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ORIGINAL ARTICLE

Shikonin Controls the Differentiation of $CD4^+CD25^+$ Regulatory T Cells by Inhibiting AKT/mTOR Pathway

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Abstract— $CD4^+CD25^+$ regulatory T (Treg) cells maintain the function of immune tolerance and the balance of immune cells. Defects in the number and function of Treg cells can induce the development and progression of inflammatory disease. Shikonin, the main active ingredient of *Lithospermum*, has anti-inflammatory and anti-tumor effects. Shikonin is also an effective drug for the treatment of psoriasis, which is a chronic inflammatory skin disease. However, the underlying mechanism is not yet clear. To evaluate the role of shikonin on the induction of Treg cells, we tested the number and function of Treg cells *in vivo* and *in vitro*. Shikonin can effectively promote the differentiation of iTreg cells by inhibiting the AKT/mTOR pathway *in vitro*. Moreover, *in vivo*, intragastrically administered shikonin effectively improved lesions in mice with imiquimod-induced psoriasis and increased the number of iTreg cells in the spleen and their secretion. Shikonin significantly increases the expression of Foxp3mRNA in skin of the psorisic mice. Therefore, we expect that shikonin can prevent the development of inflammation and treat psoriasis by regulating iTreg cells. Novel ideas for the treatment of psoriasis are also proposed.

KEY WORDS: $CD4^+CD25^+$ regulatory T cells; shikonin; psoriasis.

INTRODUCTION

$CD4^+CD25^+$ regulatory T (Treg) cells, as a subset of $CD4^+$ T lymphocytes, were first described as inhibitory cells in 1970 by Gershon [1]. Treg cells are characterized by continuously high levels of CD4 and CD25 expression, and the key transcription factor is the Foxkhead-family transcription factor FOXP3. Surface molecules on Treg cells include cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor-related (GITR) and so on. Regulatory T cells maintain immune homeostasis under normal physiological or pathological conditions [2–4]. Regulatory T cells are divided based on origin into natural regulatory T cells (nTreg cells) from the thymus and induced Treg (iTreg) cells derived from peripheral lymphoid tissues. Among these, iTreg cells can be induced upon stimulation by peripheral antigens and sustained stimulation by relevant cytokines such as TGF-β1 [5]. Multiple signal transduction pathways are involved in regulating this process of iTreg cell differentiation, such as the IL-2-Stat5, TGF-β-Smad3, and PI3K/AKT/mTOR pathways [6, 7].

Due to the important role of Treg cells, defects in their number and function Treg can induce the occurrence and development of inflammatory diseases, autoimmune

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diseases, and tumors [8–10]. Studies have found that Treg cells are inextricably linked to the development of chronic inflammatory skin diseases such as psoriasis. It has also been reported that the number and function of iTreg cells in peripheral blood and lesions of patients with various types of psoriasis differ from those in normal individuals [11, 12]. Overall, the potential of Treg cells on curing psoriasis presents a novel strategy for treatment of this condition. And it is also possible to control the immune response of effector T cells and the incidence of psoriasis by regulating the differentiation and function of Treg cells [13, 14].

Shikonin, the main component of *Lithospermum* herbs, has been applied to treat psoriasis due to its ability of anti-inflammatory, anti-tumor, and immunomodulatory effects on immune system. Previous studies have demonstrated that shikonin can exert anti-inflammatory effects *via* inhibition of activity of effector T cells and inflammatory cytokines [15, 16]. Because regulatory T cells play an important role in the process of inflammation, we hypothesized that shikonin exerts an anti-inflammatory effect by regulating the number and function of regulatory T cells. To verify this hypothesis, the current study investigated the effects of shikonin on the differentiation of iTreg cells *in vitro* and the production and function of Treg cells in the spleen of mice with imiquimod-induced psoriasis.

MATERIALS AND METHODS

Animals. Seven- to 8-week-old male BALB/c mice (17–20 g) were purchased from LiaoNing Changsheng Technology and housed under specific pathogen-free conditions with a 12-h light/dark cycle. All animal experimental procedures were approved and overseen by the Animal Care and Use Committee of the Shenyang Institute of Traditional Chinese Medicine.All animal procedures were performed in compliance with the institutional guidelines for animal experiments.

Drugs and Reagents. Shikonin of 98% purity, as verified by HPLC (Sigma, St. Louis, MO, USA), was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 20 mg/ml. Naïve $CD4^+$ T cell Isolation Kit II was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). LEAF™-purified anti-human CD3 and CD28 antibodies were purchased from BioLegend (San Diego, CA). FITC-conjugated anti-human CD4, PE-conjugated anti-human CD45RA, PE-conjugated anti-human CD25, PerCP/Cy5.5-conjugated anti-human FOXP3, FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD45RA, PE-conjugated anti-mouse CD25, and PerCP/Cy5.5-conjugated anti-mouse FOXP3 antibodies were purchased from Invitrogen eBioscience (Avenue Waltham, MA, USA). Anti-GAPDH, anti-AKT, anti-pAKT (Ser473), anti-P70S6K, and anti-pP70S6K (Thr389) antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). IL-2 and TGF-β1 were purchased from PeproTech (Rocky Hill, NJ, USA). IMQ (5%) cream was purchased from 3M Health Care (St. Paul, MN).

Grouping and Treatment. Seven- to 8-week-old male BALB/c mice were shaved on the back, with the exposed area being approximately 2 × 3 cm. All mice were randomized into six groups: negative control, model, positive control, low-dose shikonin, mid-dose shikonin, and high-dose shikonin groups. The mice in the negative control group received topical white petrolatum once daily on their bare back for 10 consecutive days. The mice in the other groups were treated topically with a 62.5-mg daily dose of 5% IMQ cream on their bare back for 10 consecutive days. Shikonin and methotrexate (positive control) were dissolved in an appropriate solvent (5% DMSO in oil) to obtain a suitable concentration for intragastric administration. The mice in the control and model groups received intragastric administration of 5% DMSO in oil to establish the model. The mice in the positive control group received intragastric administration of MTX at a dose of 0.5 mg/kg/day for 10 consecutive days. The mice in the low-, mid-, and high-dose treatment groups received intragastric administration of shikonin at doses of 6.25 mg/kg/day, 12.5 mg/kg/day, and 25 mg/kg/day, respectively, for 10 consecutive days to establish the model.

Scoring of Skin Inflammation Severity, Spleen Weight, and Histology. The Psoriasis Area and Severity Index (PASI) scoring system was used to measure the severity of lesions in the mice from different groups. The scoring items include erythema, thickness, and scaling of the lesion, and the scoring criteria are as follows: 0 = none; 1 = slight; 2 = moderate; 3 = severe; and 4 = very severe. On the 10th day of treatment, the mice from different groups were weighed; the spleen was removed following sacrifice, and the animal was weighed again. The dorsal skin of the mice was fixed in 5% buffered formalin, and the tissue was dehydrated and embedded in paraffin. Five-micrometer sections were cut and stained with H&E for pathological examination.

Flow Cytometry Analysis of iTreg Cells in the Spleen of Mice with IMQ-Induced Psoriasis. Mononuclear cells were immediately extracted from the spleen. To analyze the number of iTreg cells in the spleen, mononuclear cells were stained with FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD25 antibodies for extracellular fluorescence labeling. The cells were

fixed and permeabilized using intracellular fixation and permeabilization working solution and subsequently stained with a PerCP/Cy5.5-conjugated anti-mouse FOXP3 antibody for intracellular fluorescence labeling. The samples were examined using a FACSCalibur (BD Biosciences, San Jose, CA, USA).

Enzyme-Linked Immunosorbent Assay for Cytokines in the Splenic Supernatant. The levels of IL-10 and TGF-β1 secreted by iTreg cells were evaluated in splenic supernatants using IL-10 and TGF-β1 mouse enzyme-linked immunosorbent assays (ELISA) (eBioscience, Avenue Waltham, MA, USA).

Quantitative Real-Time PCR for Foxp3, IL-10, IL-6, IL-17A mRNA in the Skin of Mice with IMQ-Induced Psoriasis

Total RNA was extracted from skin tissue of the mice using TRI Reagent according to the manufacturer's instructions, and cDNA was synthesized using the SuperScript Reverse Transcription system (Promega, Beijing, China). Real-time PCR was performed using the GoTaq® Probe 2-Step RT-qPCR system. The primer sequences used to amplify the genes are listed in Table 1.

Human Naïve $CD4^+$ T Cell Isolation and In Vitro Differentiation. Human naïve $CD4^+$T lymphocytes were isolated from buffy coat blood obtained from Shenyang City Central Blood Station. The blood was mixed with PBS, transferred to lymphocyte separation medium (Solarbio, Beijing, China) in 15-ml tubes, and centrifuged at 1000×*g* for 25 min to separate the blood into layers. The layer of mononuclear cells was collected and purified using a MACS® naïve $CD4^+$ T cell kit. Human naïve $CD4^+$ T cells were cultured in RPMI 1640 medium supplemented with 15% FBS. For induction of naïve $CD4^+$ T cell differentiation into Treg cells, plate-coating anti-human CD3 (10 μg/ml) and soluble anti-human CD28 (10 μg/ml) were added to activate the cells, followed by treatment with TGF-β1 (7.5 ng/ml) and IL-2 (10 ng/ml) for 7 days. Shikonin was added to the cells at different concentrations, followed by culturing for 7 days.

The levels of cytokines secreted by iTreg cells were evaluated using IL-10 and TGF-β1 human ELISA (CUSABIO, WuHan, China). Total RNA from cultured cells was extracted on the 3rd, 5th, and 7th days of cell culture using TRI Reagent according to the manufacturer's instructions. Quantitative real-time PCR was performed for iTreg transcription factors. The primer sequences used to amplify the genes are listed in Table 2. The number of iTreg cells in each group was evaluated by flow cytometry on the 7th day of cell culture.

Western Blotting. Human naïve $CD4^+$ T cells were stimulated under the conditions described above. Total protein was extracted from the cultured cells and blotted onto PVDF membranes, which were then blocked in 5% BSA or 5% non-fat milk in TBST for 1 h. The membranes were incubated overnight at 4 °C with antibodies against pAKT (Ser473), AKT, pP70S6 kinase (Thr389), P70S6 kinase, and GAPDH. The following day, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h. Protein bands were visualized using ECL reagent (Beyotime, Shanghai, China).

Statistical Analysis. Statistical analysis was carried out using SPSS 22.0 software. All data are expressed as the mean ± SD. Statistically significant differences between groups were analyze using one-way ANOVA with Tukey's *post hoc* test. $p < 0.05$ was considered a difference, and $p < 0.01$ was considered a significant difference.

Table 1. Primers for real-time PCR

Primer name		Sequence
Mouse Foxp3	Forward	5′-CACCTATGCCACCCTTATCCG-3′
	Reverse	5′-CATGCGAGTAAACCAATGGTAGA-3′
Mouse IL-10	Forward	5′-AGCCTTATCGGAAATGATCCAGT-3′
	Reverse	5′-GGCCTTGTAGACACCTTGGT-3′
Mouse IL-17A	Forward	5′-TCAGCGTGTCCAAACACTGAG-3′
	Reverse	5′-CGCCAAGGGAGTTAAAGACTT-3′
Mouse IL-6	Forward	5′-CTGCAAGAGACTTCCATCCAG-3′
	Reverse	5′-AGTGGTATAGACAGGTCTGTTGG-3′
Mouse GAPD	Forward	5′-TGACCTCAACTACATGGTCTACA-3′
	Reverse	5′-CTTCCCATTCTCGGCCTTG-3′

Table 2. Primers for real-time PCR

Primer name		Sequence
Human Foxp3	Forward	5′-ATTCCCAGAGTTCCTCCACAAC-3′
	Reverse	5′-ATTGAGTGTCCGCTGCTTCTC-3′
Human GAPDH	Forward	5′-ATGAGCCCCAGCCTTCTCCAT-3′
	Reverse	5′-GGTCGGAGTCAACGGATTTG-3′

RESULTS

Shikonin Effectively Improves Lesions in Mice with IMQ-Induced Psoriasis

Following 10-day intragastric administration of shikonin at different concentrations, effective treatment of lesions in mice with IMQ-induced psoriasis was established (Fig. 1a). Mice in the positive control and shikonin treatment groups had a significantly lesser degree of thickness, scaling, and erythema infiltration of lesions than did those in the psoriasis model group, and lesions appeared later in the former groups. In the shikonin treatment groups, the severity of the lesions decreased as the concentration increased, indicating a dose-dependent effect. PASI scores in the shikonin treatment groups were significantly lower than those in the psoriasis model group (Fig. 1c). Moreover, pathological analysis *via* H&E staining revealed that the acanthosis of the skin lesions in the mice in the shikonin treatment groups was significantly thinner than that in the mice in the psoriasis model group; inflammatory cell infiltration was also significantly reduced (Fig. 1b). The spleen index of the mice in the psoriasis model group was significantly higher than that of the mice in the negative control group (18.13 ± 1.46 *vs* 7.26 ± 0.42, $^{**}p < 0.01$) (Fig. 2a), and the spleen index of the mice in the shikonin treatment groups was significantly reduced compared to that in the psoriasis model group (low-dose shikonin group, mid-dose shikonin group, high-dose shikonin group *vs* model group: 11.57 ± 1.69, 10.78 ± 1.60, 8.29 ± 0.86 *vs* 18.13 ± 1.46, respectively; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) (Fig. 2a).

Shikonin Promotes iTreg Cell Induction *In Vivo*

To confirm the role of shikonin in iTreg cell induction *in vivo*, shikonin at different concentrations was intragastrically administered to mice with IMQ-induced psoriasis for 10 days. On the last day of intragastric administration, the mice were sacrificed, and the spleen was removed; mononuclear cells of the spleens from each group of mice were then extracted for determining the number of iTreg cells. The number of iTreg cells in the spleens of mice treated with different concentrations of shikonin were significantly higher than those in the psoriasis model group, and the differences were statistically significant (low-dose shikonin group, mid-dose shikonin group, high-dose shikonin group *vs* model group: 15.70 ± 1.05, 16.91 ± 0.85, 18.39 ± 0.84 *vs* 10.55 ± 0.75, respectively; $^{**}p < 0.01$) (Fig. 2b, c).

Shikonin Increases the Levels of IL-10 and TGF-β1 in the Spleen *In Vivo*

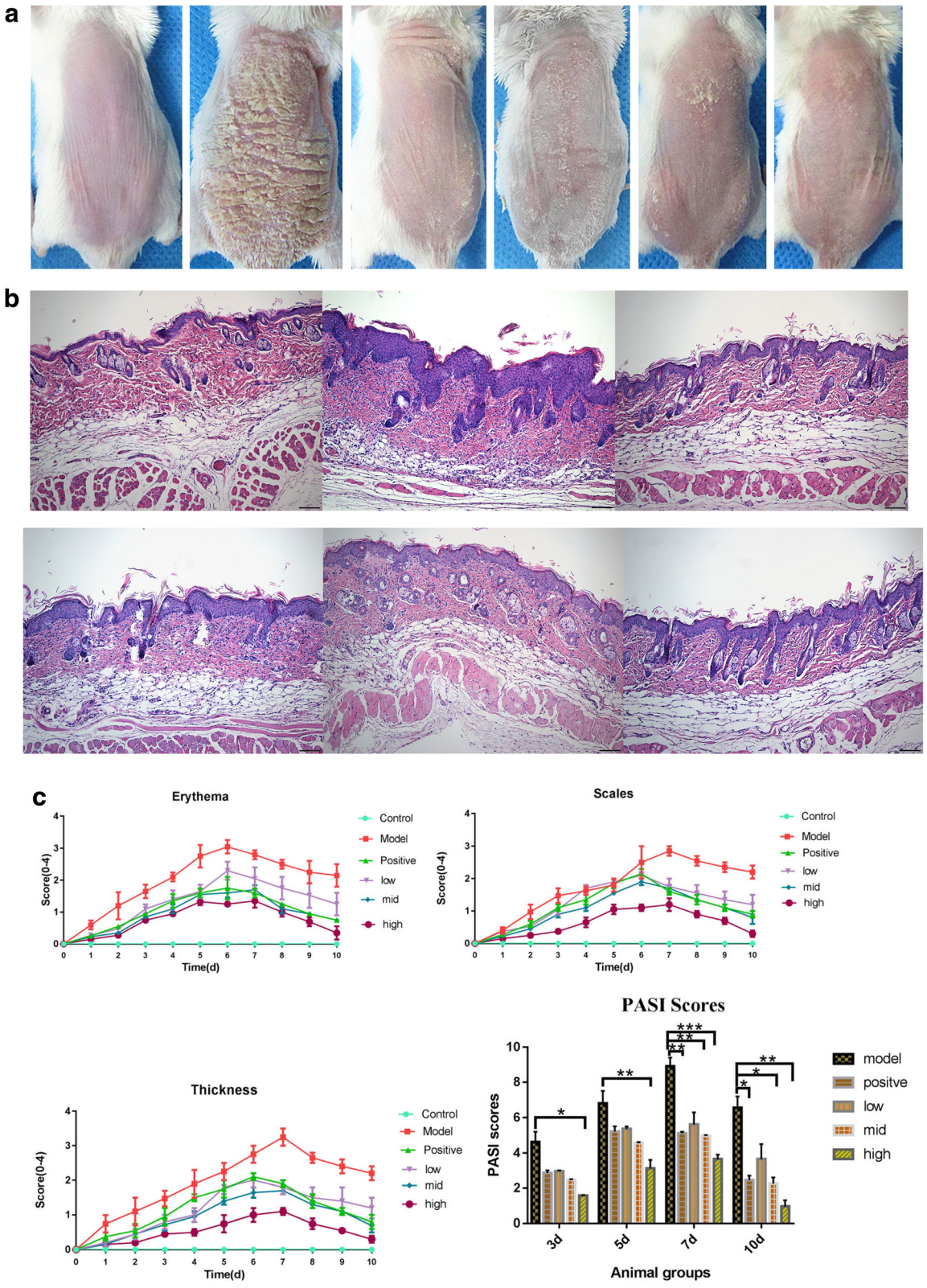
To analyze the effect of shikonin on spleen levels of IL-10 and TGF-β1, ELISA was performed to detect these cytokines in the splenic supernatant of mice in each group. The levels of IL-10 in the high-dose shikonin group were significantly higher than those in psoriasis model group (high-dose shikonin groups *vs* model groups: 413.90 ± 36.46 pg/ml *vs* 201.10 ± 28.29 pg/ml; $^{*}p < 0.05$) (Fig. 2d).

The levels of TGF-β1 in the spleen supernatant of the high-dose shikonin groups were higher than those the psoriasis model group (high-dose shikonin groups *vs* model groups: $344.1\ 0 \pm 47.14$ *vs* 72.21 ± 18.71 pg/ml; $^{*}p < 0.05$) (Fig. 2e).

Shikonin Increases the Expression of Foxp3 and IL-10mRNA, and Decreases the Expression of IL-17A and IL-6mRNA in the Skin *In Vivo*

To analyze the effect of shikonin on iTreg cell in the skin, quantitative real-time PCR was performed to detect the expression of Foxp3, IL-10, IL-17A, and IL-6 mRNA

Fig. 1Shikonin significantly improved lesions in mice with IMQ-induced psoriasis. The mice with psoriasis were treated with different concentrations of shikonin, MTX for 10 days. **a** The clinical manifestation of lesions and **b** pathological features of H&E staining (magnification × 100, scale bar = 100 μm). **c** The PASI scores of skin lesion in the mice with psoriasis during shikonin treatment for 10 days. Control (without any treatment); model (mice treated with IMQ); positive (mice treated with IMQ and MTX); shikonin low (6.25 mg/kg/day); middle (12.5 mg/kg/day); high (25 mg/kg/day). Statistical significance was determined after one-way ANOVA followed by Tukey's *post hoc* test ($n = 4$–6mice/group, $^{*}p < 0.05\%$, $^{**}p < 0.01\%$, $^{***}p < 0.001$).


a
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in the skin of mice in each group. The expression of Foxp3 mRNA in the high-dose shikonin group was significantly higher than those in psoriasis model group (high-dose shikonin groups *vs* model groups: 5.98 ± 0.78 *vs* 1.39 ± 0.32; $^{**}p < 0.01$) (Fig. 3a). The expression of IL-10mRNA in the high-dose shikonin group was significantly higher than those in psoriasis model group (high-dose shikonin groups *vs* model groups: 2.26 ± 0.34 *vs* 0.82 ± 0.12; $^{*}p < 0.05$) (Fig. 3b).

Shikonin effectively decrease the expression of the proinflammatory cytokine in the skin. The expression of IL-6mRNA in the skin of mice treated with different concentrations of shikonin were significantly lower than those in the psoriasis model group, and the differences were statistically significant (low-dose shikonin group, mid-dose shikonin group, high-dose shikonin group *vs* model group: 1.72 ± 0.45, 1.09 ± 0.19, 1.01 ± 0.13*vs* 3.97 ± 0.59; $^{*}p < 0.05$, $^{**}p < 0.01$) (Fig. 3c). The expression of IL-17A mRNA in the skin of mice treated with different concentrations of shikonin were significantly decreased (low-dose shikonin group, mid-dose shikonin group, high-dose shikonin group *vs* model group: 0.87 ± 0.22, 0.48 ± 0.11, 1.01 ± 0.13 *vs* 2.64 ± 0.45; $^{*}p < 0.05$, $^{**}p < 0.01$) (Fig. 3d).

Shikonin Promotes iTreg Cell Differentiation *In Vitro*

To examine the role of shikonin during iTreg cell differentiation, an iTreg cell differentiation system was established by incubating naïve $CD4^+$ T cells under differentiation conditions, followed by the addition of shikonin at different concentrations. Following 7-day stimulation of purified naïve $CD4^+$ T cells, relative expression levels of *Foxp3* mRNA in the 1.0 μM, 1.5 μM, and 2.0 μM shikonin groups were significantly higher than those in the control group (1.0 μM shikonin group, 1.5 μM shikonin group, 2.0 μM shikonin group *vs* control group: 1.53 ± 0.57, 1.99 ± 0.17, 1.61 ± 0.17 *vs* 1.00 ± 0.02, respectively; $^{*}p < 0.05$) (Fig. 3e). Compared with the negative control group, shikonin at 1. 5 μM significantly promoted the induction of iTreg cells on day 7, as shown by flow cytometry (1.5 μM shikonin group *vs* control group: 69.61 ± 10.24% *vs* 39.17 ± 12.42%; $^{*}p < 0.05$) (Fig. 4a, b).

Shikonin Promotes IL-10 and TGF-β1 Secretion by iTreg Cells *In Vitro*

To evaluate the effect of shikonin on the levels of IL-10 and TGF-β1 secreted *in vitro*, these cytokines were detected in the supernatant of the iTreg cell differentiation system. Shikonin at 1.0 μM and 1.5 μM could increase the levels of IL-10 on day 7 (1.0 μM shikonin group, 1.5 μM shikonin group *vs* control group: 115.20 ± 7.90, 155.38 ± 16.64 *vs* 84.66 ± 7.43 pg/ml; $^{*}p < 0.05$) (Fig. 4c).

In addition, shikonin at 0.5 μM, 1.0 μM, 1.5 μM, and 2.0 μM significantly promoted TGF-β1 secretion on day 7 (0.5 μM shikonin group, 1.0 μM shikonin group, 1.5 μM shikonin group, 2.0 μM shikonin group *vs* control group: 364.50 ± 30.10 pg/ml, 410.50 ± 41.43 pg/ml, 457.00 ± 29.90 pg/ml, 391.20 ± 40.78 pg/ml *vs* 257.70 ± 14.81 pg/ml; $^{*}p < 0.05$,$^{**}p < 0.01$) (Fig. 4d).

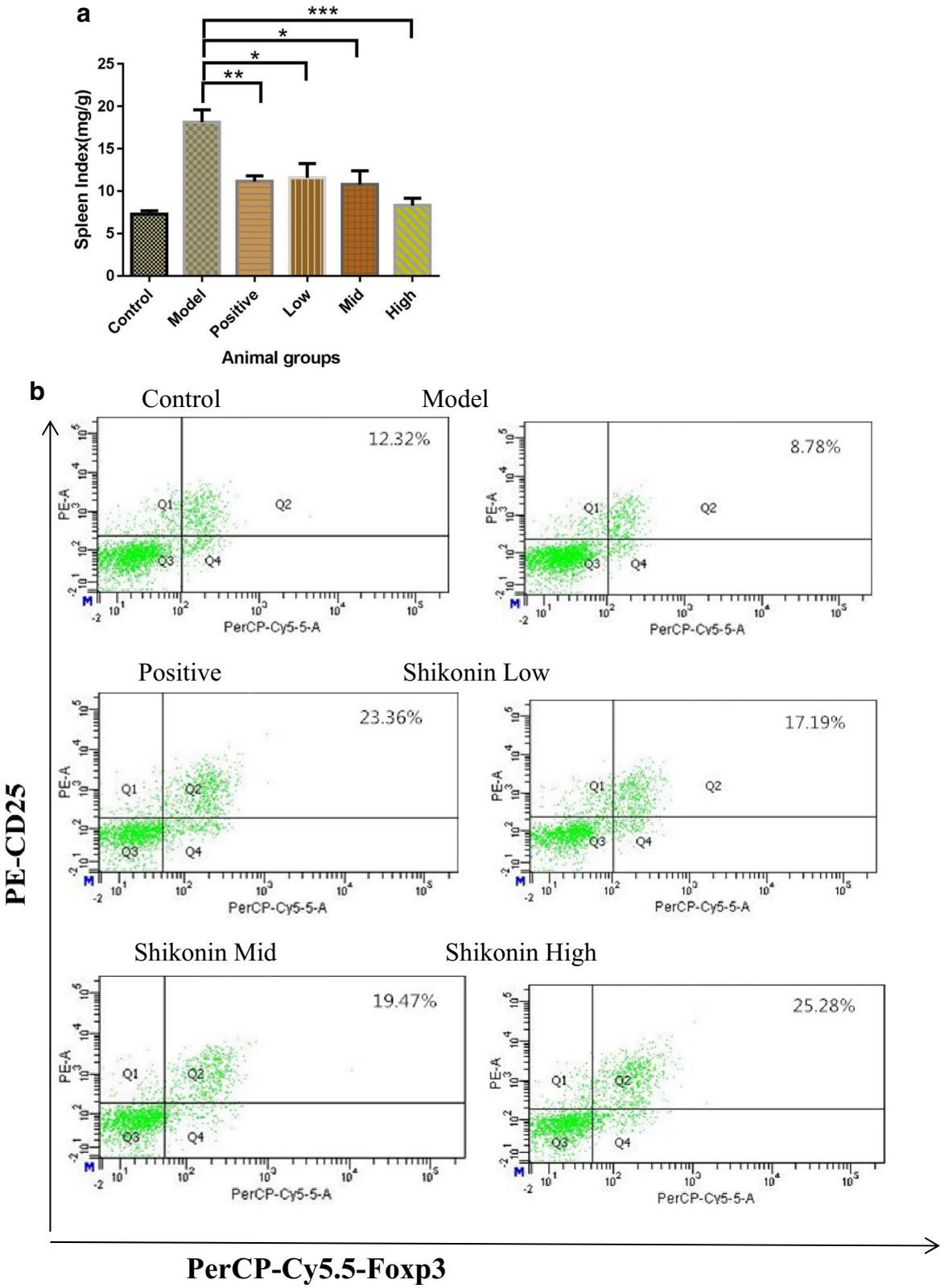
Shikonin Promotes iTreg Cell Differentiation by Inhibiting AKT/mTOR Signaling

To analyze the role of shikonin in modulating AKT/mTOR signaling, the main pathway by which iTreg cell differentiation is regulated, expression of pAKT, AKT, pP70S6K, and P70S6K was evaluated in the iTreg differentiation system. Shikonin at 1.5 μM suppressed phosphorylation of AKT (1.5 μM shikonin group *vs* control group: 0.53 ± 0.09 *vs* 0.94 ± 0.02; $^{*}p < 0.05$) (Fig. 5a, c), whereas 1.5 μM shikonin inhibited phosphorylation of P70S6K (1.5 μM shikonin group *vs* control group: 0.59 ± 0.10 *vs* 1.18 ± 0.07; $^{*}p < 0.05$) (Fig. 5b, d). Therefore, it can be suggested that shikonin promotes iTreg cell differentiation by inhibiting AKT/mTOR signaling.

DISCUSSION

Shikonin is the pharmacologically active constituent of members of the genus *Lithospermum*. Previous studies have shown that shikonin can effectively inhibit excessive activation of effector $CD4^+$ T cells and decrease levels of inflammatory cytokines [17, 18]; however, its mechanism

Fig. 2Shikonin effectively promoted the induction of iTreg cell in the spleen of mice with psoriasis. The mice with psoriasis were treated with different concentrations of shikonin and MTX. On the 10th day of treatment, the mice from different groups were weighed and the spleens were removed for weighting. **a** The spleen index of mice in the control, model, positive, and shikonin-treated groups. Mononuclear cells were extracted from the spleens of the mice with psoriasis. We examined the number of iTreg cells in the spleens by flow cytometry and gated the cells from $CD4^+$T cells. **b**, **c** The numbers of iTreg cells in the spleens of mice from the control, model, positive, and shikonin-treated groups. The levels of IL-10 and TGF-β1 secreted by iTreg cells were evaluated in splenic supernatants using ELISA. The levels of **d** IL-10 and **e** TGF-β1 in splenic supernatants of the mice from the control, model, positive, and shikonin-treated groups. Data of column graphs are presented as the mean ± SD of three independent experiments after one-way ANOVA with Tukey's *post hoc* test ($n = 4$–6mice/group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).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Spleen Index(mg/g)
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Shikonin Low
23.36%
17.19%
Shikonin Mid
Shikonin High
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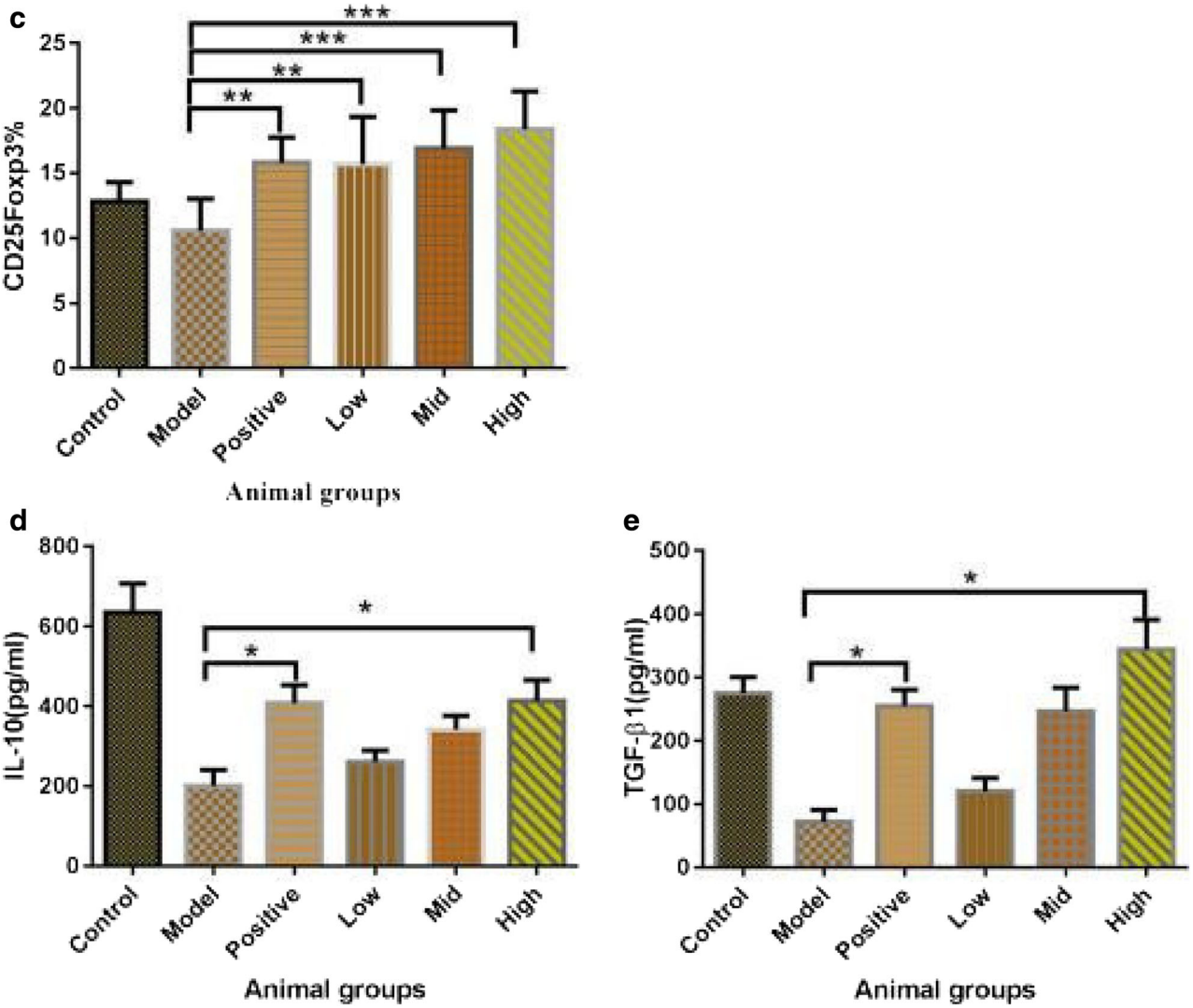
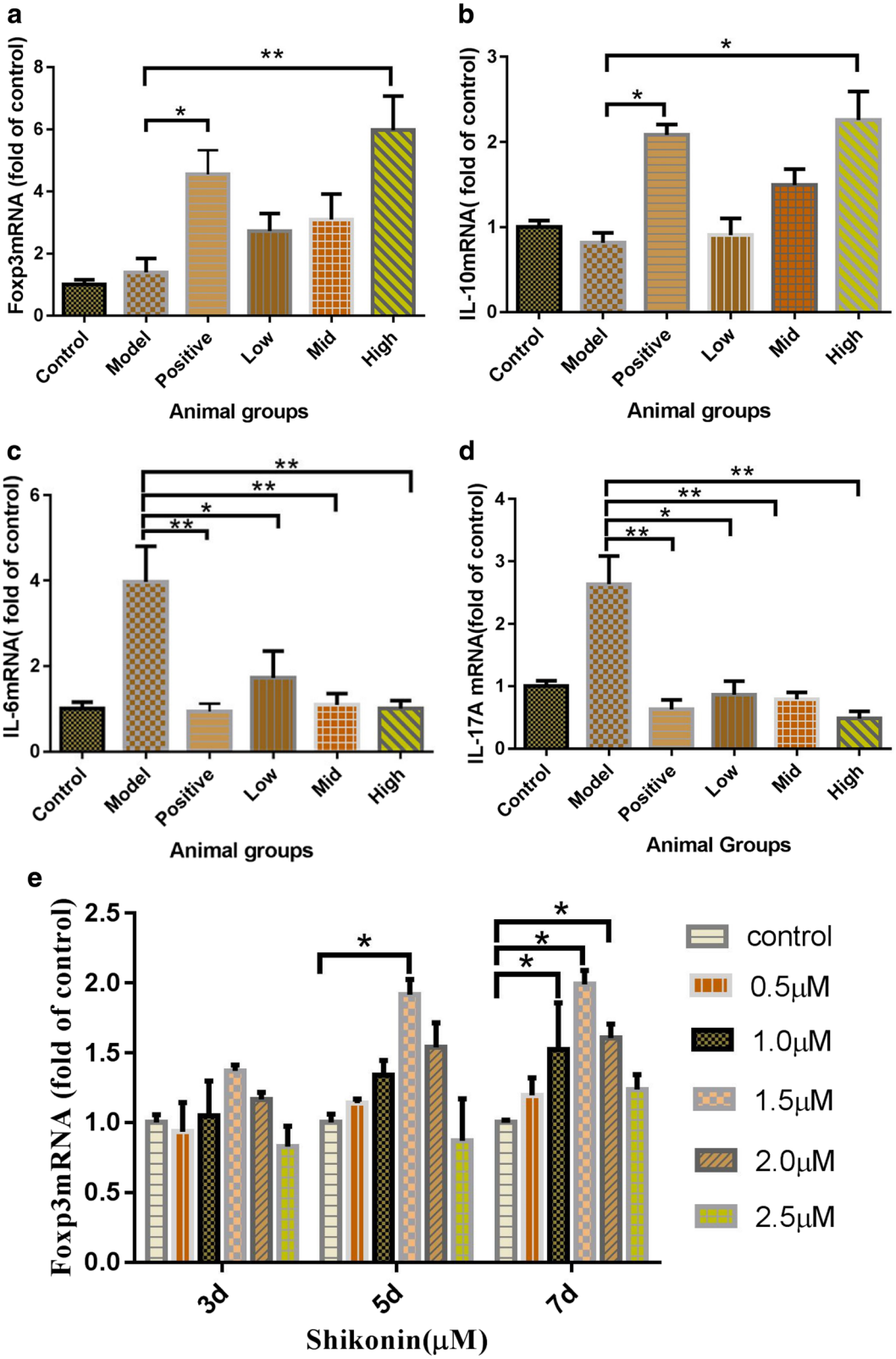


Fig. 2. continued.

remains unknown. In this study, we investigated whether shikonin acts as an anti-inflammatory and immune regulator by controlling Treg cells and found that shikonin at a concentration of 1.5 μM effectively promoted the differentiation of human naïve $CD4^+$T cells to iTreg cells *in vitro* and simultaneously facilitated the secretion of cytokines IL-10 and TGF-β1. Intragastric administration of different concentrations of shikonin effectively increased the number of iTreg cells in the mouse spleen. And shikonin also significantly increased the expression of Foxp3 mRNA in the skin of mice with psoriasis lesion. Therefore, we suggest that shikonin exerts anti-inflammatory and immunomodulatory effects by promoting the differentiation of

Fig. 3 The effects of shikonin on the key gene expression of inflammation *in vivo* and *in vitro*. On the 10th day of treatment, total RNA were extracted from skin tissues of the mice from different groups. The inflammation-associated genes (Foxp3, IL-10, IL-6, and IL-17A) were measured by qRT-PCR. All the results were normalized to GAPDH. The gene expression of **a** Foxp3, **b** IL-10, **c** IL-6, and **d** IL-17A in the skin of mice from control, model, positive, and shikonin-treated groups ($n = 4$–6mice/group). During the iTreg cell differentiation, the naïve $CD4^+$T cells were treated with different concentrations of shikonin (0.5 μM, 1.0 μM, 1.5 μM, 2.0 μM, and 2.5 μM) for indicated time points(3, 5, and 7 days). Then, the RNAs were extracted and the gene expression of Foxp3 was measured by qRT-PCR. **e** The gene expression of Foxp3 in the shikonin-treated group and the control group on the 3rd, 5th, and 7th days of differentiation ($n = 24$). The data of column graphs are presented as the mean ± SD of three independent experiments after one-way ANOVA with Tukey's *post hoc* test (*$p < 0.05$, **$p < 0.01$).


a
Foxp3mRNA (fold of control)
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Animal groups
d
IL-17A mRNA(fold of control)
Animal Groups
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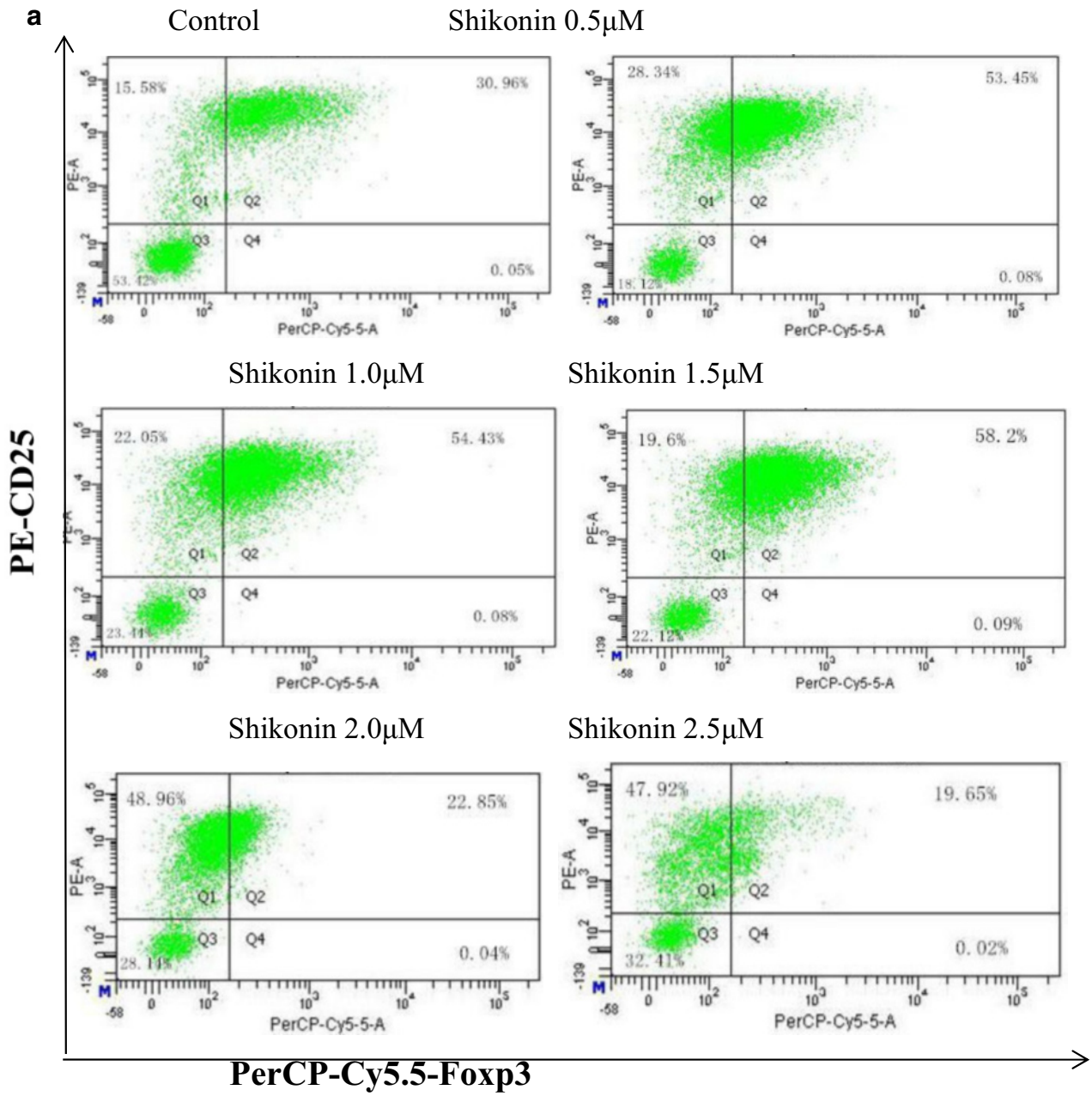

Fig. 4. Shikonin promoted the induction of iTreg cells *in vitro*. The number of iTreg cells in control and shikonin-treated group were evaluated by flow cytometry on the 7th day of cell culture. **a**, **b** The number of $CD25^{+}Foxp3^{+}$ T cells in the shikonin-treated group and the control group. The levels of cytokines secreted by iTreg cells on the 7th day were evaluated by ELISA. The levels of **c** IL-10 and **d** TGF-β1 in the shikonin-treated and control groups. Data of column graphs are presented as the mean ± SD of three independent experiments after one-way ANOVA with Tukey's *post hoc* test ($n = 24$,$^{*}p < 0.05$, $^{**}p < 0.01$).

iTreg cells [6, 7]. These data suggest a novel avenue for the treatment of inflammatory autoimmune diseases such as psoriasis.

Previous studies have shown that the PI3K/AKT/mTOR pathway is negatively correlated with the differentiation of iTreg cells. When AKT is continuously activated,

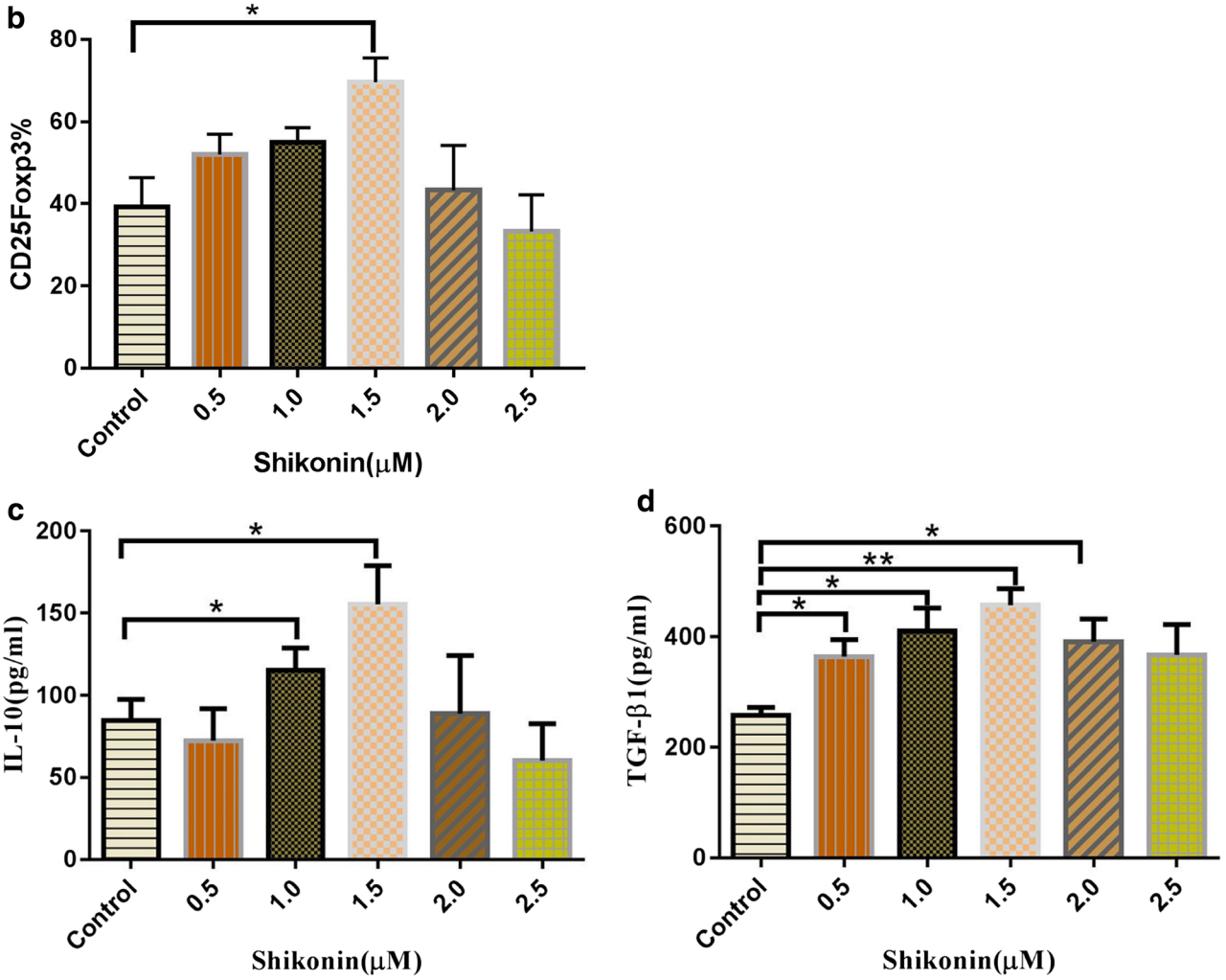

Fig. 4. continued.

expression of FOXP3 is significantly downregulated, which in turn inhibits the differentiation of Treg cells. However, the opposite effect is observed when PI3K/AKT/mTOR signaling is suppressed; for instance, PI3K or AKT inhibitors can enhance iTreg cell differentiation [19, 20]. In the present study, shikonin at a concentration of 1.5 μM significantly inhibited AKT and P70S6K phosphorylation. Thus, we speculate that shikonin regulates the differentiation of iTreg cells in peripheral tissues by inhibiting PI3K/AKT/mTOR signaling.

Treg cells are closely related to the pathogenesis of psoriasis. Previous studies have shown that the number of iTreg cells in the peripheral blood of patients with severe, advanced psoriasis was significantly lower than that of healthy controls [13], and psoriasis can be significantly improved when the number of iTreg cells in peripheral tissues increases [21, 22]. Shikonin has anti-inflammatory and immunomodulatory effects and is one of the most promising traditional Chinese medicines used to treat psoriasis; nonetheless, the therapeutic mechanism is not yet clearly understood. In the present study, mice with IMQ-induced psoriasis were treated with different concentrations of shikonin, which effectively improved the lesions and simultaneously increased the number of iTreg cells in the spleen. Our data show that shikonin significantly increased levels of IL-10 and TGF-β1 in the supernatant of splenic cells from mice with IMQ-induced psoriasis, indicating that shikonin promotes secretion by iTreg cells. Combined with *in vitro* experiments, our experimental *in vivo* results show that shikonin can effectively promote the differentiation of iTreg cells in peripheral tissues

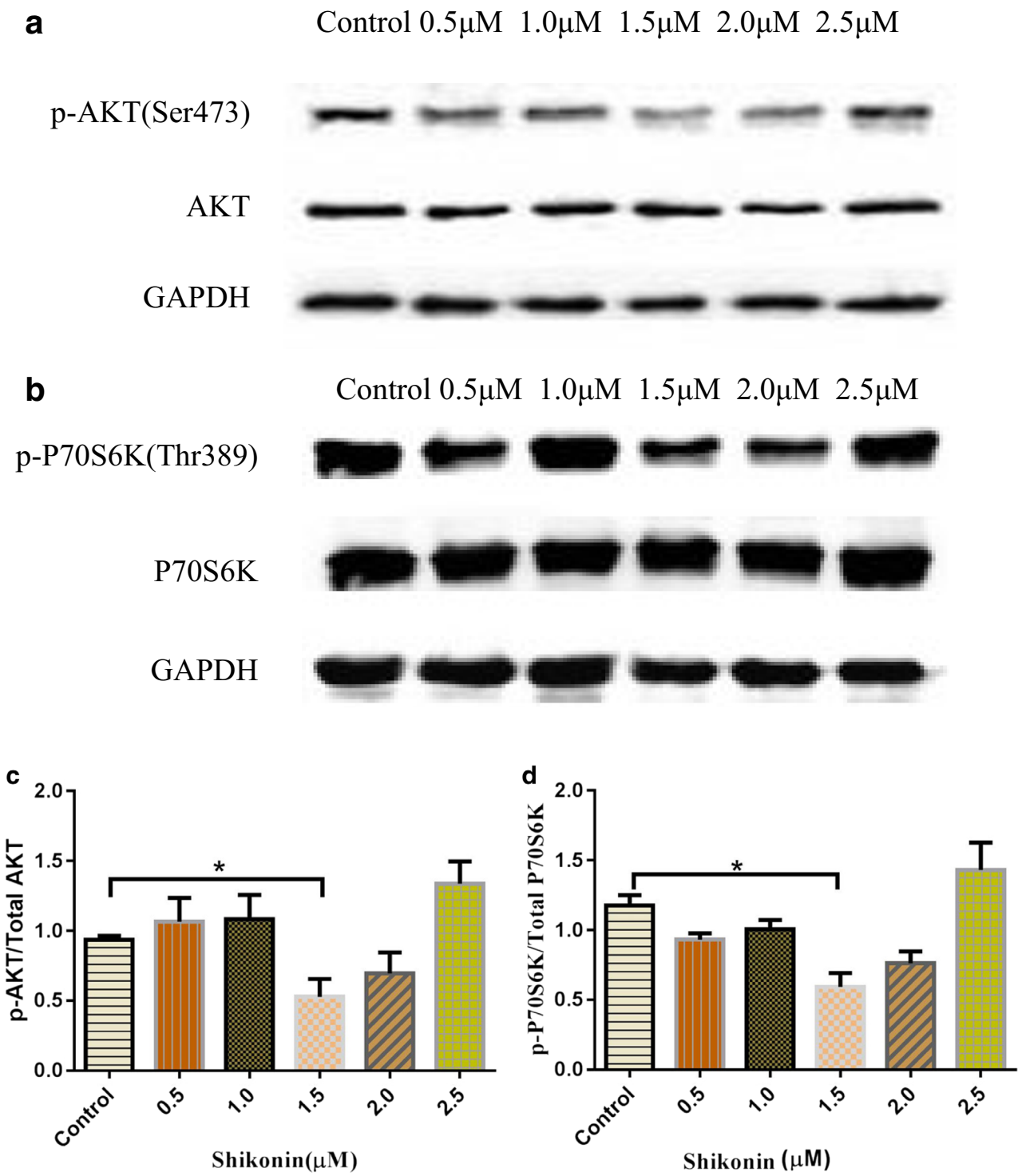

Fig. 5. Shikonin inhibited the phosphorylation of AKT and P70S6K. The protein expression of total AKT, p-AKT, P70S6K, and p-P70S6K in the iTreg cells on the 7th day were detected by western blotting. **a**, **c** The ratio of phosphorylated AKT to total AKT in the control and shikonin-treated groups. **b**, **d** The ratio of phosphorylated P70S6K to total P70S6K in the control and shikonin-treated groups. Data of column graphs are presented as the mean ± SD of three independent experiments after one-way ANOVA with Tukey's *post hoc* test ($n = 24$, $^*p < 0.05$).

and simultaneously increase iTreg cell secretion. Overall, intragastric administration of shikonin significantly improved skin lesions in mice with IMQ-induced psoriasis. The results of this study explored the mechanism related to shikonin treatment of psoriasis provide a theoretical basis and novel strategy for the treatment of psoriasis with Chinese medicine.

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COMPLIANCE WITH ETHICAL STANDARDS

All animal experimental procedures were approved and overseen by the Animal Care and Use Committee of the Shenyang Institute of Traditional Chinese Medicine.

Conflict of Interest. The authors declare that they have no conflict of interest.

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