.05% Tween 20 solution, followed by incubation for 1 hour at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology). Finally, they were developed in LumiGLO reagent (Cell Signaling Technology, Danvers, MA).

Statistical analysis

All data are shown as mean $\pm$ SE of mean. Statistical comparison was performed using one-way ANOVA followed by the Fisher's test. Significant differences between the groups were determined using the unpaired Student's *t*-test. A value of $P < 0.05$ was accepted as an indication of statistical significance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (no. 2011-0016423).

REFERENCES

- Abraham SM, Lawrence T, Kleiman A *et al.* (2006) Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. *J Exp Med* 203:1883–9
- Ardeshtna KM, Pizzey AR, Devereux S *et al.* (2000) The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood* 96:1039–46
- Arrighi JF, Rebsamen M, Rousset F *et al.* (2001) A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. *J Immunol* 166:3837–45
- Brondello JM, Pouyssegur J, McKenzie FR (1999) Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. *Science* 286:2514–7
- Chen P, Li J, Barnes J *et al.* (2002) Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. *J Immunol* 169:6408–16
- Chen Z, Gibson TB, Robinson F *et al.* (2001) MAP kinases. *Chem Rev* 101:2449–76
- Chi H, Barry SP, Roth RJ *et al.* (2006) Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. *Proc Natl Acad Sci USA* 103:2274–9
- Crawley JB, Rawlinson L, Lali FV *et al.* (1997) T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. *J Biol Chem* 272:15023–7
- Craxton A, Shu G, Graves JD *et al.* (1998) p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocytes. *J Immunol* 161:3225–36
- Cynober LA (2002) Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. *Nutrition* 18:761–6
- Encarnacion S, Calderon J, Gelbard AS *et al.* (1998) Glutamine biosynthesis and the utilization of succinate and glutamine by *Rhizobium etli* and *Sinorhizobium meliloti*. *Microbiology* 144(Part 9):2629–38
- Fox RE, Hopkins IB, Cabacungan ET *et al.* (1996) The role of glutamine and other alternate substrates as energy sources in the fetal rat lung type II cell. *Pediat Res* 40:135–41
- Franklin CC, Kraft AS (1997) Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. *J Biol Chem* 272:16917–23
- Funding AT, Johansen C, Gaestel M *et al.* (2009) Reduced oxazolone-induced skin inflammation in MAPKAP kinase 2 knockout mice. *J Invest Dermatol* 129:891–8
- Griffiths RD, Jones C, Palmer TE (1997) Six-month outcome of critically ill patients given glutamine-supplemented parenteral nutrition. *Nutrition* 13:295–302
- Hammer M, Mages J, Dietrich H *et al.* (2005) Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. *Eur J Immunol* 35:2991–3001
- Hammer M, Mages J, Dietrich H *et al.* (2006) Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. *J Exp Med* 203:15–20
- Hildesheim J, Awwad RT, Fornace AJ Jr (2004) p38 Mitogen-activated protein kinase inhibitor protects the epidermis against the acute damaging effects of ultraviolet irradiation by blocking apoptosis and inflammatory responses. *J Invest Dermatol* 122:497–502
- Hu JH, Chen T, Zhuang ZH *et al.* (2007) Feedback control of MKP-1 expression by p38. *Cell Signal* 19:393–400
- Johansen C, Kragballe K, Westergaard M *et al.* (2005) The mitogen-activated protein kinases p38 and ERK1/2 are increased in lesional psoriatic skin. *Br J Dermatol* 152:37–42
- Kang NI, Kim HK, Ko HM *et al.* (2008) Tumor necrosis factor-alpha develops late anaphylactic reaction through cytosolic phospholipase A(2) activation. *Int Arch Allergy Immunol* 147:315–22
- Kim YS, Kim GY, Kim JH *et al.* (2006) Glutamine inhibits lipopolysaccharide-induced cytoplasmic phospholipase A₂ activation and protects against endotoxin shock in mouse. *Shock* 25:290–4
- Kita Y, Ohto T, Uozumi N *et al.* (2006) Biochemical properties and pathophysiological roles of cytosolic phospholipase A₂s. *Biochim Biophys Acta* 1761:1317–22
- Ko HM, Kang NI, Kim YS *et al.* (2008) Glutamine preferentially inhibits T-helper type 2 cell-mediated airway inflammation and late airway hyperresponsiveness through the inhibition of cytosolic phospholipase A₂ activity in a murine asthma model. *Clin Exp Allergy* 38:357–64
- Ko HM, Oh SH, Bang HS *et al.* (2009) Glutamine protects mice from lethal endotoxin shock via a rapid induction of MAPK phosphatase-1. *J Immunol* 182:7957–62
- Kovacevic Z, McGivan JD (1983) Mitochondrial metabolism of glutamine and glutamate and its physiological significance. *Physiol Rev* 63:547–605
- Kracht M, Saklatvala J (2002) Transcriptional and post-transcriptional control of gene expression in inflammation. *Cytokine* 20:91–106
- Kumar S, Boehm J, Lee JC (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov* 2:717–26

- Kyriakis JM, Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81:807–69
- Maneechotesuwan K, Xin Y, Ito K *et al.* (2007) Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. *J Immunol* 178:2491–8
- Masuda K, Shima H, Watanabe M *et al.* (2001) MKP-7, a novel mitogen-activated protein kinase phosphatase, functions as a shuttle protein. *J Biol Chem* 276:39002–11
- Misra-Press A, Rim CS, Yao H *et al.* (1995) A novel mitogen-activated protein kinase phosphatase. Structure, expression, and regulation. *J Biol Chem* 270:14587–96
- Mori T, Kabashima K, Yoshiki R *et al.* (2008) Cutaneous hypersensitivities to haptens are controlled by IFN- γ -upregulated keratinocyte Th1 chemokines and IFN- γ -downregulated Langerhans cell Th2 chemokines. *J Invest Dermatol* 128:1719–27
- Newsholme EA, Crabtree B, Ardawi MS (1985) Glutamine metabolism in lymphocytes: its biochemical, physiological and clinical importance. *Q J Exp Physiol* 70:473–89
- Noé JE (2009) L-glutamine use in the treatment and prevention of mucositis and cachexia: a naturopathic perspective. *Integr Cancer Ther* 8:409–15
- Noordegraaf M, Flacher V, Stoitzner P *et al.* (2010) Functional redundancy of Langerhans cells and Langerin $^{+}$ dermal dendritic cells in contact hypersensitivity. *J Invest Dermatol* 130:2752–9
- Novak F, Heyland DK, Avenell A *et al.* (2002) Glutamine supplementation in serious illness: a systematic review of the evidence. *Crit Care Med* 30:2022–9
- Orlowski RZ, Small GW, Shi YY (2002) Evidence that inhibition of p44/42 mitogen-activated protein kinase signaling is a factor in proteasome inhibitor-mediated apoptosis. *J Biol Chem* 277:27864–71
- Rincon M, Enslen H, Raingeaud J *et al.* (1998) Interferon- γ expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. *EMBO J* 17:2817–29
- Saklatvala J (2004) The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Curr Opin Pharmacol* 4:372–7
- Schieven GL (2005) The biology of p38 kinase: a central role in inflammation. *Curr Top Med Chem* 5:921–8
- Sun H, Charles CH, Lau LF *et al.* (1993) MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell* 75:487–93
- Takanami-Ohnishi Y, Amano S, Kimura S *et al.* (2002) Essential role of p38 mitogen-activated protein kinase in contact hypersensitivity. *J Biol Chem* 277:37896–903
- Tanoue T, Moriguchi T, Nishida E (1999) Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5. *J Biol Chem* 274:19949–56
- Wischmeyer PE, Kahana M, Wolfson R *et al.* (2001a) Glutamine reduces cytokine release, organ damage, and mortality in a rat model of endotoxemia. *Shock* 16:398–402
- Wischmeyer PE, Lynch J, Liedel J *et al.* (2001b) Glutamine administration reduces Gram-negative bacteremia in severely burned patients: a prospective, randomized, double-blind trial versus isonitrogenous control. *Crit Care Med* 29:2075–80
- Xiao YQ, Malcolm K, Worthen GS *et al.* (2002) Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor- β . *J Biol Chem* 277:14884–93
- Yuan XY, Liu W, Zhang P *et al.* (2010) Effects and mechanisms of aloperine on 2, 4-dinitrofluorobenzene-induced allergic contact dermatitis in BALB/c mice. *Eur J Pharmacol* 629:147–52
- Zarubin T, Han J (2005) Activation and signaling of the p38 MAP kinase pathway. *Cell Res* 15:11–8
- Zhang J, Shen B, Lin A (2007) Novel strategies for inhibition of the p38 MAPK pathway. *Trends Pharmacol Sci* 28:286–95
- Zhao Q, Shepherd EG, Manson ME *et al.* (2005) The role of mitogen-activated protein kinase phosphatase-1 in the response of alveolar macrophages to lipopolysaccharide: attenuation of proinflammatory cytokine biosynthesis via feedback control of p38. *J Biol Chem* 280:8101–8
- Zhao Q, Wang X, Nelin LD *et al.* (2006) MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock. *J Exp Med* 203:131–40