**INTRODUCTION**

Psoriasis vulgaris is a common chronic skin disease that is characterized by red-colored plaques and skin lesions covered by slivery-white dry scales. The visible disfiguration and related symptoms are itching, pain, and bleeding (Scho¨ n and Boehncke, 2005). Worse, emerging evidence has shown an association between psoriasis, psoriatic arthritis, and cardiovascular diseases (de Oliveira et al., 2015). Although studies on the pathogenesis of psoriasis have been extensively developed, the treatments are still not satisfactory. Therefore, it was meaningful to investigate the underlying molecular mechanisms and search for new therapeutic strategies.

Keratinocyte movement from the basal layer to the spinous layer has been shown to be accelerated by approximately 10-fold in psoriatic lesions (Weinstein et al., 1985; Weinstein and Van Scott, 1963), which led to a massive thickened epidermis. The epidermis showed high amounts of proliferation, which resulted in thickened stratum corneum (hyperkeratosis) and flattened nuclei that remained in corneocytes in the upper layers (parakeratosis). There was substantial infiltration by immunocytes observed in the dermis, as well as dilated blood vessels. However, whether the dominant process in psoriasis involved primary immune activation with secondary hyperplastic keratinocytes or vice versa has been debated for many years. Emerging evidence has supported the notion that the immune system plays a more integral role in psoriasis. Although it was accepted that psoriasis is a T cell-dependent disease (Prinz, 2003), recruitment and activation of macrophages in psoriatic skin have been proven to be key pathogenic events (Scho¨ n and Erpenbeck, 2018; Stratis et al., 2006; Wang et al., 2006). The contribution of macrophages was further confirmed by the phenomenon that a small number of patients do not respond to treatment with T cell-immunosuppressive medications (Clark and Kupper, 2006). Based on these reports, targeting macrophages and modulating their functional properties might be a potential therapeutic strategy for this disease.

As the main component of the innate immune system, macrophages are activated by sensing both endogenous and exogenous signals to gain different or even opposite functional properties. The current classification of macrophage phenotypes, M1 and M2 macrophages, refers to the...
polarization of T cells (Gordon and Martinez, 2010; Sica and Mantovani, 2012). Researchers showed the key role of autophagy in suppressing proinflammatory macrophage polarization, although the detailed underlying mechanism remains unclear (Chang et al., 2013; Chen et al., 2014; Liu et al., 2015; Zhou et al., 2017). Previous articles reported that the autophagy activator, rapamycin, ameliorated imiquimod (IMQ)-induced psoriasis in mice by blocking the mTOR signal (Shirsath et al., 2016). Therefore, targeting autophagy may be a potential strategy for psoriatic inflammation; meanwhile, the signal mechanisms need to be studied further.

Khellactone is a natural pyranocoumarin that is widely used in traditional herbal medicine as a secondary metabolite. A few articles reported the anticancer effect of khellactone by inducing cell apoptosis (Jung et al., 2018; Lee et al., 2007). The use of khellactone as a precursor has become popular for synthesizing derivatives with anti-HIV activity (Jing et al., 2016; Lee, 2010; Ren et al., 2013). In this study, we found anti-psoriasis activity for cis-khellactone in IMQ-induced mice. cis-Khellactone inhibited the activation of NF-κB and subsequently regulated the balance of macrophage phenotypes. Furthermore, we showed that the anti-inflammation of cis-khellactone both in macrophages and in psoriatic skin lesions was largely dependent on inducing autophagy.

RESULTS

cis-Khellactone alleviated IMQ-induced psoriasis

We evaluated the progression of disease via an adapted human clinical Psoriasis Area and Severity Index and monitored it for 4 days. As shown in Figure 1c, the scores of the IMQ-treated group continuously increased. However, cis-khellactone administration apparently improved skin lesions, and the score remained stable at a low level after day 2 (Figure 1b and c). The mouse body weight was maintained, and both skin and epidermal thickness were significantly reduced in cis-khellactone-treated groups compared with the control (Figure 1d–f). Histopathological detection of skin sections further supported the amelioration properties of cis-khellactone (Figure 1g). The 30-mg/kg dose of cis-khellactone obviously inhibited acanthosis, parakeratosis, and...
Figure 2. *cis*-Khellactone affected the cytokines profile in lesion skin. (a, b) mRNA and (c) protein levels of cytokines in back skin were measured by quantitative PCR and ELISA, respectively. Mean ± standard deviation, n = 8. *P < 0.05 vs. IMQ-treated group. IMQ, imiquimod; KL, *cis*-khellactone.

thickening of the stratum corneum (hyperkeratosis) (Figure 1g, the up-line images). In the dermis, dilatation of blood vessels and infiltration of immune cells was also reduced in the treatment group compared with the control (Figure 1g, the bottom line images). IMQ also induced an aberrant cytokine profile in lesioned skin. We first detected the alarmins, S100A8/S100A9, and found that *cis*-khellactone suppressed these two factors (Figure 2a). In addition, *cis*-khellactone also apparently inhibited mRNA expression of cytokines IL-23, TNF-α, IL-1β, and IL-6 (Figure 2b). Only the IL-23 and TNF-α protein levels were significantly down-regulated (Figure 2c). However, one of the important factors, IL-17A, was slightly down-regulated in the *cis*-khellactone treatment group (Figure 2a). These data suggested that *cis*-khellactone improved the IMQ-induced psoriasis skin disorder in mice.

*cis*-Khellactone selectively blocked the infiltration of proinflammatory macrophages and inhibited the activation of p65

We detected infiltrating immune cells in the skin tissue of the treatment group, including macrophages, T cells, neutrophils, and monocytes. Skin sections were stained with F4/80, which is the macrophage surface marker, and they were observed by confocal microscopy. Data from the immunofluorescence assay showed that the intense infiltration of macrophages, especially in the dermis, was fiercely down-regulated by *cis*-khellactone treatment (Figure 3a). Our immunohistochemistry assay with a CD11c-specific antibody further confirmed the proinflammatory macrophage infiltration, which was inhibited in *cis*-khellactone-treated mice (Figure 3b and c). *cis*-Khellactone effectively reduced the activation of phosphorylated (p-) p65 (p-p65) both in the keratinocytes of the epidermis and immune cells of the dermis (Figure 3d and e). In addition, the colocalization of F4/80 and p-p65 mainly in the dermis indicated activation of NF-kB in macrophages (Figure 3f). The number of double-fluorescence positive macrophages, as well as of single F4/80 or p-p65 positive cells, was massively decreased in the *cis*-khellactone treatment group compared with the IMQ group (Figure 3f).

We also detected the infiltrated neutrophils, monocytes, and T cells by performing an immunofluorescence assay in skin tissues. The results showed that *cis*-khellactone displayed very slight inhibition of Ly6G+ neutrophil (Figure 4a and b), Ly6C+ monocyte (Figure 4c and d), and CD4+ T cell (Figure 4e and f) infiltration. These data narrowed the target cells of *cis*-khellactone to macrophages.

*cis*-Khellactone inhibited the classical activation phenotype of macrophage polarization without affecting activation of T and B cells

We established a macrophage polarization model with lipopolysaccharide (LPS) and IMQ stimulation in Raw 264.7 cells to evaluate *cis*-khellactone activity in vitro. *cis*-Khellactone significantly inhibited the mRNA levels of proinflammatory factors, including IL-23, TNF-α, IL-1β, and IL-6, under stimulation with both LPS and IMQ in a dose-dependent manner (Figure 5a and b). In line with these results, *cis*-khellactone displayed a blockade on TNF-α and IL-6, which resulted in their secretion into the culture medium (Figure 5c and d). In addition, we monitored a
marked reduction in the phosphorylation of IkB kinase α/β and p65 in cis-khellactone pretreated macrophages compared with the LPS-stimulated group (Figure 5g and h). These data verified the inhibition of cis-khellactone in proinflammatory macrophages. In addition to suppressing proinflammatory phenotype, cis-khellactone enhanced the anti-inflammatory macrophages (see Supplementary Figure S1a online). cis-Khellactone increased mRNA levels of Cd206, Ym1, and Fizz1, which are factors expressed in alternatively activated macrophages induced by IL-4 (see Supplementary Figure S1a).

To comprehensively evaluate the characteristics of cis-khellactone, we activated B cells in the spleen with LPS and T cells in lymph nodes with concanavalin A. Expression of the activation markers CD23 and CD69 (Luo et al., 2013) in B220-gated B cells was rarely inhibited under cis-khellactone treatment (Figure 5e). T-cell activation markers (Figure 5f), CD25 and CD69 (Tao et al., 2013), and the mRNA levels of Il6-γ and Tnf-α (see Supplementary Figure S1b), were also unaffected by cis-khellactone. In addition to immunocytes, we explored the effects of cis-khellactone on keratinocytes, which typically overproliferated in psoriasis patient skin. Data in Supplementary Figure S1c shown that cis-khellactone failed to rescue HaCaT cell proliferation promoted by TNF-α. Therefore, we focused on macrophages to further investigate the underlying mechanism of cis-khellactone.

In addition, methotrexate is the recommended first-line systemic treatment for moderate to severe plaque psoriasis patients in accordance with guidelines from the United Kingdom, United States, and Europe (Menter et al., 2009). Although it is efficient (Otero et al., 2017; Raaby et al., 2017), methotrexate has been reported to have hepatotoxicity (Maybury et al., 2014). We found that methotrexate significantly inhibited the viability of resting/LPS-stimulated macrophages (see Supplementary Figure S2a and b) and primary hepatocytes (Supplementary Figure S2c). However, unlike methotrexate, cis-khellactone modulated only the macrophage function phenotype without affecting cell viability.

**cis-Khellactone regulated macrophage phenotypes by inducing autophagy**

We further explored how cis-khellactone regulated macrophage phenotypes. As shown in Figure 5g and h, in addition to inhibiting p-IkB kinase α/β and p-p65, cis-khellactone significantly down-regulated the protein level of p62 but promoted LC-3BII, which suggested that cis-khellactone induced autophagy in LPS-stimulated macrophages. This phenomenon was further manifested by additional LC-3B (microtubule-associated proteins 1A/1B light chain 3B).
punctate observed through an immunofluorescence assay (Figure 5i) and autophagic vacuoles observed under transmission electron microscope (Figure 5j) in cis-khellactone—treated cells. As a result, reactive oxygen species accumulation was cleared partially by cis-khellactone enhanced autophagy (Figure 5k). Autophagy proteins, including Beclin1, Atg7, and LC-3BII, in psoriatic skin tissue were also partially up-regulated when treated with cis-khellactone (see Supplementary Figure S3 online). To determine the involvement of autophagy in the bioactivity of cis-khellactone on macrophage polarization, we transfected Raw 264.7 cells with shBecn1 or siAtg7 to establish autophagy-knockdown macrophages. The silencing efficiency was detected by quantitative PCR and immunoblotting, as shown in Figure 5l and n. Compared with cells transfected with negative control short hairpin or small interfering RNA, inhibition of TNF-α and IL-6 by cis-khellactone was almost completely reversed in Beclin1- and Atg7-silenced macrophages (Figure 5m and o). Knockdown autophagy remarkably increased both mRNA and protein levels of TNF-α and IL-6, which resulted in an exacerbated proinflammatory phenotype in macrophages. All of these results suggested that cis-khellactone induced autophagy against proinflammatory macrophages.

**cis-Khellactone failed to ameliorate psoriasis when autophagy was restrained in mice**

To confirm the mechanism of cis-khellactone in vivo, we used an autophagy inhibitor, chloroquine (CQ), in psoriatic mice. Compared with IMQ treatment alone in Figure 1, administration of CQ (intragastric) exacerbated psoriasis, as indicated by Psoriasis Area and Severity Index scores (Figure 6a), skin thickness (Figure 6c), and epidermal thickness (Figure 6d) while the body weight was stable (Figure 6b). Psoriasiform inflammation induced by the infiltration of immune cells in the dermis and keratinocyte hyperproliferation in the epidermis were much more severe in the CQ-treated group (Figure 6e and f). More importantly, cis-khellactone failed to ameliorate skin lesions in the presence of CQ (Figure 6a to f). Infiltrated T cells and monocytes were also unaffected in the CQ-treated mice (Figure 6g and h). In addition, IL-23, TNF-α, IL-1β, and IL-6 in lesion skin were also increased in the CQ treatment group, but they were hardly affected by cis-khellactone (Figure 6i). These
Figure 5. Effects of cis-khellactone on immune cells. Raw 264.7 were pretreated with cis-khellactone for 1 hour and then with (a) 20 ng/ml LPS or (b) 10 μmol/L IMQ for 6 hours. mRNA levels were measured by quantitative PCR. (c, d) Secreted cytokines were measured by ELISA. Activations of (e) B and (f) T cells were analyzed. (g) Raw 264.7 were treated as in a. Protein expression was detected by Western blot. (h) Data summary of g. (i) LC3B staining was observed by confocal microscopy. (j) Autophagic vesicle was observed by transmission electron microscopy. (k) Reactive oxygen species level was measured. Raw 264.7 transfected with (l) shNC/shBecn1 or (n) siNC/siAtg7 and silence efficiency was measured. Silenced cells were treated as in g. (m, o) mRNA and protein levels were measured. *P < 0.05, **P < 0.01. ConA, concanavalin A; IKK, IκB kinase; IMQ, imiquimod; KL, cis-khellactone; LPS, lipopolysaccharide; M, mol/L; NC, noncoding; ns, not significant; sh, short hairpin; si, small interfering; SSC, side-scattered light.
observations suggested that the amelioration of khellactone on psoriasis in vivo was dependent on inducing autophagy.

**DISCUSSION**

Although there has been substantial research on investigating molecular mechanisms and searching for new drugs, the current treatments for patients with psoriasis are still unsatisfactory (Mease and Armstrong, 2014; Menter et al., 2011). In this study, we identified alleviation of IMQ-induced psoriasis with a small compound, cis-khellactone, and investigated the underlying mechanism. cis-Khellactone inhibited the proinflammatory cytokines and the infiltration of proinflammatory macrophages in an autophagy-dependent manner both in vitro and in vivo.

An aberrant cytokine profile in skin lesions has been shown to be an important initiator and accelerator for the pathogenesis of psoriasis. The calprotectin heterocomplex consisting of S100A8 and S100A9 has been identified as a biomarker for inflammatory disorders, including rheumatoid arthritis and psoriatic arthritis (Hansson et al., 2014; Vogl et al., 2014). cis-Khellactone showed obvious inhibition of the expression of S100a8 and S100a9 in skin lesions (Figure 2a), which indicated that cis-khellactone produced a preliminary improvement in psoriatic inflammation. Recently, the IL-17A/IL-23 axis has emerged as a major player in various autoimmune diseases (Grine et al., 2015; Teng et al., 2015). cis-Khellactone suppressed both IL-23 and IL-17a mRNA levels and was stronger against IL-23 than IL-17a (Figure 2a and b). We also
observed inhibition of TNF-α, IL-1β, and IL-6 in cis-khellactone–treated skin; this set of cytokines is expressed in proinflammatory phenotype macrophages and is dependent on NF-κB (Figure 2b and c). Results from an immunohistochemistry assay showed that cis-khellactone inhibited macrophage infiltration in the dermis, especially CD11c-positive M1 macrophages (Figure 3). CD11c was a well-known surface marker for dendritic cells, but it was also used to characterize proinflammatory macrophages (Satoh et al., 2013). The phenotype of infiltrating macrophages needed to be further confirmed by co-staining for CD11c with F4/80 or other related markers. Along with the relatively slight inhibition of IL-17a, we speculated that cis-khellactone had a bias for targeting macrophages.

In addition, there was a small population of macrophages (F4/80 positive) without staining for p-p65 in 30-mg/kg cis-khellactone–treated mice, which might be the alternatively activated macrophages with anti-inflammatory properties (Figure 3f). To confirm this speculation, we polarized bone marrow–derived macrophages with IL-4 and measured the effects of cis-khellactone. The results in Supplementary Figure S1a display the obviously increased mRNA level of M2-associated factors in cis-khellactone–treated cells, which indicated that cis-khellactone might regulate the balance of macrophage phenotypes.

Articles have reported the increased number of T lymphocytes, macrophages, and neutrophilic granulocytes within the dermis and epidermis of psoriasis patients (Boehncke and Schön, 2015). Among these infiltrated inflammatory cells, neutrophils could be rapidly recruited to the damage site to produce proinflammatory cytokines (Lin et al., 2011; Steffen et al., 2018). We investigated the effects of the compound on these cells and observed unaffected neutrophil, T-cell, and monocyte infiltration or activation, as well as the proliferation of keratinocytes in the cis-khellactone treatment group (Figure 4, Figure 5e and 5f, and Supplementary Figure S1b and c). These results further confirmed the key role of macrophages in our study.

Targeting macrophages to inhibit the proinflammatory phenotype via blocking NF-κB signaling has been shown to be effective for alleviating psoriasis (Wang et al., 2009). Currently, biological drugs have been developed and
approved for the treatment of various diseases, such as Crohn’s disease, rheumatoid arthritis, ankylosing spondylitis, and psoriasis (Grine et al., 2015). However, long-term administration of biological drugs, such as TNF antagonists, would cause serious adverse effects, including enhanced susceptibility to bacterial infections or reactivation of latent tuberculosis (Moustou et al., 2009). Unlike TNF antagonists, cis-khellactone is a natural compound that might avoid these serious adverse effects. Improvement of inflammatory diseases with natural compounds has been reported (Feng et al., 2014; Xu et al., 2016). However, there are still limitations of these coumarin compounds, such as poor oral bioavailability and unclear detailed targets.

The role of autophagy in the immune system has been intensively investigated. Researchers reported that NF-κB degraded in hepatoma-derived M2 macrophages via TLR-2-dependent selective autophagy (Chang et al., 2013). In this study, we found that cis-khellactone down-regulated the phosphorylation of p65 without affecting the protein levels of p65, although cis-khellactone strengthened autophagy (Figure 5g–k). Additionally, in vitro and in vivo observations further confirmed that cis-khellactone exerted its bioactivity in an autophagy-dependent way (Figure 5 and Figure 6). The role of autophagy was investigated for IMQ-induced psoriasis (Chamcheu et al., 2017; Shao et al., 2016; Shirsath et al., 2016). When treated with CQ to restrict the system autophagy level, cis-khellactone failed to ameliorate IMQ-induced psoriasis (Figure 6). Under these circumstances, the autophagy level of both T cells, B cells, monocytes, neutrophils, and macrophages might be inhibited. However, the suppressed autophagy, as well as the infiltration of T cells, monocytes, and neutrophils in psoriasis skin tissue, and

Figure 6. Effect of cis-khellactone on IMQ-induced psoriasis was hampered when treated with autophagy inhibitor. IMQ-treated mice received daily administration with 30 mg/kg cis-khellactone and 50 mg/kg CQ (intragastric). (a) Clinical scores. (b) Body weight change. (c) Skin thickness. (d) Epidermal thickness. (e) Hematoxylin and eosin staining of back skin section. (f) Infiltrated macrophages, (g) T cells, and (h) monocytes were stained with F4/80, CD4, and Ly6C, respectively, and detected by confocal microscopy. (i) Cytokine levels in the skin were measured by ELISA. n = 8. Scale bar = 50 μm. CQ, chloroquine; IMQ, imiquimod; KL, cis-khellactone.
activation of T cells and B cells were unaffected when treated with cis-khellactone (Figure 4 and Figure 5e and f). Based on these observations, we surmised that cis-khellactone needs to induce autophagy to protect mice against the disease. We analyzed the anti-inflammatory activity of cis-khellactone and detected the underlying mechanism. Cis-Khellactone suppressed the expression of proinflammatory factors and activation of NF-kB signal in macrophages in an autophagy-dependent manner, which consequently ameliorated IMQ-induced psoriasis in mice. Our study provided an effective candidate for the treatment of psoriasis, although there is still a need for additional research into how autophagy is induced by cis-khellactone-mediated inactivation of NF-kB signaling.

MATERIALS AND METHODS

Mice
Female C57BL/6 mice (8 weeks old, 18–22 g) were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the National Institutes of Medicine Guidelines for the Care and Use of Laboratory Animals, the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, and the Nanjing University Animal Care and Use Committee. The protocol was approved by the Nanjing University Animal Care and Use Committee.

IMQ-induced psoriasis
Mice adapted to laboratory for 1 week and then were shaved on the back. One day later, mice were randomly assigned to different experimental groups and applied with 62.5 mg Aldara cream (3M Health Care, Leicestershire, UK) topically daily on shaved skin for 4 constitutive days, except for mice in the sham group. Various doses of cis-khellactone were dissolved in 100 μl sodium carboxymethyl cellulose and administrated daily intragastrically for each mouse. The CQ-treated group received an additional 50 mg/kg CQ dissolved in 100 μl water (intragastric). Severity of disease was measured with the adapted human clinical Psoriasis Area and Severity Index, which was scored on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; and 4, very marked (Shao et al., 2016). Mice were scored daily, and body weight was monitored. Mice were killed on day 4 when the score reached a plateau. Back skin was obtained, and the thickness was measured with a digimatic micrometer (0.001 mm; Mitutoyo Company, Tokyo, Japan). Then, the skin samples were cut into several parts for the following detections.

Real-time quantitative PCR
Total RNA was extracted from skin or cells, reverse transcribed to cDNA, and subjected to quantitative PCR via the BioRad CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA) with iQ SYBR Green Supermix (BioRad), and threshold cycle numbers were obtained with BioRad CFX manager software.

Cytokine evaluation by ELISA
Cytokines in psoriatic skin or cell culture medium were measured with ELISA kits from Dakewe Biotech Co. (Shenzhen, China).

T- and B-cell activation was measured by flow cytometry
Splenocytes were incubated with cis-khellactone and 1 μg/ml LPS for 24 hours. CD25 and CD69 expression in B220-gated B cells was analyzed by flow cytometry. Lymph node cells were incubated with cis-khellactone and 5 μg/ml concanavalin A for 24 hours. CD25 and CD69 expression levels in CD4-gated T cells were analyzed by flow cytometry.

Lentivirus-based short hairpin RNA transfection and RNA interference
Raw 264.7 cells were infected with lentivirus-based short hairpin RNA-beclin 1 for 24 hours. The stably silenced cells were selected with 5 μg/ml puromycin for 2 weeks.

Atg7 was silenced by RNA interference. Raw 264.7 was transfected with small interfering RNA-noncoding or siRNA-Atg7 for 36 hours using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Waltham, MA).

Supplementary methods
Detailed methods can be found in the Supplementary Methods online.

Statistical analysis
Results were displayed as mean ± standard deviation of five independent experiments. The Student t test was used to evaluate the differences when two groups were compared. One-way analysis of variance analysis and post hoc Dunnett tests (IMQ- or LPS-treated group was set as control) were applied when there were more than two groups in the independent variable. The level of significance was set at a P-value of 0.05.

Data availability statement
Data sets related to this article can be found at https://data.mendeley.com/datasets/n5jchbt3r14/1, hosted at Mendeley.

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Author contributions
Study conception and design: LF, XW, and QX; acquisition, analysis, and interpretation of data: LF, PS, FX, LX, FS, MG, WH, LK; drafting/revision of the work for intellectual content and context: LF and XW; final approval and overall responsibility for the published work: XW and QX; LF and PS contributed equally.

Supplementary material
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.02.021.

References


SUPPLEMENTARY METHODS

Cells

Murine Raw 264.7 cells were obtained from Cell Bank, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO₂ at 37 °C. Bone marrow-derived macrophages were established as previously described (Feng et al., 2014). Briefly, mice femurs were flushed with phosphate buffered saline, and the harvested cells were cultured in RPMI 1640 containing 10% fetal bovine serum in the presence of 10 ng/ml macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ) in 5% CO₂ at 37 °C for 7 days. Mouse B cells from the spleen of C57BL/6 mice were stimulated by 1 mg/ml concanavalin A. Primary hepatocytes were isolated from adult male C57BL/6 mice (20–25 g) with the collagenase-perfusion technique of Bissell and Guzelian (Bissell et al., 1973). Hepatocytes were cultured in serum-free Williams’ E medium containing dexamethasone (0.1 μmol/L), insulin (100 nmol/L), penicillin (100 units/ml), and thyroxine (1 μmol/L) in a 5% CO₂ environment at 37 °C.

Reagents

cis-Khellactone was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Aldara (5% IMQ cream) was purchased from 3M Health Care Limited. IMQ, sodium pentobarbital, DAPI, 3-(4,5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazoli-um bromide (i.e., MTT), LPS, methotrexate, and CQ were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant murine IL-4 (catalog no. 214-14), macrophage colony-stimulating factor (catalog no. 315-02), and recombinant human TNF (catalog no. 300-01) were purchased from Peprotech (Rocky Hill, NJ). FITC-anti-F4/80, APC-anti-Ly-6G (clone 1A8-Ly6g, catalog no. 17-9668-82), PerCP-Cy5.5-anti-Ly-6C (clone HK1.4, catalog no. 45-5932-82), goat anti-rabbit IgG (H + L) secondary antibody, Alexa Fluor 594 (catalog no. R37117, RRID: AB_2556545) and MitoSOX (catalog no. M36008) were purchased from Thermo Fisher Scientific. APC-anti-B220 (catalog no. 103211), FITC-anti-CD69 (catalog no. 104505), and PE-anti-CD23 (catalog no. 101607) were purchased from BioLegend (San Diego, CA). Anti-CD11c (catalog no. ab52632, RRID: AB_2129793) and Anti-Agt (catalog no. ab52472) were purchased from Abcam (Burlingame, CA). Antibodies for p-IKK α/β (catalog no. 2697), IKK (catalog no. 2682, RRID:AB:10694848), p-p65 (catalog no. 3033, RRID: AB_331284), p65 (catalog no. 8242, RRID: AB_10859369), p62 (catalog no. 8833, RRID: AB:2728820), LC-3B (catalog no. 3868, RRID: AB:2137707), and Beclin1 (catalog no. 3738) were purchased from Cell Signaling Technology (Beverly, MA). The short hairpin RNA- scramble and short hairpin RNA-Becln1 were purchased from Shanghai Neuro-nBiotech Company. SiNC (catalog no. 6560) and siAgt7 (catalog no. 6604) were purchased from Cell Signaling Technology. Lipofectamine RNAiMAX Transfection Reagent (catalog no. 13778150) was purchased from Thermo Fisher Scientific.

The primer sequences used in this study

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Immunohistochemistry assay

Paraffin-embedded skin sections were heat-fixed, deparaffinized, rehydrated, antigen retrieved, blocked with 3% goat serum, and incubated with anti-CD11c or anti-p-p65 overnight at 4 °C. Then, the slides were detected using Real Envision Detection kit (GeneTech, Shanghai, China) according to the manufacturer’s instructions.

Immunofluorescence assay

Paraffin-embedded skin sections were heat-fixed, deparaffinized, rehydrated, antigen retrieved, blocked with 3% goat serum, and incubated with FITC-anti-F4/80, FITC-anti-CD4, APC-anti-Ly-6G or PerCP-Cy5.5-anti-Ly-6C for 2 hours at room temperature, respectively. Then, the slides were counterstained with DAPI and imaged with a confocal laser scanning microscope (Olympus, Lake Success, NY). For the double-staining immunofluorescence assay, after blocking with 3% goat serum, the slide was incubated with anti-p-p65 overnight at 4 °C. Skin sections were stained with secondary antibody (Alexa Fluor 594) and FITC-anti-F4/80 for 2 hours at room temperature. Then, the slides were counterstained with DAPI.

Transmission electron microscopy

Raw 264.7 cells were treated with cis-khellactone and LPS for 6 hours. After washing the medium with cold phosphate buffered saline, cells were fixed in 0.25% glutaraldehyde solution (BD Pharimagen diluted by fix buffer; BD Biosciences, San Jose, CA) over 24 hours. The autophagic vesicle was observed by transmission electron microscopy.

Western blot

Obtained cell lysates were separated by 10% SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). Immunoblotting was
performed with specific primary antibodies on the membranes.

**Reactive oxygen species detection**
Mitochondria-associated reactive oxygen species levels were measured by staining cells with MitoSOX (Thermo Fisher Scientific, Waltham, MA). Cultured cells were incubated with 2.5 mmol/L MitoSOX for 15 minutes at 37 °C in a cell incubator. Then, cells were harvested and analyzed by flow cytometry.

**MTT assay**
Cells were seeded in a 96-well plate and incubated with compounds in for 24 hours. Next, 20 μl of 4 mg/ml MTT dissolved in phosphate buffered saline was added to the plate wells and cultured at 37 °C for 4 hours. The plate was centrifuged at 1,200 g for 5 minutes, the supernatant was discarded, 200 μl DMSO was added, and the plate was vibrated for 10 minutes. The absorbance was detected at 570 nm.

**REFERENCES**
Supplementary Figure S2. Effect of *cis*-khellactone on cell viability. Bone marrow-derived macrophages were cultured with various doses of *cis*-khellactone or MTX in the (a) absence or (b) presence of 20 ng/ml LPS for 24 hours. Cell viability was measured by MTT assay. (c) Primary hepatocytes were cultured with various doses of *cis*-khellactone or MTX for 24 hours. Cell viability was measured by MTT assay. BMDM, bone-marrow derived macrophage; KL, *cis*-khellactone; MTX, methotrexate; M, mol/L.

Supplementary Figure S3. Effect of *cis*-khellactone on autophagy proteins levels of skin tissue. Beclin1, Atg7, and LC3B protein levels in skin tissue were measured by Western blot. IMQ, imiquimod; KL, *cis*-khellactone.