



Herpetol ameliorates allergic contact dermatitis through regulating T-lymphocytes

Xiang Li ^{a,1}, Xingqi Wang ^{a,b,1}, Hezhong Jiang ^{a,c,1}, Guohui Zhang ^a, Rui Tan ^c, Yang Sun ^a, Xuefeng Wu ^a, Renxiang Tan ^{a,*}, Qiang Xu ^{a,*}

^a State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, China

^b Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, School of Life Sciences, Jiangsu Normal University, Xuzhou, China

^c School of Life Science and Engineering, and College of Medicine, Southwest Jiaotong University, Chengdu, China

ARTICLE INFO

Article history:

Received 27 April 2016

Received in revised form 23 August 2016

Accepted 23 August 2016

Available online xxxx

Keywords:

Herpetol

Allergic contact dermatitis

T-lymphocytes

Glycolysis

Drug candidate

ABSTRACT

An immunosuppressant with fewer adverse effects is still urgently needed for increasing numbers of patients suffering from allergic contact dermatitis. In the present study, we aimed to investigate the immunosuppressive activity of herpetol on T-lymphocytes in vitro and in vivo and explore its potential pharmacological mechanism. The results showed that herpetol could effectively inhibit the proliferation of activated T cells and reduce the production of pro-inflammatory cytokines at 5–20 μ M. Additionally, the ear swelling and inflammatory responses induced by picryl chloride were significantly ameliorated by herpetol at 20–40 mg/kg. Moreover, herpetol could cause cell cycle arrest of activated T cells in a dose-dependent manner. Furthermore, herpetol reduced the expression and activity of HIF-1 α , Glut1 and LDHA, leading to glycolysis inhibition in activated T cells. Taken together, herpetol showed an immunosuppressive activity against T-cell mediated immune responses in vitro and in vivo, and it has potential for the treatment of immune-related skin diseases.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Contact hypersensitivity (CHS) is an adverse consequence of immune system disorders. T-lymphocyte proliferation is accepted to be a hallmark of immune responses against antigens [1,2], and the development of immunosuppressive drugs traditionally has been focused on lymphocytes. However, most of the immunosuppressants, such as dexamethasone, cyclophosphamide and cyclosporine A (CsA), have been reported to inevitably possess severe side effects primarily owing to their poor selectivity [3]. Even to this day, there is still an urgent need for effective natural immunosuppressants with low toxicity to minimize the risk from side effects.

T cell growth and function must be tightly regulated to provide protection against foreign pathogens while avoiding autoimmunity and immunodeficiency [4]. It has been known that T-cell activation leads to increased glycolytic metabolism that fuels proliferation and effector function [5]. T-cell activation has an ability to increase the expression and surface localization of glucose transporter 1 (Glut1) [6] and enhances the activities of key glycolytic enzymes [4,7,8]. HIF-1 α is a metabolic checkpoint in activated T cells, and it plays an important role in T-cell activation [9]. Therefore, HIF-1 α could be a therapy target for CHS.

After screening of multiple compounds, we found that herpetol, a dimeric lignoid from *Herpetospermum caudigerum* wall [10,11], showed a potent immunomodulatory activity. It has been known that herpetol possessed neuro-protective and anti-inflammatory activities [12]. However, no studies have examined its effects on disease caused by activated T cells. In this study, we investigated the ameliorating effect of herpetol on picryl chloride (PCI)-induced contact hypersensitivity and explored its regulatory role on T lymphocytes. The underlying mechanisms that involved inhibitory regulations of proliferation, activation and glycolytic metabolism in T lymphocytes by herpetol were investigated in this study.

2. Materials and methods

2.1. Mice

Specific pathogen-free, 6–8-week-old female C57BL/6 mice were purchased from the Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Female BALB/c mice (6–8 weeks old, 18–22 g) were obtained from the Experimental Animal Center of Yangzhou University (Yangzhou, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

* Corresponding authors at: State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, 163 Xianlin Avenue, Nanjing 210046, China.

E-mail addresses: rxtan@nju.edu.cn (R. Tan), molpharm@163.com (Q. Xu).

¹ These authors contributed equally to this work.

2.2. Reagents

Herpetol was isolated and identified as reported previously [10]. Herpetol (molecular weight: 356) with 99% of purity was dissolved at a concentration of 100 mM in 100% DMSO as a stock solution, stored at -20°C , and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study (all the control groups are composed of 0.1% DMSO). PCI, concanavalin A (Con A), 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St Louis, MO). CsA was purchased from Hubei Jianyuan (Wuhan, China). Propidium iodide (PI) was purchased from BD Biosciences (San Jose, CA). Purified anti-mouse CD3 (145-2C11) and purified anti-mouse CD28 (37.51) were purchased from BD PharMingen (San Diego, CA). Dynal® mouse T cell Negative Isolation Kit (Invitrogen Dynal AS, Oslo, Norway) was purchased from the Life Technology and the CD4^{+} - and CD8^{+} -T Cell Isolation Kits were purchased from Miltenyi Biotec (Auburn, CA). The ELISA kits for murine IL-17A, IFN- γ and IL-2 were purchased from Dakewe Biotech (Beijing, China). Antibodies against phospho-Rb (Ser 807/811), Rb, cyclin D1 and p27^{kip} were purchased from Cell Signal Technology (Beverly, MA). Antibodies against HIF- α , Glut1, LDHA and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lactic dehydrogenase (LDH) activity detection kit and lactic acid detection kit were purchased from Nanjing Jiancheng Biotech Company (Nanjing China).

2.3. Cell culture and cell proliferation assay

Spleen cells isolated from female C57BL/6 mice were maintained in RPMI 1640 medium supplemented with 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 U/ml penicillin, and 10% fetal calf serum under a humidified 5% (v/v) CO_2 atmosphere at 37°C . Cell proliferation was determined by MTT assay as we previously reported [13].

2.4. T cell purification assay

Mouse lymphocytes were isolated from lymph nodes of C57BL/6 mice. T cells were purified according to standard procedures using Dynal® mouse T cell Negative Isolation Kit (Invitrogen Dynal AS, Oslo, Norway) via magnetic cell separation. The purity was higher than 97%.

2.5. CD4^{+} - and CD8^{+} -T cell sorting assays

CD4^{+} T-cell and CD8^{+} T-cell were purified by magnetic bead separation. CD4^{+} T- and CD8^{+} T-cells were purified according to standard procedures using a CD4^{+} T-cell isolation kit (Miltenyi Biotec, Auburn, CA) and CD8^{+} T-cell isolation kit (Miltenyi Biotec, Auburn, CA). The purity was higher than 85%.

2.6. Quantification of mRNA expression by means of real-time PCR

This work was performed as we previously reported. Spleen cells were isolated and collected from mice. Total RNA was homogenized and extracted with TRIzol reagent. Then total RNA was treated by DNase I and subjected to quantitative PCR, which was performed with the BioRad CFX96 Touch™ Real-Time PCR Detection System (BioRad, CA) using iQTM SYBR® Green Supermix (BioRad, CA), and threshold cycle numbers were obtained using BioRad CFX Manager Software. The program for amplification was 1 stage of 5 min at 94°C followed by 40 cycles of a 3-step loop: 5 s at 94°C , 10 s at 58°C , and 10 s at 72°C . The PCR results were normalized to actin expression and were quantified by the $\Delta\Delta\text{CT}$ method. The primer sequences used in this study were as follows: actin (163 bp), forward 5'-ACATCTGCTGGAAG GTGGAC-3', reverse 5'-GGTACCACCATGTACCCAG G-3'; IFN- γ

(456 bp), forward 5'-CTTCTTCAGCAACAGCAAGGCCGAAAA-3', reverse 5'-CCCCAGATACAACCCCGCAATCA-3'; TNF- α (175 bp), forward 5'-CATCTTCTCAAAATTTCGAGTGACAA-3', reverse 5'-TGGGAGTAGACAAGG TACAACCC-3'; IL-17A (71 bp), forward 5'-TCGAGAAGATGCTGGTGG GT-3', reverse 5'-CTCTGTTTAGGCTGCTGG C-3'; HIF-1 α (228 bp), forward 5'-ACC TTCATCGGAACTCCAAAG-3', reverse 5'-CTGTTAGGCTGG GAAAAGTTAG G-3'; Glut1 (156 bp), forward 5'-CAGTTCGGCTATAACA CTGGTG-3', reverse 5'-GCCCCGACAGAGAAGATG-3'; LDHA (113 bp), forward 5'-CATTGT CAA GTACAGTCCACACT-3', reverse 5'-TTCCAATT ACTCGGTTTTTGGGA-3'; and HKII (112 bp), forward 5'-TGATCGCCTG CTTATTCACGG-3', reverse 5'-AACCGC CTAGAAATCTCCAGA-3'.

2.7. Cell cycle assay

T cells from spleen cells of C57BL/6 mice were treated with or without herpetol for 24 h in the presence of 5 $\mu\text{g}/\text{ml}$ Con A, and then collected and washed with cold PBS and fixed with 70% ethanol at 4°C overnight. Then, the fixed cells were washed with PBS and stained with 50 $\mu\text{g}/\text{ml}$ of PI containing 100 $\mu\text{g}/\text{ml}$ of RNase A and 1% Triton X-100 in a dark at room temperature for 30 min. The DNA contents of the cells were analyzed with Modfit software (Becton Dickinson, San Jose, CA).

2.8. Western blot analysis

Proteins were extracted in lysis buffer (30 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4°C and then incubated with a HRP-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.9. PCI-induced contact hypersensitivity

PCI-induced contact hypersensitivity was investigated as described before [14,15]. On the first day (day 0), female BALB/c mice were painted on the clipped abdomen with 0.1 ml of 1% PCI in absolute ethanol. Five days after sensitization (day 5), these mice were challenged on their left ears with 30 μL of 1% PCI in olive oil. Ear swelling was evaluated by the difference in thickness between the left and the right, as measured with a digimatic micrometer 20 h after challenge. Mice in normal group were normally sensitized and challenged with olive oil without PCI. Herpetol (20, 40 mg/kg) and dexamethasone (Dex, 5 mg/kg) intragastrically were administered once a day from day 0 to day 5. Mice in the normal group and model group were given saline instead of drugs.

2.10. Histological analysis

Histological assessment was performed as described before [14]. Formalin-fixed, paraffin-embedded ear tissue was sectioned at 5 μm thickness, and the sections were stained with hematoxylin and eosin. The following parameters were assessed: (1) the level of leukocyte infiltration and vascular congestion; (2) the erosion and anabrosis of epidermal cells; and (3) affection of the other side of the ears. The histological scores were assessed from 1 to 4. Final data are the average scores of each animal in the same group, and the higher score means more serious inflammation.

2.11. Lactic acid release detection assay

Purified T cells from spleen cells of C57BL/6 mice were treated with or without herpetol for 24 h in the presence of 5 $\mu\text{g}/\text{ml}$ Con A, and then

the supernatant was harvested for lactic acid detection using the Nanjing Jiancheng lactic acid assay kit.

2.12. LDH activity detection assay

The activity of LDH was detected by LDH assay kit. Cells were cultured in a 96-well plate and treated with or without herpetol for 24 h in the presence of 5 µg/ml Con A. After 24 h of incubation, LDH in cell lysate was tested by using the Nanjing Jiancheng LDH detection kit.

2.13. Statistical analysis

All results shown represent means $\pm$ SEM from triplicate experiments performed in a parallel manner. Data were statistically evaluated by one-way ANOVA followed by Dunnett's test between control group and multiple dose groups. The level of significance was set at a *P* value of 0.05.

3. Results

3.1. Herpetol inhibits T-cell proliferation and pro-inflammatory cytokine production in vitro

The structure of herpetol was shown in Fig. 1A. As compared with the control group, T-cell proliferation induced by Con A or anti-CD3/anti-CD28 (Fig. 1B) was significantly inhibited by 5–20 µM herpetol in a concentration-dependent manner. These results suggested that herpetol exerted an inhibitory effect on proliferation of activated T cells. Compared to the cell toxicity induced by herpetol on activated T-cell, herpetol showed a much fewer inhibition of proliferation in naïve T cells (Fig. 1C). Additionally, herpetol nearly had the same inhibitory effects on activated CD4⁺ T- and CD8⁺ T-cells but barely showed inhibition on naïve macrophage, CD4⁺ T-, CD8⁺ T- and B-cells (Fig. 1D and E). On the other hand, herpetol at 10 µM also showed inhibitory effects on LPS-stimulated B cells and macrophage (Fig. 1E). It should be noted that we only studied the effects of herpetol on T cells in this

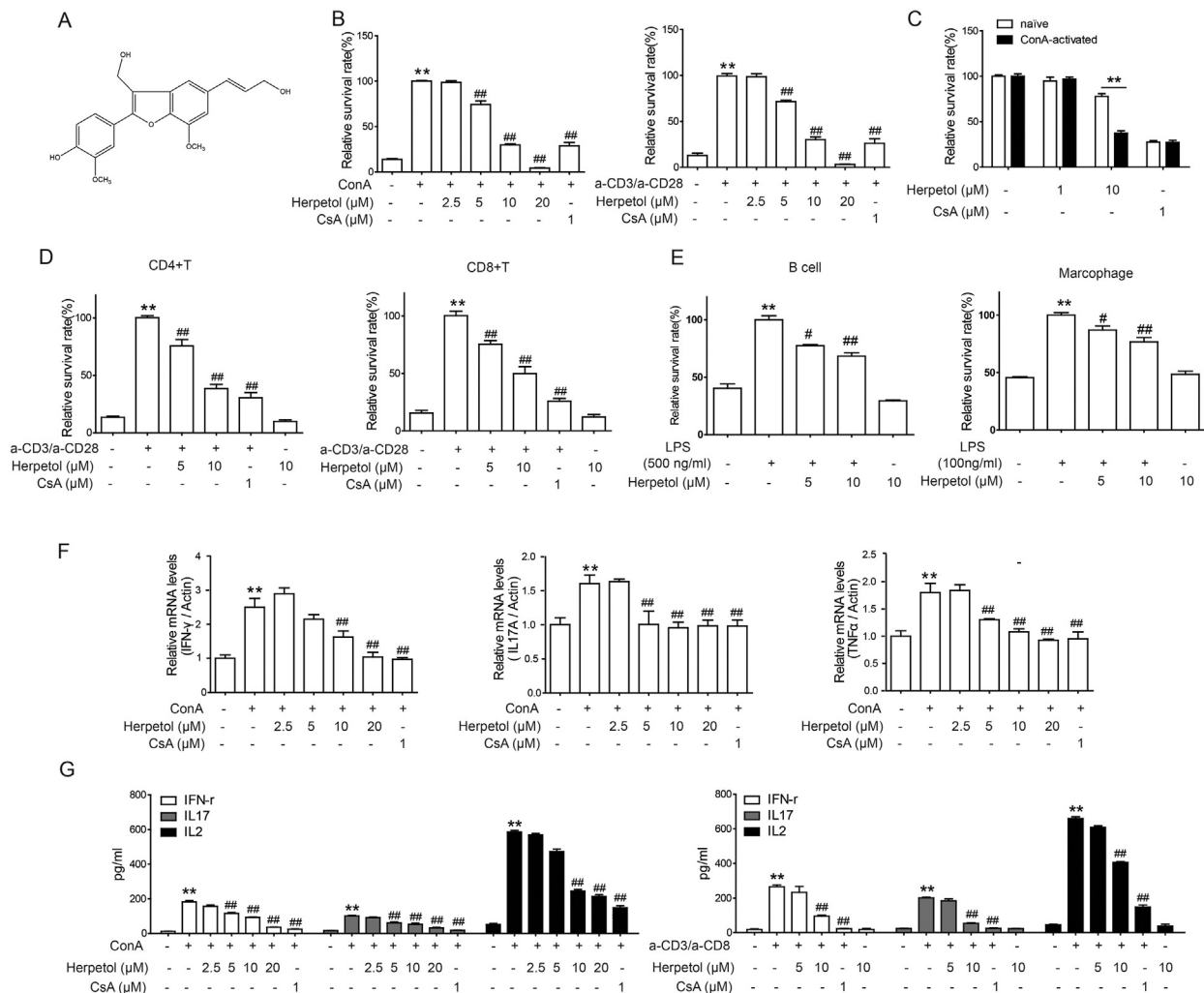


Fig. 1. Herpetol inhibited proliferation and pro-inflammatory cytokine production of activated T cells in vitro. (A) The chemical structure of herpetol. (B) Purified T cells isolated from female C57BL/6 mice were treated with 2.5, 5, 10 and 20 µM herpetol or 1 µM CsA in the presence of 5 µg/ml Con A or 2 µg/ml anti-CD3 plus 1 µg/ml anti-CD28 for 48 h. Cell proliferation was assessed by MTT assay. (C) Naïve T cells were treated with 1 and 10 µM herpetol or 1 µM CsA in the presence or absence of 5 µg/ml Con A for 48 h. Cell proliferation was assessed by MTT assay. (D) CD4⁺ T- and CD8⁺ T-cells were sorted from spleen T cells, and they were treated with 5 and 10 µM herpetol or 1 µM CsA in the presence or absence of 2 µg/ml anti-CD3 plus 1 µg/ml anti-CD28 for 48 h. Cell proliferation was assessed by MTT assay. (E) B cells isolated from spleen and RAW264.7 cells were treated with 5 and 10 µM herpetol or 1 µM CsA in the presence or absence of LPS for 48 h. Cell proliferation was assessed by MTT assay. (F) Purified T cells were stimulated with 5 µg/ml Con A for 48 h in the presence or absence of herpetol (2.5, 5, 10 and 20 µM), and the mRNA levels of pro-inflammatory cytokines in cells were measured by real-time PCR. (G) Purified T cells were stimulated with 5 µg/ml Con A or 2 µg/ml anti-CD3 plus 1 µg/ml anti-CD28 for 48 h in the presence or absence of herpetol, and the production of pro-inflammatory cytokines in cell culture supernatant was determined using ELISA kits. All data represent the means $\pm$ SEM of three independent experiments performed in triplicate. **P* < 0.05, ***P* < 0.01 versus vehicle group, ##*P* < 0.05, ###*P* < 0.01 versus Con A group or anti-CD3/anti-CD28 group.

work while the effects and mechanisms of herpetol on B cells and macrophage will be studied in other works. In order to clearly distinguish the inhibition rate of herpetol among activated T cells, naïve T cells and other cells, we used the relative survival rate to show our results.

To examine the effect of herpetol on the production of pro-inflammatory cytokines including IFN- γ , IL-17, IL-2 and TNF- α , mRNA expression in activated T cells was detected by real-time PCR, and cytokine production in culture supernatant was performed with ELISA kits. The results showed that mouse T cells stimulated by Con A or anti-CD3/anti-CD28 exhibited considerable production of these cytokines, whereas after being treated by 5–20 μ M herpetol, these pro-inflammatory cytokines in activated T cells were significantly and dose-dependently reduced by herpetol (Fig. 1F and G).

3.2. Herpetol improves contact hypersensitivity in mice

In our pre-experiment, the drug concentration in serum of mice was tested by a high performance liquid chromatography assay, and we found that the concentration reached a peak at about 6.8 μ M after i.p. administered 20 mg/kg of herpetol, which just fell within the range of 5–20 μ M as that used in vitro. Therefore, the doses of herpetol selected for in vivo experiments were 20 and 40 mg/kg. In order to create contact hypersensitivity, mice were sensitized with PCI on day 0 and challenged with the PCI on day 5. Histopathological analysis demonstrated that the ear sections of vehicle-treated model group still showed severe inflammatory cell infiltration, obvious vascular congestion and moderate edema in dermis and subcutaneous tissue. Compared with the vehicle-treated model group, these pathological reactions were much mitigated by herpetol treatment (Fig. 2A and C). In addition, ear swelling also was ameliorated by administration of herpetol to mice with contact hypersensitivity (Fig. 2B).

As shown in Fig. 3A, ear swelling and vasodilation were clearly observed in PCI-challenged ears, while these pathological reactions caused by contact hypersensitivity were relieved by herpetol in a dose-dependent manner. Mast cell infiltration also correlates with the

severity of contact hypersensitivity. Administration of herpetol to mice could reduce the number of mast cell in PCI-challenged ear tissue (Fig. 3B and C).

3.3. Herpetol causes cell cycle arrest in activated T cells

Furthermore, the mechanisms of herpetol to improve contact hypersensitivity were further investigated in this study. The cell cycle related proteins cyclin D1, CDK inhibitor p27^{kip} and p-Rb play important roles in cell cycle process. As shown in Fig. 4A and B, the increased expressions of cyclin D1 and p-Rb were suppressed by herpetol in a dose-dependent manner. Meanwhile, herpetol at 2.5–20 μ M elevated the protein level of p27^{kip}. Additionally, herpetol at 5–10 μ M also exhibited similar effects on the expression of these proteins in anti-CD3/anti-CD28-activated T cells (Fig. 4C). Con A-activated T cells enter into their cell cycle with 25.01% of them at S and G₂/M phases, whereas they showed cell cycle arrest in G₀/G₁ phase after being treated by 2.5–20 μ M herpetol, with 23.28%, 23.64%, 18.97% and 16.38% of them at the S and G₂/M phases, respectively (Fig. 4D & E). Additionally, the results of flow cytometry also showed that 16.78% of naïve T cells and 23.56% of anti-CD3/anti-CD28-activated T cells enter into their cell cycle, while herpetol at 5 and 10 μ M exhibited similar cell cycle arrest effects on anti-CD3/anti-CD28-activated T cells, with 24.62% and 19.73% of them at the S and G₂/M phases, respectively (Fig. 4F). Our results consistently indicated that herpetol caused cell cycle arrest in activated T cells.

3.4. Herpetol down-regulates glycolysis in activated T cells

T-cell activation was thought to drive increased glycolysis [16] due to the increased expression and surface localization of Glut1 [6] and the enhanced activities of key glycolytic enzymes [5,17]. Therefore, the glycolysis-related proteins and enzymes were detected in this study. As shown in Fig. 5A, the increased mRNA levels of HIF-1 α , Glut1, LDHA and HKII in Con A-activated T cells were significantly down-

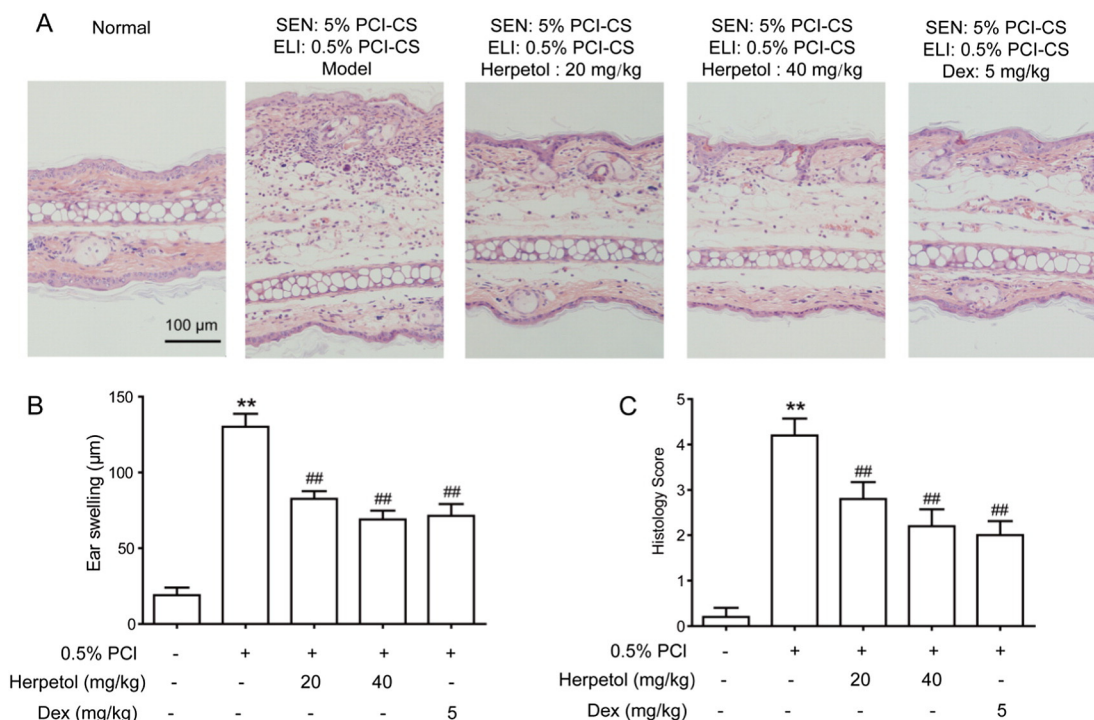


Fig. 2. Effects of herpetol on the microscopic and macroscopic appearances of mice ears with contact hypersensitivity. Samples were taken as described in the Materials and methods section. (A) Hematoxylin and eosin staining of ear sections (original magnification 200 $\times$). (B) The detection of ear swelling. (C) Histopathological scores. The data indicated the means $\pm$ SEM of experimental animals ($n = 8$), and these experiments were repeated three times. One-way ANOVA revealed a significant difference at * $P < 0.05$, ** $P < 0.01$ versus normal group, # $P < 0.05$, ### $P < 0.01$ versus model group.

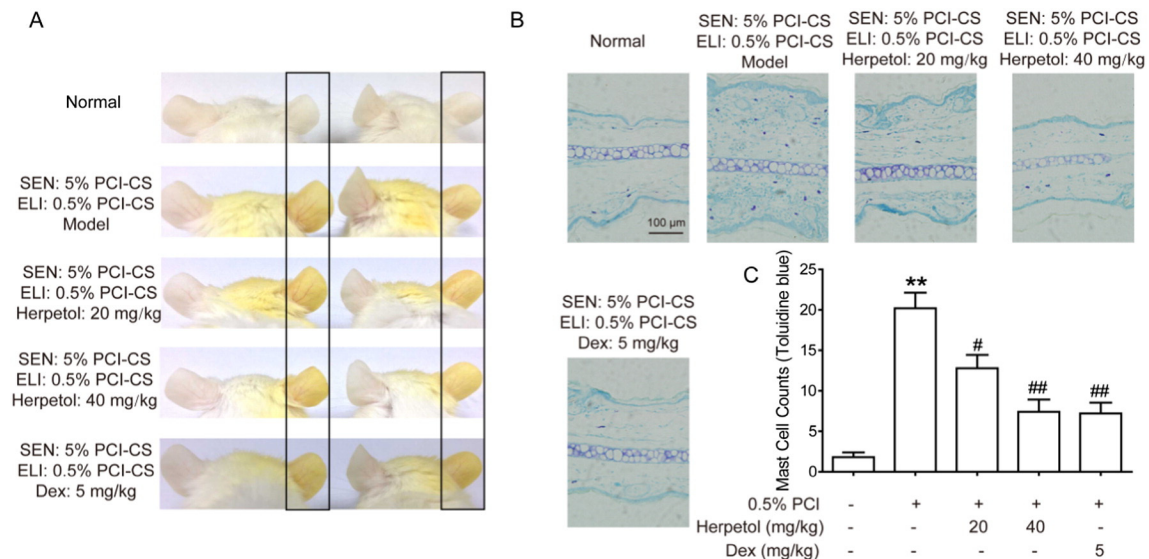


Fig. 3. Lesions and symptoms of mice with contact hypersensitivity and the mast cells staining in mice ear tissue. (A) After being anesthetized, photos of mice ears were taken by a high resolution camera. (B) Mast cells in ear tissue were stained by toluidine blue assay. (C) Quantification of the number of positive mast cells in each group, the data indicated the means $\pm$ SEM of experimental group ($n = 8$). These experiments were repeated three times and the data were presented as means $\pm$ SEM. One-way ANOVA revealed a significant difference at * $P < 0.05$, ** $P < 0.01$ versus normal group, # $P < 0.05$, ## $P < 0.01$ versus model group.

regulated by 5–20 μ M herpetol in a dose-dependent manner. Consistent with the changes of mRNA levels, herpetol also exhibited an inhibitory effect on the protein expression of HIF-1 α , Glut1 and LDHA in Con A-activated T cells (Fig. 5B). Moreover, the activity of glycolysis-related enzymes was evaluated in our study. LDHA activity in Con A-activated T cells was dose-dependently inhibited by herpetol incubation (Fig. 5C). Furthermore, lactic acid release from Con A-activated T cells also was reduced by herpetol (Fig. 5C). Additionally, the increased expression of HIF-1 α , Glut1 and LDHA and the enhanced LDHA activity in anti-CD3/anti-CD28-activated T cells also were decreased by herpetol treatment, but herpetol had no inhibitory regulations of glycolytic metabolism in naïve T cells (Fig. 5D & E).

4. Discussion

Several different classes of drugs, such as corticosteroids, NSAIDs and biologics, are used to treat inflammatory disorders [18]. These drugs can cause unwanted adverse effects while the biologics usually are too expensive for patients. The traditional medicines and natural products store huge number of alternative compounds with anti-inflammatory effect, offering a great hope for us to explore the leading compounds or potential drugs to treat inflammatory diseases. Fortunately, it has made great progress in discovery and development of immunosuppressant from Chinese herbal medicine in recent years. For example, triptolide, a diterpenoid epoxide found in the *Tripterygium wilfordii*, had been proven to have immunosuppressive activities for autoimmune diseases through inhibition of dendritic cell migration [19]. In the present study, herpetol was proved to be a potential candidate for the treatment of inflammatory diseases due to its inhibitory effects on activated T cells through regulation of multiple aspects including suppression of cell proliferation, cell cycle arrest and inhibition of glycolysis. As far as we know, it is the first time that the anti-inflammatory effect of herpetol on activated T cells was examined by our study.

Contact hypersensitivity (CHS) is a universally used animal model for studying T cell-mediated allergic contact dermatitis [20–22]. CHS is a delayed-type hypersensitivity response in the skin elicited by previously sensitized hapten such as some low molecular weight chemicals [23]. It is believed that the proliferation and activation of T-lymphocytes contributed to this acute cutaneous inflammatory reaction [24–26]. In our study, we found that herpetol dose-dependently

inhibited the proliferative response of Con A or anti-CD3/anti-CD28-activated mouse lymph cells (Fig. 1B), and herpetol showed a very similar inhibitory effect on the proliferation of CD4⁺ T- and CD8⁺ T-cells. However, herpetol at 10 μ M had a fewer inhibitory effect on the viability of naïve T-, B- and RAW264.7 cells (Fig. 1C and E). Meanwhile, the therapeutic effect of herpetol on PCI-induced contact hypersensitivity was also examined in this study. Our results revealed that the PCI-induced ear swelling and pathological responses were remarkably ameliorated by herpetol (Figs. 2 & 3). Notably, the concentration of herpetol used in vivo and in vitro experiments was processed according to its serum concentration in mice. It should be noted that the inflammatory responses modulated by T-cells mainly play a pivotal role in CHS [23]. Therefore, although herpetol could inhibit the proliferation of LPS-stimulated B cells and macrophage we only studied the inhibitory effect and mechanism of herpetol on T cells in this research.

IL-2 and IFN- γ produced from type-1 T cells exert crucial roles in PCI-induced contact hypersensitivity [27,28]. IL-2 is considered to promote the activation and proliferation responses of T cells, while IFN- γ secreted from activated T lymphocytes is able to stimulate and enhance the inflammatory reaction of macrophages [29]. In addition, TNF- α and IL-17A are also involved in CHS responses [30–32]. Our data indicated that herpetol could reduce the production of IFN- γ , IL-17A, TNF- α and IL-2 (Fig. 1F & G).

To further explore the underlying mechanisms of herpetol on cell cycle arrest, we examined the effects of herpetol on the expression of cell cycle-related proteins including p-Rb, cyclin D1 and p27^{kip} in activated T cells. Overexpression of cyclin D1 was found to shorten the G₁-phase of cell cycle in various cell types [33]. The phosphorylated Rb played a pivotal role in helping S-phase entry and cell growth [34], while p27^{kip} can inhibit cell cycle progression from G₁ to S phase transition [35]. In our present study, herpetol exerted similar effects on these cell cycle-related proteins both in Con A-activated T cells and in anti-CD3/anti-CD28-activated T cells. Herpetol suppressed the expression of p-Rb and cyclin D1, but increased the expression of p27^{kip} (Fig. 4A, B & C). Additionally, we also found that herpetol could inhibit T-cell cycle progression and cause G₀/G₁ phase arrest in Con A-activated and anti-CD3/anti-CD28-activated T cells (Fig. 4D, E & F). These results indicated that its inhibitory effects on activated T cells may be associated with a potent regulation of cell cycle. Because apoptosis is an important mechanism to reduce cell proliferation, we also investigated the pro-apoptotic effect of herpetol in activated T cells. However, it was found

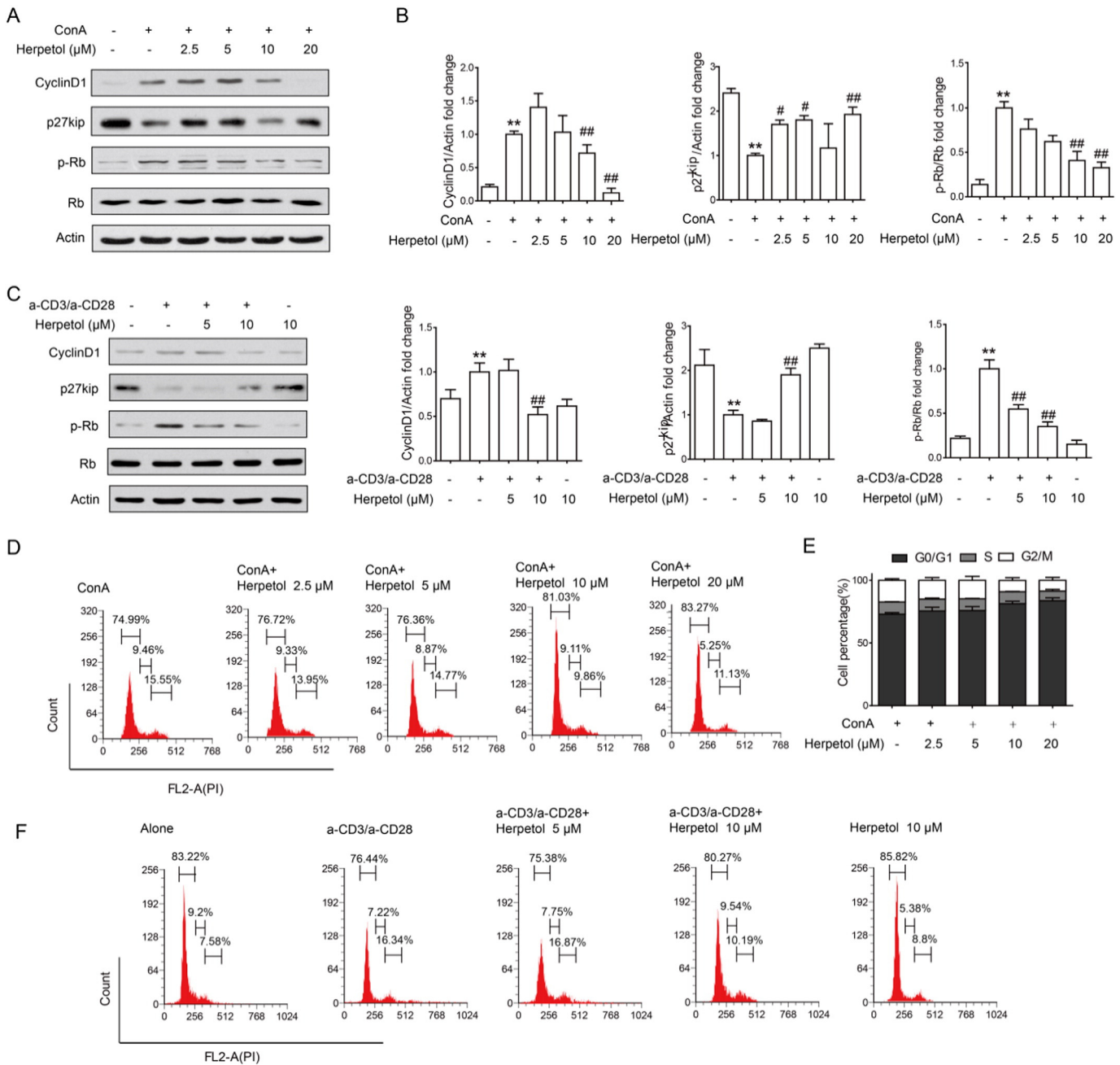


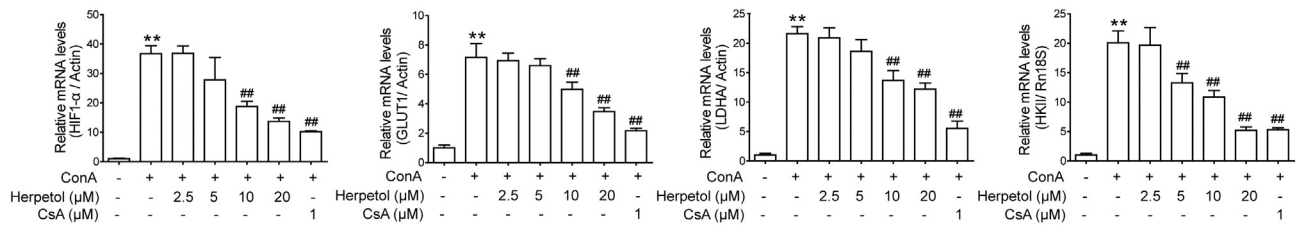
Fig. 4. Herpetol caused cell cycle arrest in activated T cells. (A) Purified T cells isolated from female C57BL/6 mice were stimulated with 5 μg/ml Con A with or without 2.5–20 μM herpetol for 48 h. Cells were harvested and lysed, and then the expression of cell cycle-related proteins was analyzed by Western blotting. (B) All data were presented as means ± SEM of three independent experiments performed in triplicate. (C) T cells were stimulated with or without 2 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 with or without 5 and 10 μM herpetol for 48 h. Cells were harvested and lysed, and then the expression of cell cycle-related proteins was analyzed by Western blotting. The quantity data were presented as means ± SEM of three independent experiments performed in triplicate. (D) Purified T cells were stimulated with 5 μg/ml Con A for 48 h in the presence or absence of 2.5, 5, 10 and 20 μM herpetol, and then they were stained by PI which was used for cycle distribution analysis by flow cytometry. (E) The data represented here are one of three independent experiments with similar results. (F) T cells were treated with or without 5 and 10 μM herpetol in the presence or absence of 2 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 for 48 h, and then they were stained by PI which was used for cycle distribution analysis by flow cytometry. The data represented here are one of three independent experiments with similar results. One-way ANOVA revealed a significant difference at * $P < 0.05$, ** $P < 0.01$ versus normal group, # $P < 0.05$, ## $P < 0.01$ versus Con A group or anti-CD3/anti-CD28 group.

that herpetol could not induce apoptosis in activated T cells (data not show). Therefore, there was no herpetol-induced apoptotic cell population in Fig. 4A and E.

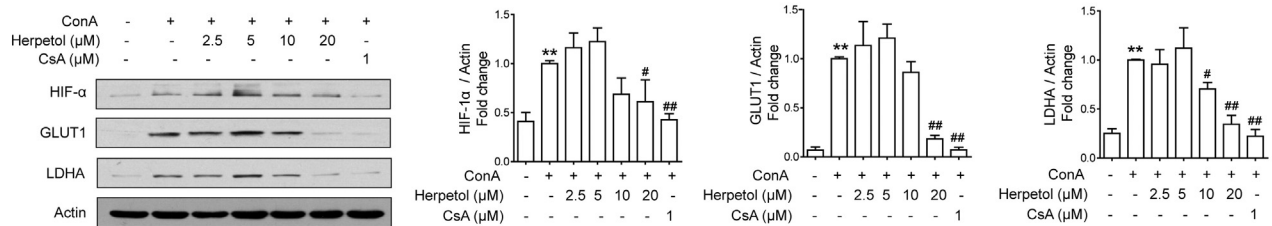
It is well known that activation-induced glucose uptake, glycolysis and lipid synthesis support effector T cell growth and proliferation [36]. Overexpression of glucose transporter Glut1 can increase the glucose uptake and glycolysis as well, thus T-cell-specific transgenic expression of Glut1 leads to increased T cell proliferation, survival and cytokine production [37]. HIF-1α is a key transcription factor orchestrating the expression of glycolytic enzymes [38], and HIF-1α can also

regulate the expression and activity of LDHA [39]. We found that herpetol could decrease the elevated expression of Glut1, HIF-1α and LDHA in activated T cells. In addition, both lactic acid release and LDHA activity in activated T cells were reduced by herpetol incubation. All of these results indicated that herpetol could down-regulate the expression of glycolysis-related proteins. Previous study has shown that the metabolism of activated lymphocyte is a promising target for selective immunomodulation [4]. Augmenting lymphocyte glycolysis might aid in Treg generation and improve immunization responses but also promote autoimmunity and inflammation, whereas inhibiting

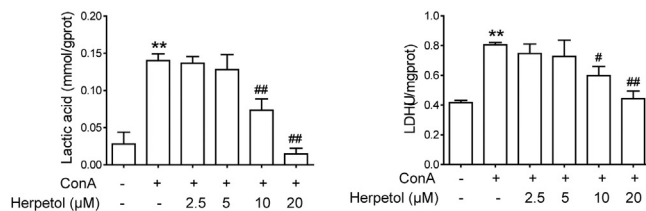
A



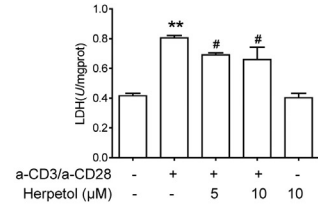
B



C



E



D

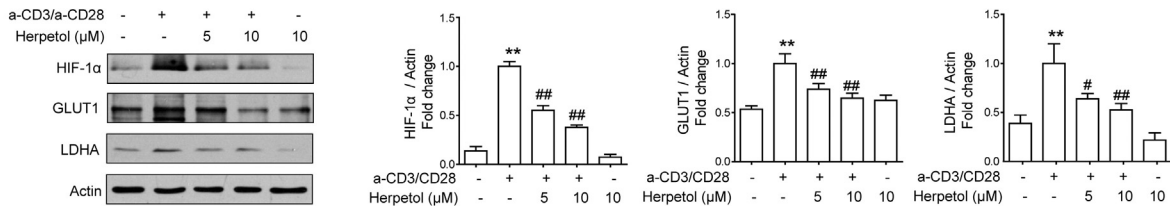


Fig. 5. Herpetol inhibited the glycolysis in activated T-cells. (A) Purified T cells isolated from female C57BL/6 mice were treated with 2.5, 5, 10 and 20 μ M herpetol or 1 μ M CsA in the presence or absence of 5 μ g/ml Con A, and the mRNA levels of glycolysis-related genes were measured by real-time PCR. (B) The expression of glycolysis-related proteins was detected by Western blotting. (C) Purified T cells were stimulated with 5 μ g/ml Con A with or without 2.5–20 μ M herpetol for 48 h. Cell culture supernatant was collected to measure the content of lactic acid, and cells were harvested and lysed to measure the content of LDH. (D) Spleen T cells were treated with or without 5 and 10 μ M herpetol in the presence or absence of 2 μ g/ml anti-CD3 plus 1 μ g/ml anti-CD28, and the expression of glycolysis-related proteins was detected by Western blotting. (E) T cells were treated with or without 5 and 10 μ M herpetol in the presence or absence of 2 μ g/ml anti-CD3 plus 1 μ g/ml anti-CD28, and they were harvested and lysed to measure the content of LDH. All of these data above were presented as means $\pm$ SEM of three independent experiments performed in triplicate. One-way ANOVA revealed a significant difference at * P < 0.05, ** P < 0.01 versus normal group, # P < 0.05, ## P < 0.01 versus Con A group or a-CD3/a-CD28 group.

glycolysis in T cells could ameliorate autoimmune and inflammatory diseases [5]. Therefore, the down-regulation of glycolysis may be an inhibitory mechanism of herpetol to reduce the proliferation of activated T cells. Furthermore, no side effects were observed after herpetol administration at these dosage levels used in this study in either model or naïve animals.

In conclusion, this study identified herpetol as a safe and therapeutic agent against contact hypersensitivity. These data collectively suggest that herpetol may be a potential candidate for T-cell-mediated skin diseases.

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

This work was supported by National Natural Science Foundation of China (nos. 91313303, 81373466, 81422050, 31200256), Natural

Science Foundation of Jiangsu Province (BK20151394) and the visiting scholars' project under KF-GN-201404 in the State Key Laboratory of Pharmaceutical Biotechnology, Nanjing Univ., China.

References

- [1] H. Hackstein, A.W. Thomson, Dendritic cells: emerging pharmacological targets of immunosuppressive drugs, *Nat. Rev. Immunol.* 4 (2004) 24–34.
- [2] T.E. Starzl, R.M. Zinkernagel, Transplantation tolerance from a historical perspective, *Nat. Rev. Immunol.* 1 (2001) 233–239.
- [3] A. Bogaczewicz, T. Sobow, J. Bogaczewicz, A. Wozniacka, Acute onset of manic episode induced by dexamethasone in a patient with atopic dermatitis, *Dermatitis* 25 (2014) 103–104.
- [4] M. Galgani, V. De Rosa, G. Matarese, T cell metabolism and susceptibility to autoimmune diseases, *Mol. Immunol.* 68 (2015) 558–563.
- [5] D.R. Wahl, C.A. Byersdorfer, J.L. Ferrara, A.W. Opipari Jr., G.D. Glick, Distinct metabolic programs in activated T cells: opportunities for selective immunomodulation, *Immunol. Rev.* 249 (2012) 104–115.
- [6] K.A. Frauwirth, J.L. Riley, M.H. Harris, R.V. Parry, J.C. Rathmell, D.R. Plas, R.L. Elstrom, C.H. June, C.B. Thompson, The CD28 signaling pathway regulates glucose metabolism, *Immunity* 16 (2002) 769–777.
- [7] A.V. Belikov, B. Schraven, L. Simeoni, T cells and reactive oxygen species, *J. Biomed. Sci.* 22 (2015) 85.

- [8] S. Zundler, M.F. Neurath, Immunopathogenesis of inflammatory bowel diseases: functional role of T cells and T cell homing, *Clin. Exp. Rheumatol.* 33 (2015) S19–S28.
- [9] R. Wang, D.R. Green, Metabolic checkpoints in activated T cells, *Nat. Immunol.* 13 (2012) 907–915.
- [10] Mourad Kaoudji, J. Favre-Bonvin, Herpetol, a new dimeric lignoid from *Herpetospermum caudigerum* wall, *Z. Naturforsch.* 39c (1984) 307–308.
- [11] H.-F. Fan, Y.-M. Ren, X.-L. Wu, Q.-A. Wang, Synthesis and cytotoxicity of novel benzofuran neolignan derivatives, *J. Chem. Res.* 34 (2010) 233–235.
- [12] S.-J. In, K.-H. Seo, D.-S. Lee, Y.-C. Kim, N.-Y. Song, N.-I. Baek, Lignans and neolignans from the stems of *Viburnum erosum* and their neuroprotective and anti-inflammatory activity, *Arch. Pharm. Res.* 38 (2015) 26–34.
- [13] X. Wang, X. Wu, A. Zhang, S. Wang, C. Hu, W. Chen, Y. Shen, R. Tan, Y. Sun, Q. Xu, Targeting the PDGF-B/PDGFR- β interface with destruxin A5 to selectively block PDGF-BB/PDGFR- $\beta\beta$ signaling and attenuate liver fibrosis, *EBioMedicine* 7 (2016) 146–156.
- [14] X. Wang, S. Wang, C. Hu, W. Chen, Y. Shen, X. Wu, Y. Sun, Q. Xu, A new pharmacological effect of levornidazole: inhibition of NLRP3 inflammasome activation, *Biochem. Pharmacol.* 97 (2015) 178–188.
- [15] T. Mori, K. Kabashima, R. Yoshiki, K. Sugita, N. Shiraishi, A. Onoue, E. Kuroda, M. Kobayashi, U. Yamashita, Y. Tokura, 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 (2008) 1719–1727.
- [16] C.J. Fox, P.S. Hammerman, C.B. Thompson, Fuel feeds function: energy metabolism and the T-cell response, *Nat. Rev. Immunol.* 5 (2005) 844–852.
- [17] K.A. Frauwirth, C.B. Thompson, Regulation of T lymphocyte metabolism, *J. Immunol.* 172 (2004) 4661–4665.
- [18] R. Gautam, S.M. Jachak, Recent developments in anti-inflammatory natural products, *Med. Res. Rev.* 29 (2009) 767–820.
- [19] X. Li, Y. Han, E. Sun, Sniping the scout: targeting the key molecules in dendritic cell functions for treatment of autoimmune diseases, *Pharmacol. Res.* 107 (2016) 27–41.
- [20] L.D. Abeynaike, J.A. Deane, C.L. Westhorpe, Z. Chow, M.A. Alikhan, A.R. Kitching, A. Issekutz, M.J. Hickey, Regulatory T cells dynamically regulate selectin ligand function during multiple challenge contact hypersensitivity, *J. Immunol.* 193 (2014) 4934–4944.
- [21] X. Wang, A. Zhang, J. Gao, W. Chen, S. Wang, X. Wu, Y. Shen, Y. Ke, Z. Hua, R. Tan, Y. Sun, Q. Xu, Trichomide A, a natural cyclodepsipeptide, exerts immunosuppressive activity against activated T lymphocytes by upregulating SHP2 activation to overcome contact dermatitis, *J. Invest. Dermatol.* 134 (2014) 2737–2746.
- [22] X. Wang, C. Hu, X. Wu, S. Wang, A. Zhang, W. Chen, Y. Shen, R. Tan, X. Wu, Y. Sun, Q. Xu, Roseotoxin B improves allergic contact dermatitis through a unique anti-inflammatory mechanism involving excessive activation of autophagy in activated T lymphocytes, *J. Invest. Dermatol.* 136 (2016) 1636–1646.
- [23] I.C. Allen, Contact hypersensitivity models in mice, *Methods Mol. Biol.* 1032 (2013) 139–144.
- [24] R. Cornely, A.H. Pollock, C. Rentero, S.E. Norris, A. Alvarez-Guaita, T. Grewal, T. Mitchell, C. Enrich, S.E. Moss, R.G. Parton, J. Rossy, K. Gaus, Annexin A6 regulates interleukin-2-mediated T-cell proliferation, *Immunol. Cell Biol.* (2016), <http://dx.doi.org/10.1038/icb.2016.15>.
- [25] F. Petta, C. De Luca, M. Triggiani, V. Casolaro, Fragments of truth: T-cell targets of polyclonal immunoglobulins in autoimmune diseases, *Curr. Opin. Pharmacol.* 17 (2014) 1–11.
- [26] H. Nivarthi, M. Prchal-Murphy, A. Swoboda, M. Hager, M. Schleder, L. Kenner, J. Tuckermann, V. Sexl, R. Moriggl, O. Ermakova, Stat5 gene dosage in T cells modulates CD8 + T-cell homeostasis and attenuates contact hypersensitivity response in mice, *Allergy* 70 (2015) 67–79.
- [27] Y. Yin, Y. Sun, L. Gu, W. Zheng, F. Gong, X. Wu, Y. Shen, Q. Xu, Jaceosidin inhibits contact hypersensitivity in mice via down-regulating IFN- γ /STAT1/T-bet signaling in T cells, *Eur. J. Pharmacol.* 651 (2011) 205–211.
- [28] F. Fang, Y. Tang, Z. Gao, Q. Xu, A novel regulatory mechanism of naringenin through inhibition of T lymphocyte function in contact hypersensitivity suppression, *Biochem. Biophys. Res. Commun.* 397 (2010) 163–169.
- [29] S. Pastore, A. Cavani, G. Girolomoni, Epidermal cytokine and neuronal peptide modulation of contact hypersensitivity reactions, *Immunopharmacology* 31 (1996) 117–130.
- [30] Q. Jia, D. Zang, J. Yi, H. Dong, Y. Niu, Q. Zhai, Y. Teng, P. Bin, W. Zhou, X. Huang, H. Li, Y. Zheng, Y. Dai, Cytokine expression in trichloroethylene-induced hypersensitivity dermatitis: an in vivo and in vitro study, *Toxicol. Lett.* 215 (2012) 31–39.
- [31] M. Peiser, Role of Th17 cells in skin inflammation of allergic contact dermatitis, *Clin. Dev. Immunol.* 2013 (2013) 261037.
- [32] Z. Gao, Y. Ma, D. Zhao, X. Zhang, H. Zhou, H. Liu, Y. Zhou, X. Wu, Y. Shen, Y. Sun, J. Li, X. Wu, Q. Xu, Neochromine S5 improves contact hypersensitivity through a selective effect on activated T lymphocytes, *Biochem. Pharmacol.* 92 (2014) 358–368.
- [33] R. Tapia, M. Huerta, S. Islas, A. Avila-Flores, E. Lopez-Bayghen, J. Weiske, O. Huber, L. Gonzalez-Mariscal, Zona occludens-2 inhibits cyclin D1 expression and cell proliferation and exhibits changes in localization along the cell cycle, *Mol. Biol. Cell* 20 (2009) 1102–1117.
- [34] A. Kawatsuki, J.I. Yasunaga, Y. Mitobe, P.L. Green, M. Matsuoka, HTLV-1 bZIP factor protein targets the Rb/E2F-1 pathway to promote proliferation and apoptosis of primary CD4 T cells, *Oncogene* (2016), <http://dx.doi.org/10.1038/onc.2015.510>.
- [35] J. Iacovelli, J. Lopera, M. Bott, E. Baldwin, A. Khaled, N. Uddin, C. Fernandez-Valle, Serum and forskolin cooperate to promote G1 progression in Schwann cells by differentially regulating cyclin D1, cyclin E1, and p27Kip expression, *Glia* 55 (2007) 1638–1647.
- [36] V.A. Gerriets, J.C. Rathmell, Metabolic pathways in T cell fate and function, *Trends Immunol.* 33 (2012) 168–173.
- [37] R.D. Michalek, V.A. Gerriets, S.R. Jacobs, A.N. Macintyre, N.J. MacIver, E.F. Mason, S.A. Sullivan, A.G. Nichols, J.C. Rathmell, Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4 + T cell subsets, *J. Immunol.* 186 (2011) 3299–3303.
- [38] L.Z. Shi, R. Wang, G. Huang, P. Vogel, G. Neale, D.R. Green, H. Chi, HIF1 α -dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells, *J. Exp. Med.* 208 (2011) 1367–1376.
- [39] C.J. Valvona, H.L. Fillmore, P.B. Nunn, G.J. Pilkington, The regulation and function of lactate dehydrogenase A: therapeutic potential in brain tumor, *Brain Pathol.* 26 (2016) 3–17.