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Shikonin inhibited mitogen-activated IL-4 and IL-5 production on EL-4 cells through downregulation of GATA-3 and c-Maf induction

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ABSTRACT

Aim: To investigate the effects of shikonin on phorbol myristate acetate (PMA) plus cyclic adenosine monophosphate (cAMP)-induced T helper (T_H) 2 cell cytokine production, and the underlying mechanism.
Main methods: We used activated EL-4 murine T-lymphoma cells, which produce interleukin (IL)-4 and IL-5, but not interferon (IFN)-γ, as T_H2 cell-like cells and treated them with PMA + cAMP to investigate the effects of shikonin on T_H2 cytokines, transcriptional factors, and the related mitogen-activated protein kinase (MAPK)/nuclear factor (NF)-κB signaling pathway.
Key findings: The data show that shikonin inhibited the PMA + cAMP-induced mRNA and protein expression of IL-4 and IL-5 via the downregulation of GATA-binding protein-3 (GATA-3) and c-musculoaponeurotic fibrosarcoma (Maf) but not T-box expressed in T cells (T-bet). Moreover, shikonin suppressed the phosphorylation of p38, inhibitor of κB (IκB) kinase (IKK)-β and IκB-α, and the subsequent IκB-α degradation induced by PMA + cAMP; however, the PMA + cAMP-induced phosphorylation of extracellular signal-related kinase (ERK), which resulted in minor inhibition and phosphorylation of c-Jun N-terminal kinase (JNK), seemed to be unaffected by shikonin treatment.
Significance: This study suggests that downregulation of GATA-3 and c-Maf via the suppression of p38, IKK-β and IκB-α phosphorylation might contribute to the inhibitory effect of shikonin on mitogen-induced IL-4 and IL-5 production in EL-4T cells. Furthermore, shikonin is a potential drug for treating allergic diseases.

Introduction

Asthma is a chronic inflammatory disease affecting about 300 million people worldwide, with 255,000 people dying of the disease in 2005 (World Health Organization). Studies on patients and animal models of asthma suggest that in allergic asthma, $CD4^+$ T helper (T_H) 2 lymphocytes induce an inflammatory cascade via cytokine production comprising eosinophil action, IgE production, and mast cell activation — all of which in turn produce the necessary mediators causing airway hyperresponsiveness (Chung and Barnes, 1999; Wills-Karp, 1999; Umetsu and DeKruyff, 2006). The pathologic role of T_H2 cells is mediated through the release of T_H2 cytokines such as interleukin (IL)-4, IL-5, and IL-13. IL-4 induces IgE isotype switching and is implicated in stimulating VCAM-1 expression (Schnyder et al., 1996) and enhancing eosinophil recruitment to the lungs (Venkayya et al., 2002). IL-5 is the key cytokine involved in eosinophil growth and differentiation in bone marrow (Sanderson, 1988, 1992; Yamaguchi et al., 1988) and the subsequent release of eosinophils into peripheral circulation (Wiktor-Jedrzejczak, 1993; Collins et al., 1995).

Shikonin and its derivatives are analogs of naphthoquinone pigments, the major components of root extracts of a Chinese medicinal herb, *Lithospermum erythrorhizon* (Chen et al., 2002). Treatment indications claimed for *L. erythrorhizon* roots include burns, anal ulcers, hemorrhoids, infected crusts, bedsores, external wounds, and oozing dermatitis (Papageorgiou et al., 1999). Multiple pharmacological actions of these compounds have been documented, including (1) the inhibition of vascular permeability and acute edema induced by histamine upon topical application of shikonin (Hayashi, 1977) and (2) the inhibition of cyclooxygenase-2 transcription through the downregulation of extracellular signal-regulated kinase-1/2 (ERK1 and ERK2) and activation protein-1 (AP-1) activities (Subbaramaiah et al., 2001). Other pharmacological actions include the suppression of mast cell degranulation (Wang et al., 1995), protection of vasculature, inhibition of the neutrophil respiratory burst (Kawakami et al., 1996), and blocking CCL5 (RANTES) and CCL4 (MIP-1α) binding to human monocytes (Das et al., 2001). In addition,

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shikonin exhibits anti-cancer effects (Guo et al., 1991; Hisa et al., 1998). Although shikonin exhibits a broad range of biological and pharmacological activities, there is little information regarding its effects on allergic diseases. In the present study, we explored the possible effects of shikonin on T cells. T-cell activation in vitro can be mimicked by phorbol myristate acetate (PMA) and calcium ionophores, or by anti-CD3 antibodies and lectins (Boonyaratanakornkit et al., 2005; Hughes-Fulford et al., 2005); however, this mimicking effect induces T cells to produce more IFN-γ than IL-4 or IL-5, which drives the T cells to develop into T_H1 cells rather than T_H2 cells. Therefore, we used PMA combined with dibutyryl-cyclic adenosine monophosphate (cAMP)-activated EL-4 murine T-lymphoma cells, which produce IL-4 and IL-5 (Lee et al., 1993) but not IFN-γ, as T_H2-like cells, to investigate the effects of shikonin on T_H2 cytokines, transcriptional factors, and the related mitogen-activated protein kinase (MAPK)/nuclear factor (NF)-κB signaling pathway.

Materials and methods

Drugs and chemicals

Shikonin was purchased from EMD Chemical Inc. (Darmstadt, Germany); its chemical structure is shown in Fig. 1A. PMA and cAMP were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM, Hank's balanced salt solution (HBSS), penicillin, streptomycin, L-glutamine, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture

EL-4 murine T-lymphoma cells were purchased from the ATCC (Manassas, VA, USA). EL-4 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. Confluent cells were subcultured at a ratio of 1:3, and media were changed twice a week.

Cytotoxicity assay

EL-4 T cells were pretreated with various concentrations of shikonin for 10 min and cultured with or without PMA (5 ng mL^{-1}) plus cAMP (250 μM) for 24 h. At this point, the number of viable cells was determined using trypan blue staining (Sugiura et al. 2007). Q1

Quantitative real-time PCR

Cells were collected 24 h after different drug treatments, and RNA was isolated using RNA TRIzol reagent was purchased from Invitrogen

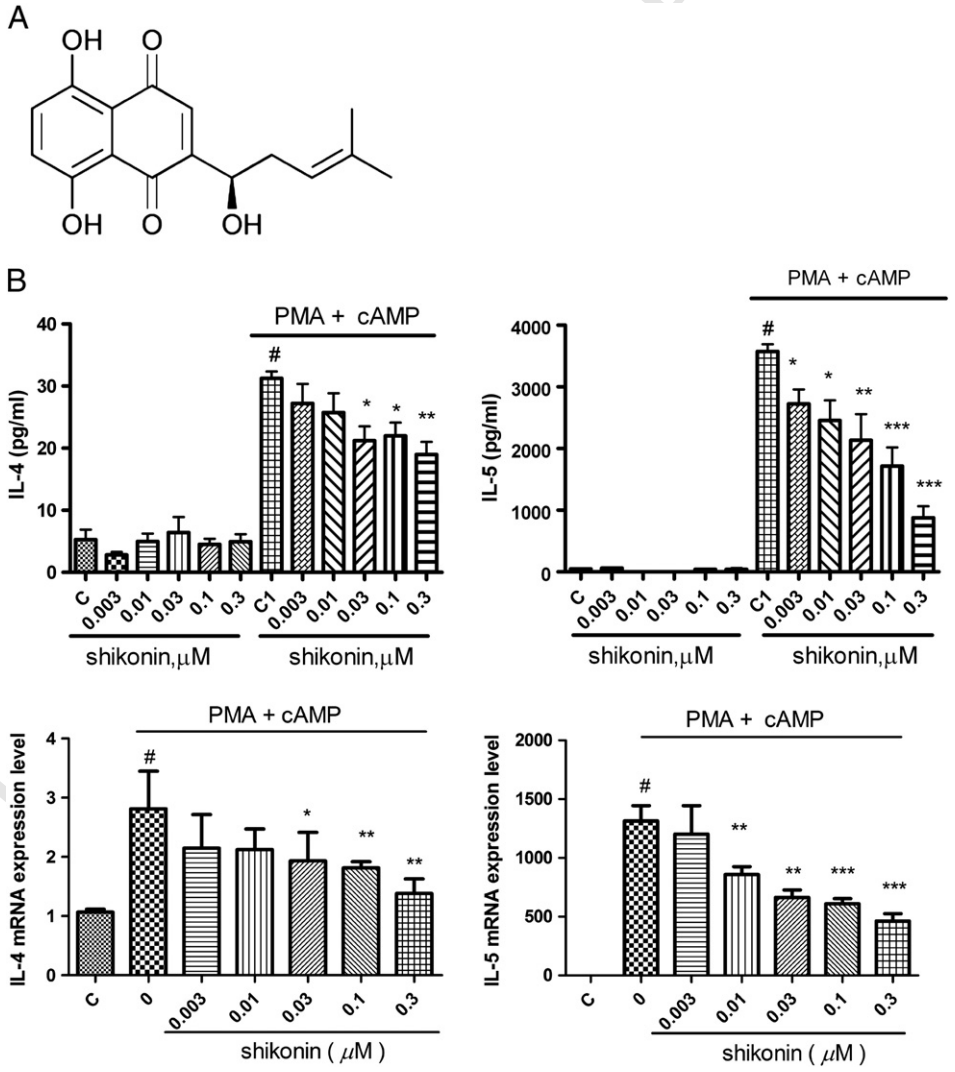

Fig. 1. Shikonin inhibited PMA + cAMP-induced IL-4 and IL-5 expression in EL-4 T cells. (A) Chemical structure of shikonin. (B) IL-4 and IL-5 production detected by ELISA, and mRNA expression detected by real-time PCR. Data are expressed as mean ± SEM (n = 6). $^{\#}p<0.001$, compared to the control group without PMA + cAMP treatment. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, compared to the control group with PMA + cAMP treatment.

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(Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was converted into cDNA and subsequently quantified by quantitative real-time PCR using an ABI PRISM 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The partial cycles that resulted in statistically significant increases in IL-4, IL-5, GATA-3, c-Maf, and T-bet expression were determined (threshold cycle, Ct) and normalized to the Ct for β-actin. IL-4, IL-5, GATA-3, c-Maf, T-bet, and β-actin were amplified using an SYBR Green kit (Applied Biosystems). The primer sequences used were as follows: IL-4, sense 5′-CTCATGGAGCTGCAGAGACTCTT-3′, antisense 5′-CATTCATGGTGCAGCTTATC-GA-3′; IL-5, sense 5′-TGACCGC-CAAAAAGAGAAGTG-3′, antisense 5′-GAACTCTTGC-AGGTAATCCAGGAA-3′; GATA-3, sense 5′-CAGAACCGGCCCCTTATCA-3′, antisense 5′-ACAGTTCGCGCAGGATGTC-3′; c-Maf, sense 5′-AGAGGCGGACCCT-GAAAAA-3′, antisense 5′-GTGTCTCTGCTGCACCCTCTT-3′; T-bet, sense 5′-CTGGATGCGCCAGG-AAGT-3′, antisense 5′-TGTTGGAAGCCCCCTTGTT-3′; and β-actin, sense 5′-ACTGCCGCATCCTCTTCCT-3′, antisense 5′-ACCGCTCGTTGCCAATAGTG-3′.

Cytokine assays

Cell culture supernatants were collected 24 h after different drug treatments and stored at −20 °C before analysis by ELISA, according to the manufacturer's instructions. Standard samples were prepared from recombinant mouse IFN-γ, IL-4, and IL-5 (R&D Systems, Minneapolis, MN, USA).

Western blotting

Cells were collected after incubation with different drugs in 6-well plates for the indicated durations. Total cell lysates were separated using 10% SDS-PAGE gels, and electrophoresed proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween and incubated with a primary antibody. Horseradish peroxidase-labeled secondary antibody was used; bands were detected with chemiluminescence reagents, according to the manufacturer's instructions (PerkinElmer Life Science, Boston, MA, USA), and subsequently exposed to an X-ray film. The bands were scanned and analyzed using Image J software. Monoclonal antibodies against β-actin and polyclonal antibodies against phosphorylated IκB-α, ERK, JNK, and p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibody against phosphorylated IKK-β was purchased from Abcam plc. (Cambridge, UK). Monoclonal antibodies against GATA-3, c-MAf, and T-bet and polyclonal antibodies against ERK, JNK, and p38 MAPK were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Statistical analysis

All experimental data are expressed as mean ± SEM using one-way ANOVA followed by the Newman–Keuls post-hoc test. Statistical significance was set at $p<0.05$.

Results

Shikonin inhibited the mitogen-induced expression of IL-4 and IL-5 in EL-4 T cells

First, we evaluated the possible cytotoxic effects of shikonin on EL-4 cells. After treatments with 0.003, 0.01, 0.03, 0.1, or 0.3 μM shikonin for 24 h, EL-4 cells did not exhibit any cytotoxicity, as shown by the trypan blue exclusion assay (data not shown). The 50% of lethal concentration (LC_{50}) of shikonin in EL-4 cells was 1.33 ± 0.13 μM. Next, we investigated the production of IL-4 and IL-5. EL-4 cells treated with different concentrations of shikonin did not exhibit any apparent changes with respect to IL-4 or IL-5 production (Fig. 1B). Because PMA activates protein kinase C (PKC) and cAMP activates protein kinase A (PKA) – all of which are involved in EL-4 T-cell activation and the release of IL-4 and IL-5 (Lee et al., 1993) – we used a mixture of 5 ng mL^{-1} PMA and 250 μM cAMP to drive EL-4 T cells to behave like T_H2 cells. Shikonin inhibited the PMA + cAMP-induced IL-4 and IL-5 production and mRNA expression in a dose-dependent manner. Compared to treatment with PMA + cAMP alone in the control group, treatment with 0.03, 0.1, and 0.3 μM shikonin reduced IL-4 production by 32.1%, 29.7%, and 39.6%, respectively. Treatment with 0.003, 0.01, 0.03, 0.1, and 0.3 μM shikonin reduced IL-5 production by 23.8%, 31.3%, 40.2%, 52%, and 75.5%, respectively. The 50% of inhibitory concentration (IC_{50}) of shikonin in IL-5 production was 0.13 ± 0.04 μM. The IC_{50} values of shikonin in IL-4 and IL-5 mRNA expression were 0.08 ± 0.04 and 0.15 ± 0.07 μM, respectively.

Shikonin suppressed the mitogen-induced expression of GATA-3 and c-Maf in EL-4 T cells

Next, we analyzed the T_H2- and T_H1-related transcription factors and found that shikonin inhibited PMA + cAMP-induced GATA-3 and c-Maf mRNA (Fig. 2A) and protein (Fig. 2B) expression in a dose-dependent manner. However, T-box expressed in T cells (T-bet) was not induced by PMA + cAMP treatment, and shikonin treatment did not result in any obvious changes in T-bet mRNA expression.

Shikonin decreased mitogen-induced MAPK activation

To further investigate the mechanism underlying the shikonin-mediated inhibition of IL-4 and IL-5 production, we focused on MAPK pathways, which are known to play critical roles in the activation of T cells (Boulton et al., 1991; Kyriakis et al., 1994; Lee et al., 1994; Su et al., 1994). We found that PMA + cAMP induced ERK, JNK, and p38 activation from 5 to 60 min, peaking 30 min after treatment (Fig. 3A). Thus, we investigated the effects of shikonin on MAPK after 30 min of treatment with PMA + cAMP (Fig. 3B). We found that 0.03 and 0.3 μM shikonin inhibited the PMA + cAMP-induced ERK and p38 activation but had no obvious effect on JNK activation.

Shikonin inhibited mitogen-induced IKK-β and IκB-α activation

NF-κB activation is involved in the initiation and amplification of the inflammatory response (Handel and Girgis, 2001; Andujar et al., 2010) and is also involved downstream of MAPK signaling (Dhawan and Richmond, 2002). The nuclear translocation and DNA binding of NF-κB are preceded by the phosphorylation of IKK-β, IκB-α and subsequent degradation of IκB. We found that the PMA + cAMP-induced phosphorylation of IKK-β and activation and degradation of IκB-α were inhibited by treatment with 0.03 and 0.3 μM shikonin (Fig. 4). Expression of phosphorylated IKK-β and IκB-α proteins and the level of IκB-α degradation induced by PMA + cAMP were blocked after 0.3 μM shikonin treatment.

Discussion

In a previous study, we found that shikonin impaired IL-4 and IL-5 production in lung cells and mediastinal lymph nodes in a murine model of asthma with antigen-induced airway inflammation (Lee et al., 2010); however, the mechanism underlying this impairment remains unclear. In the present study, we aimed to determine whether shikonin directly inhibits T_H2 cell function; therefore, we used PMA + cAMP-activated EL-4 T cells as T_H2-like cells, which induce IL-4 and IL-5 production. We found that shikonin inhibited the PMA + cAMP-induced IL-4 and IL-5 expression in a dose-dependent manner. Because PMA + cAMP drastically increased IL-5 mRNA and protein expression, the inhibition level of IL-5 expression was higher

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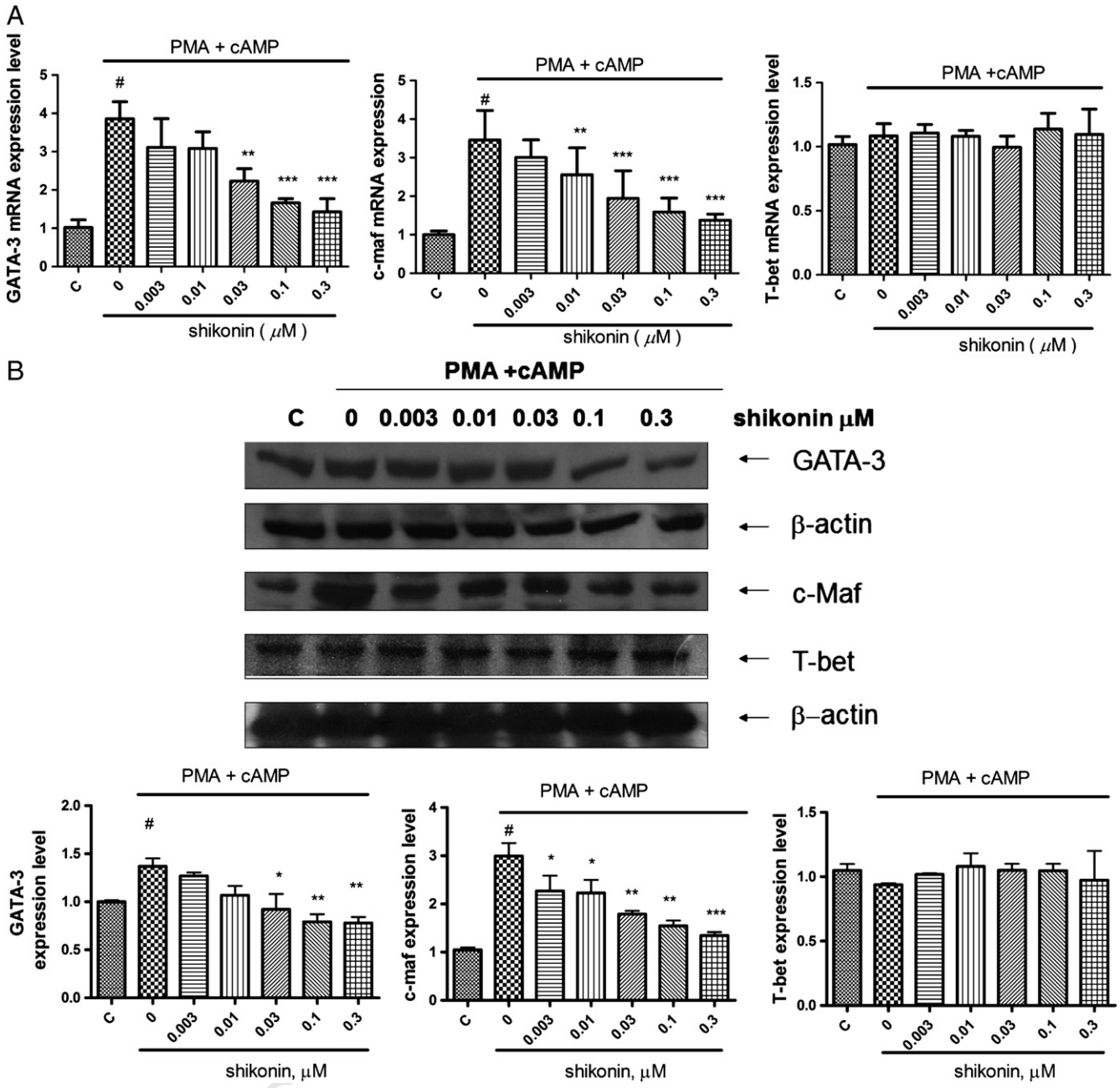

Fig. 2. Shikonin inhibited the PMA + cAMP-induced GATA-3 and c-Maf expression in EL-4 T cells. (A) GATA-3 and c-Maf mRNA expression were detected by real-time PCR. Data are expressed as mean ± SEM (n = 6). (B) The protein expression of GATA-3, c-Maf, and T-bet was detected by western blotting. Histograms represent quantifications of protein expression by western blotting. Data are expressed as mean ± SEM (n = 3). $^{\#}p<0.001$, compared to the control group without PMA + cAMP treatment. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to the control group with PMA + cAMP treatment.

than that of IL-4 expression with shikonin treatment. Since the expression of T-cell-specific transcription factors and components of MAPK pathways, which are known to play critical roles in T-cell activation (Boulton et al., 1991; Kyriakis et al., 1994; Lee et al., 1994; Su et al., 1994), affects IL-4 and IL-5 gene expression, we further investigated the possible role of shikonin on related signaling pathways.

The gene expressions of all 3 T_H2 cytokines are regulated by the transcriptional factor GATA-3 (Lee et al., 2008). The GATA site located upstream of the IL-4 and IL-5 promoters is important in regulating IL-4 and IL-5 expression, respectively (Ray and Cohn, 1999; Zhu et al., 2006). Shikonin inhibited PMA + cAMP-induced GATA-3 expression at both the mRNA and protein level. During T_H2 cell differentiation, GATA-3 can be activated through the activation of the IL-4 receptor, notch receptor, TCR, or IL-2 receptor (Ho et al., 2009). In our study, PMA + cAMP-induced GATA-3 activation mimicked the TCR activation pathway. Furthermore, NF-κB is reported to play a role in the antigen TCR-activated GATA-3 signaling pathway. Two separate research groups report the requirement of NF-κB1/p50 for optimal GATA-3 induction in T cells, based on the fact that GATA-3 expression and Th2 differentiation are specifically abrogated in $p50^{-/-}$ T cells and in $SAP^{-/-}$ cells in which the nuclear translocation of NF-κB is inhibited (Das et al., 2001; Cannons et al., 2004). The GATA-3 promoter region contains several consensus-potential NF-κB binding sites (Das et al., 2001; Cannons et al., 2004). Direct binding of NF-κB subunits may control the transcriptional activation of GATA-3 as well as the subsequent development of the T_H2 lineage. We found that shikonin inhibited IκB-α phosphorylation and degradation, which

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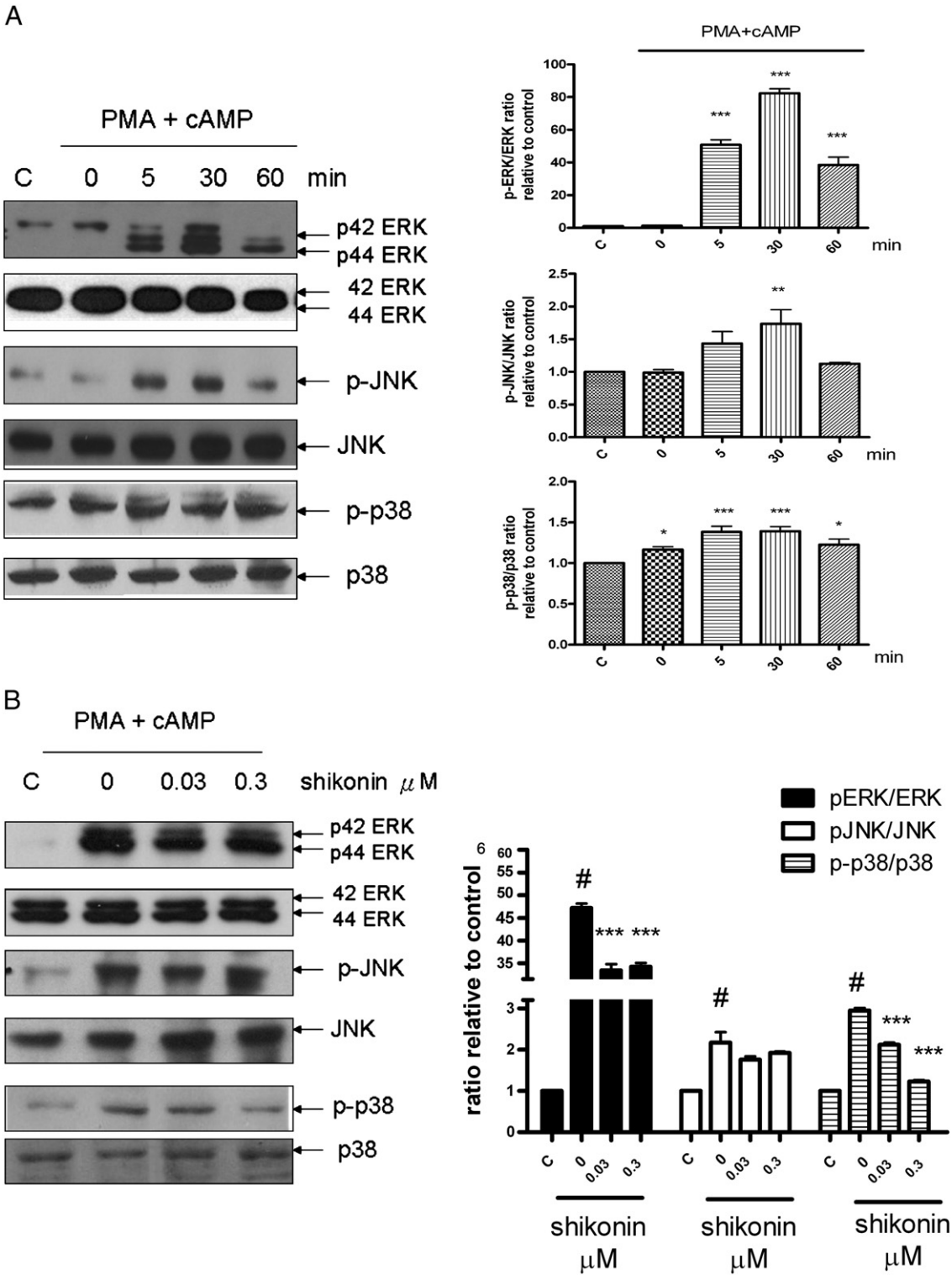

Fig. 3. Effects of shikonin on the PMA + cAMP-induced phosphorylation of MAPKs in EL-4 T cells. (A) Time dependence of MAPK activation by 5 ng mL^{-1} PMA + 250 μM cAMP treatment. Phosphorylated (p)-ERK, p-JNK, p-38, ERK, JNK, and p38 proteins were detected by western blotting. Histograms represent quantifications of protein expression by western blotting. Data are expressed as mean ± SEM (n = 3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$, compared to the control without the PMA + cAMP group. (B) Cells were treated with 0.03 or 0.3 μM shikonin for 10 min followed by 5 ng mL^{-1} PMA + 250 μM cAMP for 30 min. Cell lysates were analyzed by western blotting using antibodies specific for p-ERK, p-JNK, p-38, ERK, JNK, and p38. Histograms represent quantifications of protein expression by western blotting. Data are expressed as mean ± SEM (n = 3). # $p<0.001$, compared to the control group without PMA + cAMP treatment. *** $p<0.001$, compared to the control group with PMA + cAMP treatment.

might in turn downregulate NF-κB activation; therefore, shikonin might suppress GATA-3 mRNA expression by inhibiting NF-κB activation. In addition, Andujar et al. (2010) also found that shikonin reduces phorbol ester-induced IκB degradation, thus inhibiting the translocation of NF-κB.

In addition to GATA-3, the proto-oncogene c-Maf is a potent and specific transactivator of the *Il4* gene (Ho et al., 1996; Tanaka et al., 2005). c-Maf binds to a half Maf recognition element (MARE) site and transactivates the IL-4 promoter. The forced expression of c-Maf is sufficient to drive endogenous IL-4 production in M12 B cells or

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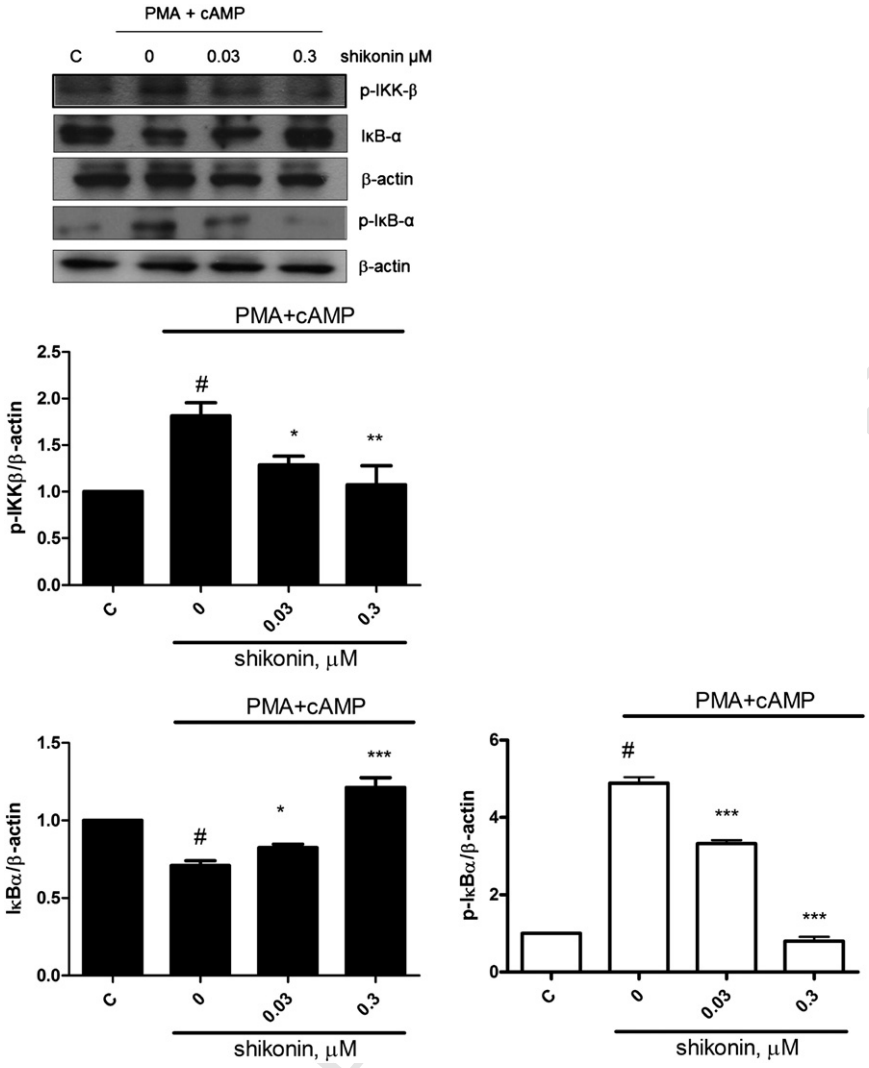

Fig. 4. Shikonin suppressed the PMA + cAMP-induced activation of IKK-β and IκB-α in EL-4 T cells. Cells were treated with 0.03 or 0.3 μM shikonin for 10 min followed by 5 ng mL^{-1} PMA + 250 μM cAMP for 30 min. Cell lysates were analyzed by western blotting using antibodies specific to p-IKK-β, IκB-α, p-IκB-α, and β-actin. Histograms represent quantifications of protein expression by western blotting. Data are expressed as mean ± SEM (n = 3). $^{\#}p<0.05$, compared to the control group without PMA + cAMP treatment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to the control group with PMA + cAMP treatment.

differentiating T_H1 cells (Ho et al. 1998). We found that PMA + cAMP-induced c-Maf expression was inhibited as a result of shikonin treatment, suggesting that shikonin inhibits IL-4 production via the downregulation of c-Maf.

Since T-bet is expressed predominantly in T_H1 cells and exhibits reciprocal inhibitory effects with GATA-3 in T-cell differentiation (Szabo et al., 2000), shikonin might affect T-bet activation to inhibit GATA-3 expression. However, we found that treatment with shikonin alone did not have any obvious effect on T-bet expression in EL-4 T cells. Furthermore, T-bet was not activated after PMA + cAMP treatment. Thus, the effect of T-bet activation after shikonin treatment requires further investigation.

Previous studies report that p38 MAPK is involved in T-cell activation and development, and that the inhibition of p38 activity reduces IL-2, IL-4, and IFN-γ production (Rincon et al., 1998; Zhang et al., 1999). Chen et al. (2000) found that GATA-3 phosphorylation by p38 may be important for the activation of IL-5 and IL-13 gene expression. In the present study, we found that shikonin inhibited mitogen-induced p38 phosphorylation and that p38 was barely activated with 0.3 μM shikonin treatment. This might indicate that p38 is involved in shikonin-inhibited GATA-3 expression. In addition to the regulation of IL-4 transcription, the activation of p38 also induced IL-4 mRNA stability (Dean et al., 2004; Guo et al., 2008). However, whether shikonin regulates IL-4 mRNA stability via p38 activation requires further investigation.

We also found that PMA + cAMP induced phosphorylation of ERK and JNK in EL-4 T cells. According to previous studies, both ERK and JNK are involved in T-cell activation. TCR engagement activates the ERK pathway, and co-stimulation through CD28 causes JNK activation, which is required for the complete activation of T cells (Su et al., 1994; Ho et al., 1996). The ERK pathway has been found to cause IκB phosphorylation and degradation, which lead to NF-κB activation. In contrast, other studies found that ERK and JNK do not have positive roles in T_H2 cytokine production. Dumont et al. (1998) found that the ERK inhibitor PD98059 enhances T_H2 cytokine production. Furthermore, using JNK-1-deficient mice, Dong et al. (1998) found enhanced T_H2 responses. However, another previous study shows that pulsed human myelin-reactive T cells with different myosin basic protein peptides induce T_H1 and T_H2 deviation via the activation of JNK and ERK, respectively (Singh and Zhang, 2004). Therefore, the roles of the ERK and JNK pathways in T_H2 cytokine production remain unclear. In our study, we found that shikonin slightly inhibited PMA + cAMP-induced ERK activation and had no inhibitory effects on JNK activation as a result of PMA + cAMP treatment. This suggests that ERK plays a minor role and that JNK has no obvious effect in shikonin-suppressed IL-4 and IL-5 production induced by PMA + cAMP treatment.

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Conclusion

Our data show that shikonin directly inhibits T_H2 responses in T cells by reducing the expression of the cytokines IL-4 and IL-5 and the transcription factors GATA-3 and c-Maf. Suppression of the phosphorylation of IKK-β and activation of IκB-α and p38 might play an important role in the shikonin-induced inhibition of GATA-3 expression in EL-4 cells. Our findings provide useful and novel mechanistic explanations for the anti-allergic inflammatory effect of shikonin, and highlight its pharmaceutical value.

Acknowledgments

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