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Herpetol ameliorates allergic contact dermatitis through regulating T-lymphocytes

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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 μM. 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-1α, 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.

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-1α is a metabolic checkpoint in activated T cells, and it plays an important role in T-cell activation [9]. Therefore, HIF-1α 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 (PCl)-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.

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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). PCl, 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 μg/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 ΔΔCT 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′-CTTCTTCAGCAACAGCAAGGCGAAAA-3′, reverse 5′-CCCCCAGATACAACCCCGCAATCA-3′; TNF-α (175 bp), forward 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, reverse 5′-TGGGAGTAGACAAGG TACAACCC-3′; IL-17A (71 bp), forward 5′-TCGAGAAGATGCTGGTGG GT-3′, reverse 5′-CTCTGTTTAGGCTGCCTGG C-3′; HIF-1α (228 bp), forward 5′-ACC TTCATCGGAAACTCCAAAG-3′, reverse 5′-CTGTTAGGCTGG GAAAAGTTAG G-3′; Glut1(156 bp), forward 5′-CAGTTCGGCTATAACA CTGGTG-3′, reverse 5′-GCCCCCGACAGAGAAGATG-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 μg/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 μg/ml of PI containing 100 μg/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. PCl-induced contact hypersensitivity

PCl-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% PCl in absolute ethanol. Five days after sensitization (day 5), these mice were challenged on their left ears with 30 μL of 1% PCl 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 PCl. 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 μg/ml Con A, and then