

Accepted Article Preview: Published ahead of advance online publication



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Cite this article as: Xingqi Wang, Aihua Zhang, Jian Gao, Wei Chen, Shiyu Wang, Xuefeng Wu, Yan Shen, Yuehai Ke, Zichun Hua, Renxiang Tan, Yang Sun, Qiang Xu, Trichomides A, a Natural Cyclodepsipeptide, Exerts Immunosuppressive Activity Against Activated T Lymphocytes by Upregulating SHP2 Activation to Overcome Contact Dermatitis, *Journal of Investigative Dermatology* accepted article preview 16 June 2014; doi: [10.1038/jid.2014.252](https://doi.org/10.1038/jid.2014.252).

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Received 28 September 2013; accepted 25 April 2014; Accepted article preview online 16 June 2014

Trichomides A, a natural cyclodepsipeptide, exerts immunosuppressive activity against activated T lymphocytes by upregulating SHP2 activation to overcome contact dermatitis

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Running title: Trichomides A ameliorates dermatitis via activating SHP2

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Abstract

Increasing numbers of people are suffering from allergic contact dermatitis. However, the immunosuppressive drug candidate with negligible toxicity is still deficient. In the present study, we identified a natural cyclodepsipeptide named trichomides A that effectively inhibited the proliferation of activated T cells and reduced the production of proinflammatory cytokines but had almost no toxic effect on naive T cells at 0.3-3 μ M. In addition, trichomides A caused G0/G1 phase arrest, suppressed the activation of AKT and STAT3, and increased the level of phosphorylated SHP2 in activated T cells in dose- and time-dependent manners. Furthermore, an *in vivo* experiment demonstrated that trichomides A significantly ameliorated picryl chloride-induced contact hypersensitivity in mice. Such effects of trichomides A in the aforementioned experiments were significantly reversed by the inhibition of SHP2 activity using the SHP2-specific inhibitor PHPS1 or conditional SHP2 knockout mice in T cells, suggesting the SHP2-dependent action of trichomides A. Taken together, trichomides A showed an immunosuppressive activity against T cell-mediated immune responses both *in vitro* and *in vivo*, which is potential for the treatment of immune-related skin diseases.

Key words: cyclodepsipeptide; SHP2; contact dermatitis; AKT; STAT3

INTRODUCTION

It has been reported that allergic contact dermatitis is a delayed-type hypersensitivity reaction mediated by T cells (Kaplan *et al.*, 2012; Kimber *et al.*, 2012). Increasing numbers of people are suffering from this type of skin disease caused by exposure to a large variety of compounds present in our surroundings (Elliott and Das, 2010). Activation of the immune system can play a role in protecting organisms when the body is exposed to such chemicals. During immune system activation, some T cells proliferate and differentiate into effector cells that help to kill pathogens (Mueller *et al.*, 2013). However, excessive immune responses can cause damage to healthy tissue. Shutting down the unwanted activated T cells may be a strategy to limit damage to the host (Shen *et al.*, 2011). Immunosuppressants can effectively regulate the overwhelmed immune responses, but most of the currently available immunosuppressive drugs, and even cyclosporine A, often demonstrate some adverse reactions primarily due to poor selectivity (Kahan, 2003; Hackstein and Thomson, 2004; Halloran, 2004). Therefore, potent agents with negligible or acceptable toxicity will provide a better strategy for the treatment of immune-related diseases.

To avoid being infected with phytopathogenic microbes, plants resist infections through their own immune systems, which are similar to those of animals (Lee *et al.*, 2006; Hamel *et al.*, 2012). However, some metabolites produced from these phytopathogenic microbes effectively disturb the immune system of their host, which weakens the host's scavenging effects and causes pathogenesis in plants (Silverman and Reiner, 2011). Previously, we have obtained some natural compounds from a

culture of a mantis-associated fungus with an immunosuppressive activity (Zhang *et al.*, 2008; Zhang *et al.*, 2011), which led to the discovery of a class of immunosuppressants from phytopathogenic microbes. Cyclopeptides have shown antiplasmodial, antibacterial, sedative, antifungal, and immunomodulatory activities (Hwang *et al.*, 2001; Kunze *et al.*, 2008; Panseeta *et al.*, 2011; Bucci *et al.*, 2013). In this study, we examined the immunomodulatory activity and mechanism of trichomides A, a natural cyclodepsipeptide isolated from the fermentation products of *Trichothecium roseum*. Our findings herein revealed that trichomides A could ameliorate picryl chloride-induced contact hypersensitivity in mice through up-regulating SHP2 function in activated T cells.

RESULTS

Trichomides A inhibits T cell proliferation and proinflammatory cytokine production *in vitro*

The structure of trichomides A is shown in Figure 1a. As compared with the control group, T cell proliferation induced by Con A (Figure 1b) and anti-CD3/anti-CD28 (Figure 1c) was significantly inhibited by 0.3-30 μ M trichomides A in a concentration-dependent manner, respectively. The inhibitory effect of trichomides A on Con A-induced T cell proliferation was further confirmed by a CFSE assay (Figure 1e). It should be noted that trichomides A, at doses of 0.3, 1 and 3 μ M, had little inhibitory effect on naïve T cells, while 1 μ M CsA exerted a toxicity on naïve T cells (Figure 1d). Moreover, trichomides A influenced neither CD25 nor

CD69 expression in Con A-activated T cells (Supplementary Figure S1). These results suggest that trichomides A inhibited T cell proliferation without influence on naïve T cells and their activation at less than 3 μ M, which may be distinct from cyclosporine A. Thus, the concentrations less than 3 μ M of trichomides A were used in the next *in vitro* experiments.

To examine the effect of trichomides A on the production of proinflammatory cytokines, including IL-1 β , IFN- γ , IL-6, IL-17, TNF- α and IL-2, ELISA was performed to measure the level of cytokines in the culture supernatant of Con A-activated T cells. The results showed that mouse T cells stimulated by Con A exhibited considerable production and secretion of these cytokines into the culture medium. After treatment with 0.3-3 μ M trichomides A, these proinflammatory cytokines were significantly and dose-dependently reduced in Con A-activated T cells (Figure 1f).

Trichomides A causes G0/G1 phase arrest in Con A-activated T cells

As shown in Figure 2a and b, activated T cells enter into the cell cycle with 22.7% of T cells at S and G2/M phase under Con A stimulation, while the PI-stained cells showed a significant arrest in the G0/G1 phase of the cell cycle under trichomides A treatment (0.3, 1 and 3 μ M), with only approximately 13.3%, 10.1% and 6.9% of T cells at the S and G2/M phases, respectively. It has been reported that the proteins p-Rb, cyclin D1 and the CDK inhibitor p27^{kip} play major roles in the cell cycle of T cells (Luo *et al.*, 2011). We then examined the influence of trichomides A on these

proteins. The results were shown in Figure 2c and d. In consistent with the observations of cell cycle distribution, the increased expressions of p-Rb and cyclin D1 were inhibited by 0.3-3 μ M trichomides A in a concentration-dependent manner. Meanwhile, trichomides A at 1 and 3 μ M increased the protein level of p27^{kip}. These results indicated that trichomides A caused G0/G1 phase arrest in Con A-activated T cells.

Trichomides A inhibits AKT signaling and STAT3 signaling but not ERK signaling in Con A-activated T cells

To further examine the molecular mechanism underlying the action of trichomides A against T-cell-mediated immune response, the levels of p-AKT, p-ERK and p-STAT3 were explored by Western blotting. As shown in Figure 3a-d, trichomides A reduced the levels of p-AKT and p-STAT3 in dose- and time-related manners, but it did not inhibit that of p-ERK. It should be noted that trichomides A had no effect on the protein expressions of total AKT, STAT3 or ERK.

Trichomides A initiates the tyrosine phosphorylation of SHP2 in Con A-activated T cells

As shown in Figure 3e-h, trichomides A enhanced the level of p-SHP2 in a dose- and time-related manner in T cells activated by Con A. Furthermore, SHP2 phosphatase activity was increased by 3 μ M trichomides A treatment in a time-dependent manner, while the increased phosphatase activity was markedly inhibited by a SHP2-specific

inhibitor PHPS1 (Figure 3i). In addition to T lymphocytes, we also found that trichomides A could induce SHP2 activation in human monocytic THP1 cell line and rat hepatic stellate CFSC-8B cells (Supplementary Figure S2). To examine whether trichomides A directly interacts with SHP2 protein and exerts its activating effect, we performed surface plasmon resonance assay by Biacore T200, which can directly measure the binding of small molecules to target protein. As a result, the K_D value for trichomides A binding to SHP2 exceeded 1 mM, indicating that trichomides A does not directly bind to SHP2 (Supplementary Figure S3). To confirm the effect of trichomides A on SHP2 phosphatase activity, SHP2 activity in cell-free assay was determined by commercial kit. Analysis for SHP2 activity showed that trichomides A had little inhibitory effect on the phosphatase activity of SHP2, while SHP2-specific inhibitor PHPS1 significantly inhibited SHP2 phosphatase activity (Supplementary Figure S4).

The SHP2-specific inhibitor PHPS1 reverses the effect of trichomides A in Con A-activated T cells

To determine whether trichomides A suppressed the T-cell-mediated immune response via regulating SHP2, the effects of trichomides A were examined in the presence of SHP2 specific inhibitor PHPS1. As the result, the inhibitory effects of trichomides A on T cell proliferation (Figure 4a) and proinflammatory cytokine production (Figure 4c) were partially attenuated by PHPS1. The similar result on the cell proliferation was seen in the CFSE assay (Figure 4b). Furthermore, using T cells from conditional

SHP2 knockout mice, we found that inhibitory effect of trichomides A on T cell proliferation was significantly weakened (Figure 4e). In addition, G0/G1 phase arrest caused by trichomides A in Con A-activated T cells was partially blocked by PHPS1 (Supplementary Figure S5 and Figure 4d). Moreover, the regulation on the expressions of p-Rb, cyclin D1, p27^{kip}, p-AKT and p-STAT3 in T cells treated with trichomides A was almost completely reversed by PHPS1 (Figure 4f-4i). As we mentioned in previous work, the percentage of apoptotic T cells was increased by trichomides A in a concentration-dependent manner (Zhang *et al.*, 2013). In this study, the pro-apoptotic effect of trichomides A on activated T cells was also decreased by PHPS1 treatment (Supplementary Figure S6).

Trichomides A ameliorates PCI-induced contact hypersensitivity in mice

In the pre-experiment, we found that the drug concentration in the serum of mice with intraperitoneally administered 10 mg/kg of trichomides A reached a peak at about 0.9 μ M by a high performance liquid chromatography assay, which just fell within the range of the drug concentrations (0.3-3 μ M) *in vitro*. So 10 and 30 mg/kg of trichomides A were used for *in vivo* experiments to further assess the ameliorative property of trichomides A in PCI-induced contact dermatitis in BALB/c mice. Administration of trichomides A to BALB/c mice significantly inhibited the ear swelling, and the positive control, dexamethasone, also showed a strong inhibition (Figure 5). Figure 5a is a representative image of H&E staining of ear tissues from various groups of mice, which revealed that there was vascular congestion, severe

inflammatory infiltration and moderate edema in the dermis and subcutaneous tissue. The mice treated with trichomides A only showed mild vasodilatation and cellular infiltration and had minimal edema (Figure 5a, b and c). However, PHPS1, a specific SHP2 inhibitor, markedly attenuated the protective effect of trichomides A on PCl-induced contact dermatitis.

SHP2 contributes to the improvement of contact dermatitis observed in trichomides A-treated mice

In a previous study, we established a mouse model with conditional SHP2 knockout in T cells (Wu *et al.*, 2012). The efficacy of trichomides A *in vivo* was further confirmed using T cells conditional SHP2 knockout mice. The resolution of dermatitis by trichomides A observed by macroscopic and histological analysis was significantly blocked in conditional SHP2 knockout mice (Figure 6a-6c). Furthermore, the decrease in the expressions of p-AKT, p-STAT3, p-Rb and cyclin D1 in lymph node T cells treated with trichomides A was mostly reversed in conditional SHP2 knockout mice (Figure 6d and Supplementary Figure S7). Thus, the improvement of PCl-induced contact dermatitis by trichomides A depends on SHP2.

In summary, trichomides A triggered SHP2 activation in activated T cells, subsequently leading to G0/G1 phase arrest as well as the inactivation of AKT and STAT3. T cell proliferation and proinflammatory cytokine production were also inhibited, and PCl-induced contact hypersensitivity in mice was ameliorated by trichomides A (Figure 6e).

DISCUSSION

The mechanisms underlying allergic contact dermatitis have been extensively studied using contact-hypersensitivity mouse models, and these investigations have supplied many potential therapeutic strategies for this type of skin disease. Excessive activation of T lymphocytes has a strong relationship with the occurrence of allergic-contact dermatitis (Kaplan *et al.*, 2012; Kimber *et al.*, 2012). Previous investigations have found that stimulation of the T lymphocyte attenuator, a CD28 family coinhibitory receptor, with agonistic agents could suppress DNFB-induced contact hypersensitivity (Nakago *et al.*, 2013). Our recent study revealed that erlotinib, which has been approved for the treatment of several types of cancer as an inhibitor of multiple tyrosine kinases, also possessed a potential immunosuppressive effect against T lymphocytes and therefore ameliorated picryl chloride-induced contact hypersensitivity in mice (Luo *et al.*, 2011). In clinical applications, many immunosuppressive agents, including glucocorticoids and CsA, are usually used for the treatment of diseases mediated by the excessive activation of T lymphocytes (Minguillón *et al.*, 2005; Fischer *et al.*, 2013). In this study, we found that trichomides A, a natural cyclodepsipeptide, dose-dependently inhibited the proliferative response of mouse lymph node cells to Con A with an IC_{50} value of $1.81 \pm 0.22 \mu\text{M}$, which was comparable to that of CsA ($0.74 \pm 0.04 \mu\text{M}$). However, the IC_{50} ($9.33 \pm 0.24 \mu\text{M}$) of this compound for normal T cells (without Con A or anti-CD3/anti-CD28 stimulation) was much higher than that of CsA ($1.50 \pm 0.10 \mu\text{M}$) (Figure 1), suggesting its higher

selectivity.

In addition to the inhibition of multiple cytokines' production including IL-1 β , IFN- γ , IL-6, IL-17, TNF- α , and IL-2 (Figure 1f), the cyclodepsipeptide also inhibited T cell cycle progression and caused G0/G1 phase arrest (Figure 2). To explore the underlying mechanisms of the effect of trichomides A on T cell progression through the cell cycle, we further examined the effect of trichomides A on p-Rb, cyclin D1 and CDK inhibitor p27^{kip} in activated T cells. These three proteins regulate phase transitions in the cell cycle. Cyclin D1 is a G1-phase cell cycle molecule that can shorten the G1 phase when overexpressed (Tapia *et al.*, 2009). For entry into the S phase p-Rb is usually required as an important regulator (Liu and Lee, 2006), and the CDK inhibitor p27^{kip} also controls the G1 to S phase transition and inhibits cell growth (Iacovelli *et al.*, 2007). The effect of trichomides A on these proteins indicates that its action may be related to the regulation of cell cycle. In addition to regulating the phosphorylation of cell cycle-related proteins, trichomides A also suppressed the phosphorylation of AKT and STAT3 in activated T cells in a dose- and time-dependent manner (Figure 3a-d), suggesting that the inhibition of trichomides A against T cell responses involved the suppression of the AKT and STAT3 signaling pathways. Moreover, a phosphorylated SHP2 was significantly enhanced by trichomides A treatment in dose- and time-dependent manners (Figure 3e-h). The phosphorylation of this phosphatase has been reported to be required for conformational alterations and activation of its PTP enzyme site (Araki *et al.*, 2003; Xu and Qu, 2008). The above findings in protein regulation caused by trichomides A

hinted at some connection between these signaling pathways.

To further explore the underlying mechanisms of trichomides A and the possible connection in signaling pathways influenced by the cyclodepsipeptide, a specific SHP2 inhibitor PHPS1 was used in the next experiments (Hellmuth *et al.*, 2008). The above inhibitory effects of trichomides A on the various aspects of activated T cell functions were significantly blocked by the treatment with PHPS1 (Figures 3-4 and Supplementary Figure S5). For example, the inhibitor significantly blocked the enhancement of pSHP2 by trichomides A. Also, the inhibitions on the survival rate and cytokine production, and G0/G1 phase arrest as well as the expressions of cell cycle-related proteins caused by trichomides A were significantly reversed by PHPS1. PHPS1 also disrupted the inhibitory effect of trichomides A on the AKT and STAT3 signaling pathways. Furthermore, experiments with conditional SHP2 knockout T cells confirmed that trichomides A can regulate the p-AKT, p-STAT3, p-Rb and cyclin D1 signaling pathways by increasing the phosphorylation level of SHP2, in agreement with the results of experiments with PHPS1. These findings suggest that trichomides A exerts inhibitory effects on activated T cells in a SHP2-dependent manner.

Because delayed-type hypersensitivity relies entirely on the effects of T cells, it has been used as a classic model for the evaluation of *in vivo* immunocompetency and the effect of immunosuppressants (Feng *et al.*, 2013). Contact dermatitis is known to occur through a mechanism similar to delayed-type hypersensitivity (Luo *et al.*, 2011). In our *in vivo* experiments, trichomides A significantly blunted ear inflammation in response to picryl chloride (Figure 5). Against such effect of trichomides A, inhibition

of SHP2 activity using the SHP2-specific inhibitor PHPS1 (Figure 5) or conditional SHP2 knockout mice in T cells (Figure 6 and Supplementary Figure S7) significantly blocked the effects of trichomides A both in vitro and in vivo, indicating the SHP2-dependent action of trichomides. Previous studies have demonstrated that SHP2 plays a negative-regulation role in T-cell activation and differentiation processes (Salmond and Alexander, 2006). This result further confirmed that SHP2 might be a potential therapeutic strategy for this type of skin disease.

In conclusion, this study identified a therapeutic agent against contact hypersensitivity in mice through a SHP2-dependent immunosuppressive activity. Our findings suggest that the cyclodepsipeptide trichomides A may be a potential candidate for immune-related skin diseases.

MATERIALS AND METHODS

Mice

Specific pathogen-free, six- to eight-week-old female C57BL/6 mice were purchased from 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). Shp2-floxed mouse was a kind gift from Dr. Gen-Sheng Feng at Department of Pathology, School of Medicine, University of California, San Diego. 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.

Reagents

Trichomides A was isolated and identified as reported by us previously (Zhang *et al.*, 2013). Trichomides A (molecular weight: 637.81) with 99% of purity was dissolved at a concentration of 30 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). Picryl chloride (PCl), concanavalin A (Con A), ionomycin, 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), SHP2 specific inhibitor PHPS1 and carboxyfluorescein diacetate succinimidyl ester (CFSE) were purchased from Sigma-Aldrich (St. Louis, MO). Cyclosporine A (CsA) was purchased from Hubei Jianyuan Co. Ltd, (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). ELISA kits for murine TNF- α , IL-1 β , IL-17A, IFN- γ , IL-10, IL-2 and IL-6 were purchased from Dakewe Biotech Co. Ltd (Beijing, China). Antibodies against phospho-SHP2 (Tyr 580), phospho-STAT3 (Tyr 705), phospho-Rb (Ser 807/811), Rb, ERK, phospho-ERK (Thr 202/Tyr 204), phospho-AKT (Thr 308), Cyclin D1 and p27^{kip} were purchased from Cell Signal Technology (Beverly, MA). Antibodies against AKT, STAT3, GAPDH and SHP2 were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). Human/Mouse/Rat active SHP2 activity assay was purchased from R&D Systems (Minneapolis, MN). Recombinant mouse SHP2/PTPN11 protein (His tag) was purchased from Sino Biological Inc. (Beijing, China). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and cell proliferation assay

Lymph node cells isolated from female C57BL/6 mice were maintained in RPMI 1640 medium supplemented with 100 µg/mL of streptomycin, 100 U/mL of penicillin and 10% fetal calf serum under a humidified 5% (v/v) CO₂ atmosphere at 37 °C. Cell proliferation was determined by MTT and CFSE assay as we previously reported (Sun *et al.*, 2010).

Cell cycle assay

T cells from lymph nodes of C57BL/6 mice were treated with or without trichomides A 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 the dark at room temperature for 45 min. The DNA contents of the cells were analyzed with Modfit software (Becton Dickinson, San Jose, CA).

Western blot analysis

Proteins were extracted in lysis buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L 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 horse radish peroxidase-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

Picryl chloride (PCI)-induced contact hypersensitivity

PCI-induced contact hypersensitivity was investigated as described before (Mori T *et al.*, 2008). On the first day (day 0), female BALB/c mice were painted on the clipped abdomen with 50 µl of 5% PCI in ethanol/acetone (3:1). Five days after sensitization (day 5), trichomides A (10, 30 mg/kg), Dexamethasone (Dex, 5 mg/kg) and SHP2 inhibitor PHPS1 (1 mg/kg) dissolved in olive oil without DMSO were given intraperitoneal (i.p.) for one time, and mice were challenged by painting both sides of each ear with 20 µl of 0.5% PCI in ethanol/acetone (3:1). Twenty-four hours later, ear thickness of mice was measured using a digimatic micrometer (0.001 mm, Mitutoyo Co., Tokyo, Japan) before and 24 hours after challenge. Ear swelling was calculated as (ear thickness after challenge) - (ear thickness before challenge). The sensitization, elicitation and model controls mice were run parallel with other groups except for i.p. administration of same volume of olive oil (Luo *et al.*, 2011).

Histological analysis

Histological assessment was performed as described before (Luo *et al.*, 2011). Formalin-fixed, paraffin-embedded ear tissue was sectioned at 5 mm in thickness, and the sections were stained with hematoxylin and eosin. Following parameters were assessed: 1) the level of leukocyte infiltration and vascular congestion; 2) the erosion and anabrosis of epidermal cells; 3) the affection of 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.

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.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We greatly thank Dr. Gen-Sheng Feng at University of California, San Diego, who kindly presented us the Shp2-floxed mice as a gift for generating Shp2-floxed/CD4-Cre mice. This work was supported by National Natural Science

Foundation of China (NSFC) (Nos. 81330079, 81173070, 91229109) and Science Fund for Creative Research Groups of NSFC (No. 81121062), and Ministry of Science and Technology of China (2013AA092901).

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Accepted manuscript

FIGURE LEGENDS

Figure 1. Trichomides A inhibited T cell proliferation and proinflammatory cytokine production in activated mouse T cells *in vitro*. (a) The chemical structure of trichomides A. (b-e) Lymph node cells isolated from female C57BL/6 mice were treated with 0.3, 1, 3, 10 and 30 μ M trichomides A in the presence of 5 μ g/ml Con A (b) or 10 μ g/ml anti-CD3 plus 1 μ g/ml anti-CD28 (c) or RPMI 1640 medium (d) for 48 h. Cell proliferation was assessed by the MTT (b, c and d) and CFSE assays (e). (f) Purified T cells were stimulated with 5 μ g/ml Con A for 48 h in the presence or absence of trichomides A (0.3, 1 and 3 μ M), and the cytokines in the cell culture supernatant were determined using ELISA kits. All data represent the means $\pm$ SEM of three independent experiments in triplicate. * P <0.05, ** P <0.01 vs. Con A or anti-CD3/anti-CD28 or vehicle group.

Figure 2. Trichomides A caused T cell arrest in G0/G1 phase of the cell cycle. (a) Purified T cells isolated from the lymph node of female C57BL/6 mice were stimulated with 5 μ g/ml Con A for 48 h in the presence or absence of 0.3-3 μ M trichomides A, and then cells were stained with PI, which was used for cell cycle distribution analysis by flow cytometry. (b) The data represented here are one of three independent experiments with similar results. (c) T cells were stimulated with 5 μ g/ml Con A without or with 0.3-3 μ M trichomides A for 48 h. Cells were harvested and lysed. The expression of cell cycle-related proteins was analyzed by Western blotting. (d) Data are presented as means $\pm$ SEM. ** P <0.01 vs. Con A group.

Figure 3. Trichomides A inhibited the activation of AKT and STAT3 but increased the phosphorylated SHP2 level in Con A-activated T cells. (a-d)

Purified T cells were stimulated with 5 µg/ml Con A in the presence or absence of 0.3-3 µM trichomides A for 12 h (a, b) or in the presence or absence of 3 µM trichomides A for 0-12 h (c-d). (e-h) Purified T cells were stimulated with 5 µg/ml Con A in the presence or absence of 0.3-3 µM trichomides A for 3 h (e, f) or in the presence or absence of 3 µM trichomides A for 0-12 h (g, h). Cells were harvested and lysed, and the levels of p-AKT, p-STAT3 and p-SHP2 were analyzed by Western blotting. (i) T cells from lymph nodes of C57BL/6 mice were treated with or without 3 µM trichomides A or 1 µM PHPS1 for 0-12 h in the presence of 5 µg/ml Con A, and then collected and washed with cold TBS. Mouse active SHP2 activity was determined using SHP2 activity assay kit. Data are presented as means ± SEM of three different experiments. * $P < 0.05$, ** $P < 0.01$ vs. Con A group.

Figure 4. The SHP2-specific inhibitor PHPS1 reversed the effects of trichomides A on activated T cells. (a-d)

Purified T cells from C57BL/6 mice were stimulated with 5 µg/ml Con A and cultured with the indicated concentration of trichomides A in the presence or absence of 1 µM PHPS1. Cell proliferation was assessed by the MTT (a, 24 h) and CFSE assay (b, 48 h). The levels of cytokines in cell culture supernatants were determined using ELISA kits after 24 h of incubation (c). Cell cycle was analyzed by PI staining (d, 24 h). (e) Purified T cells from WT and conditional

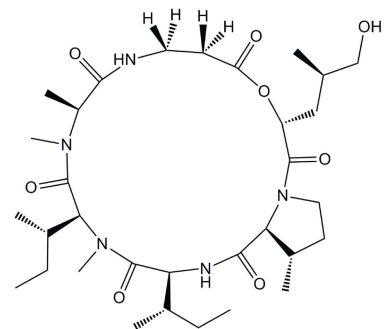
SHP2 KO mice were stimulated with 5 µg/ml Con A in the presence of the indicated concentration of trichomides A for 48 h. Cell proliferation was assessed by the MTT assay. **(f, g)** The expressions of cell cycle-related proteins were analyzed by Western blotting after 24 h of treatment. **(h, i)** The activation of AKT and STAT3 were analyzed by Western blotting after 12 h of treatment. The data are presented as the means $\pm$ SEM of three different experiments. * P <0.05, ** P <0.01, NS means no significance.

Figure 5. Trichomides A suppressed picryl chloride-induced contact hypersensitivity in mice. Mice were sensitized by painting 50 µl of 5% PCl in ethanol/acetone (3:1) onto the shaved skin of their abdomens. On day 5, trichomides A (10, 30 mg/kg), dexamethasone (Dex, 5 mg/kg) or the SHP2 inhibitor PHPS1 (1 mg/kg) were given i.p. for one time, and the mice were challenged by painting both sides of each ear with 20 µl of 0.5% PCl in ethanol/acetone (3:1). **(a)** Hematoxylin and eosin staining of ear sections (original magnification 200 $\times$). **(b)** Twenty-four hours after the challenge, the thickness of the right and left ears was measured. Ear swelling is presented as the increase in ear thickness. **(c)** Ear histological scoring. The data are presented as the means $\pm$ SEM of 8 mice. * P <0.05, ** P <0.01, NS means no significance.

Figure 6. SHP2 contributed to the improvement of contact dermatitis in mice by trichomides A. **(a)** Hematoxylin and eosin staining of ear sections (original

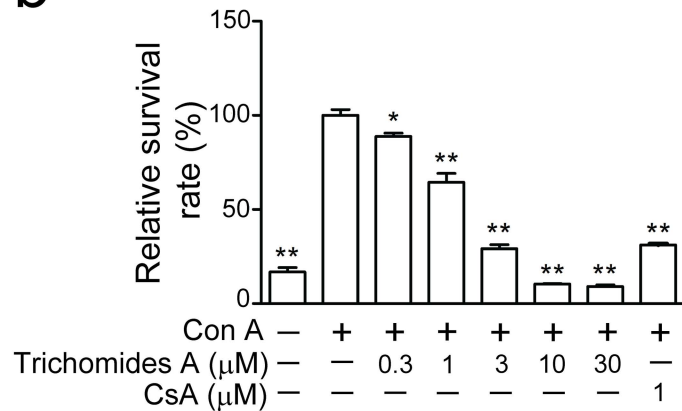
magnification 200×). **(b)** Twenty-four hours after the challenge, the thickness of the right and left ears was measured. Ear swelling was presented as the increase in ear thickness. **(c)** Ear histological scoring. The data are presented as the means $\pm$ SEM of 8 mice. * $P < 0.05$, ** $P < 0.01$, NS means no significance. **(d)** Purified T cells isolated from the lymph node of wild-type or conditional SHP2 knockout female C57BL/6 mice were stimulated with 5 $\mu\text{g/ml}$ Con A for 24 h in the presence or absence of 3 μM trichomides A. Cells were harvested and lysed, and the levels of p-AKT, p-STAT3, p-Rb and cyclin D1 were analyzed by Western blotting. **(e)** Schematic diagram of the mechanism underlying the effect of trichomides A on activated T cells via triggering SHP2 activation, which contributes to the improvement of contact hypersensitivity.

a

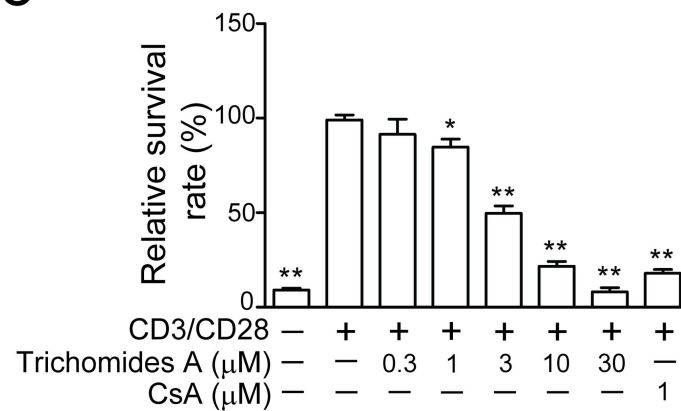


Trichomides A ($C_{32}H_{55}N_5O_8$)
Molecular Weight: 637.81

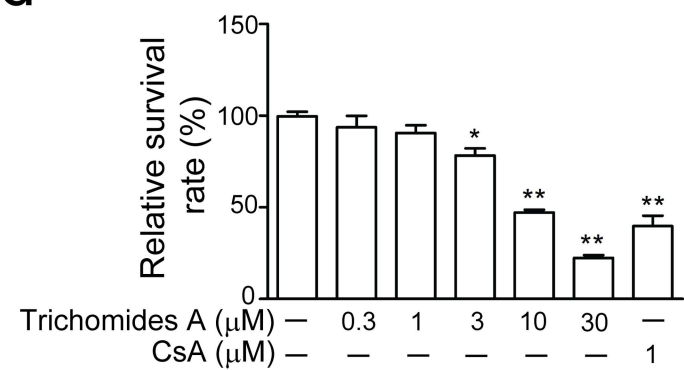
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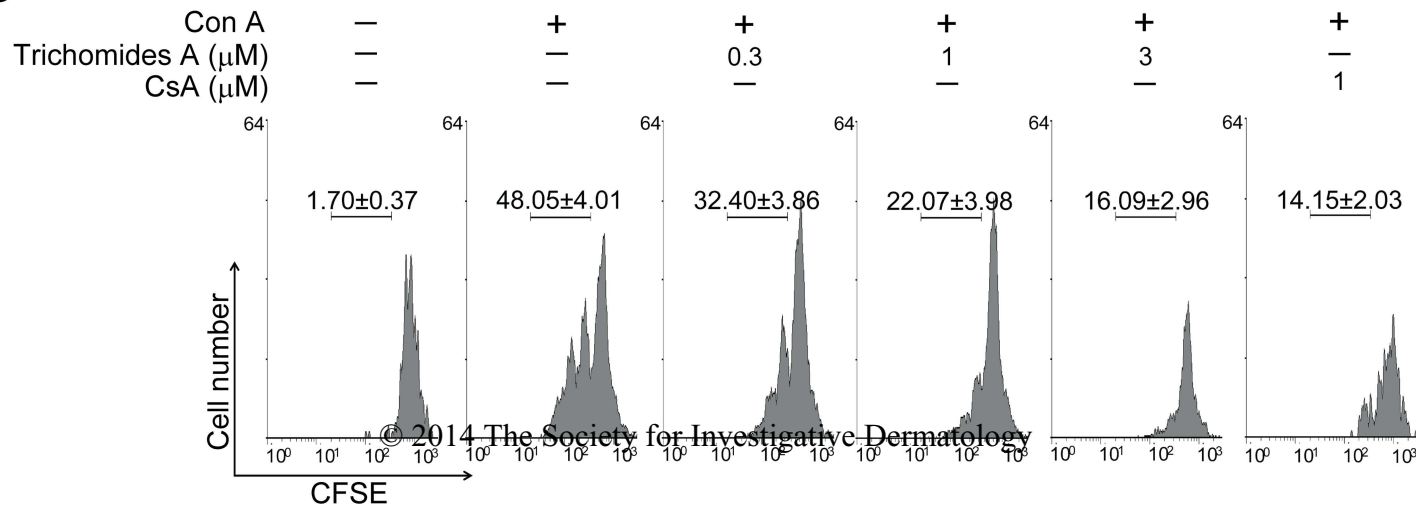
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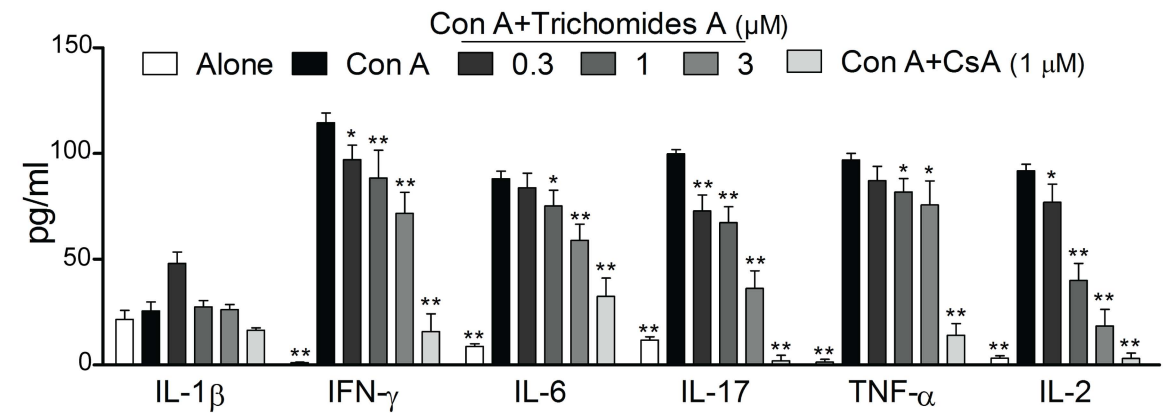
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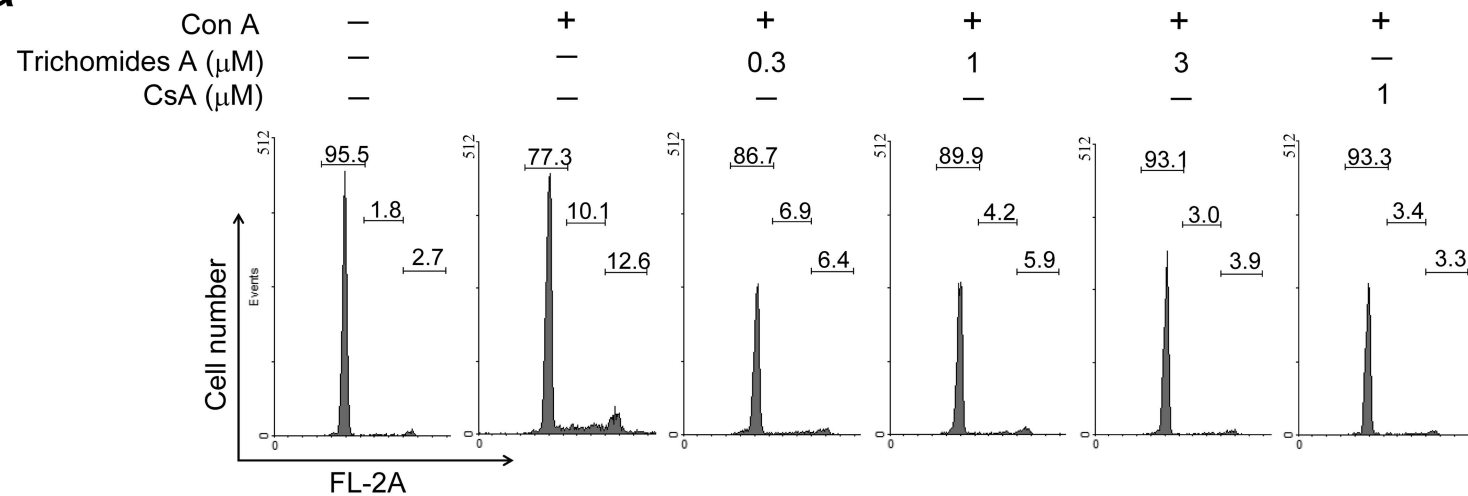
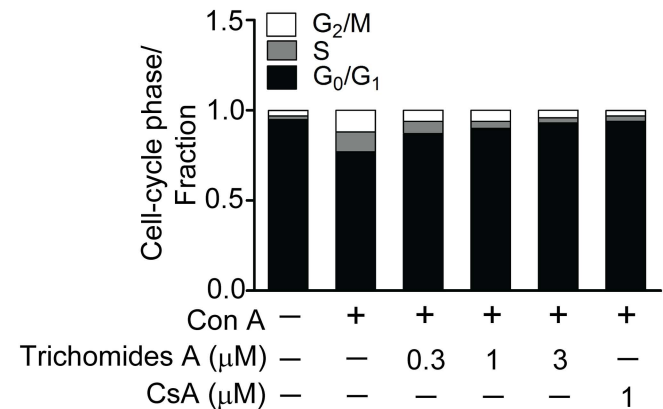
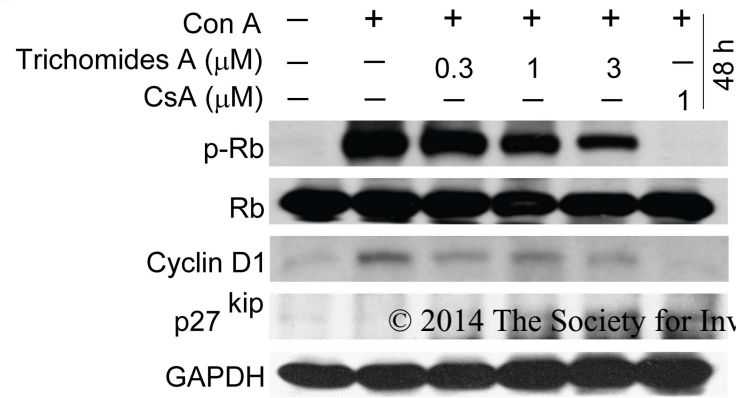
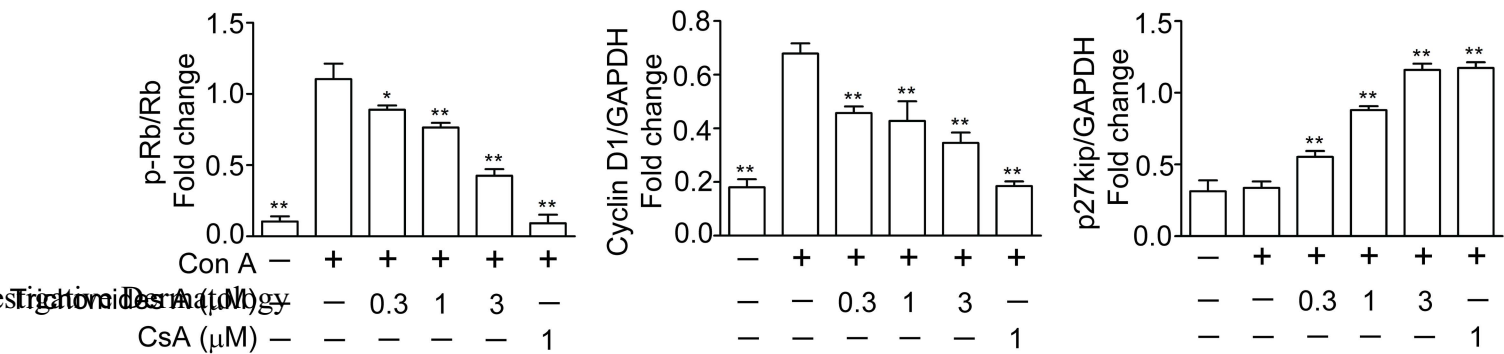


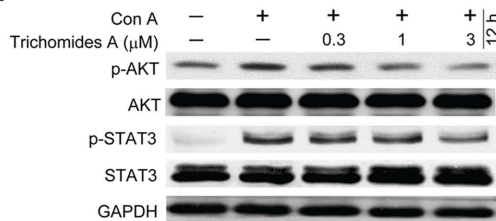
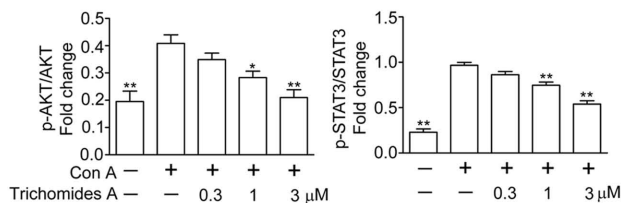
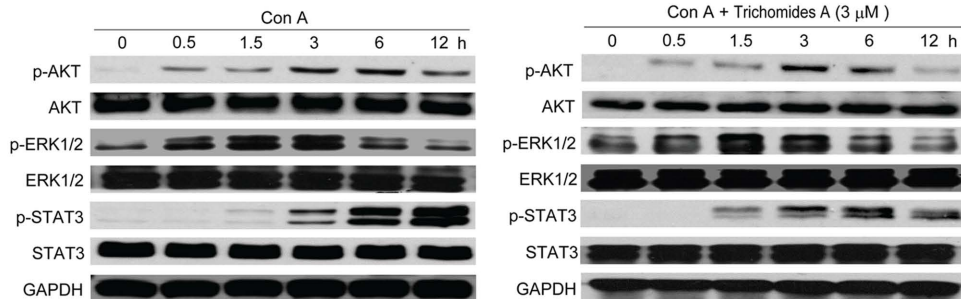
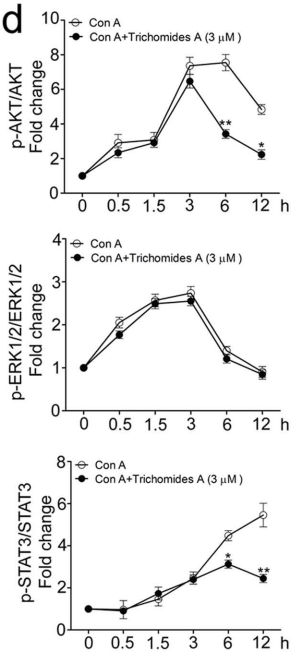
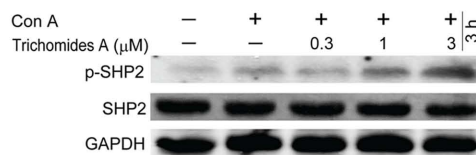
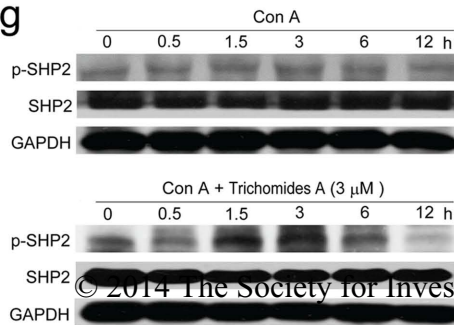
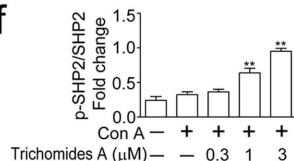
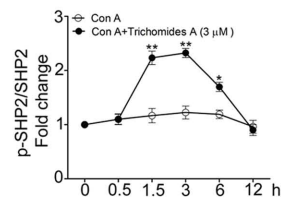
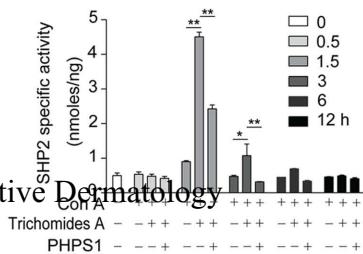
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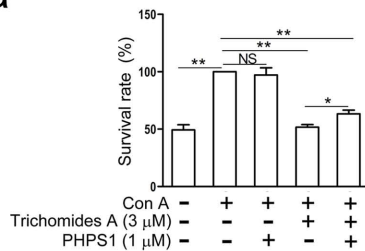
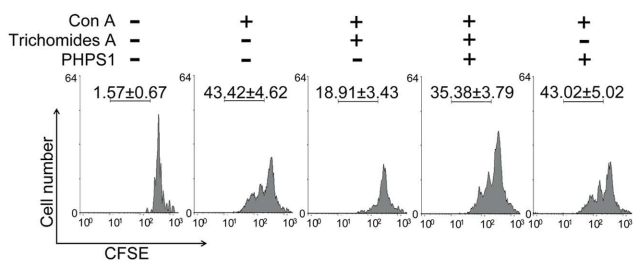
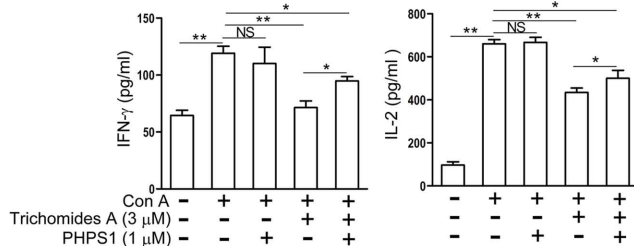
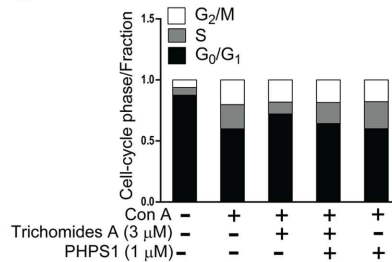
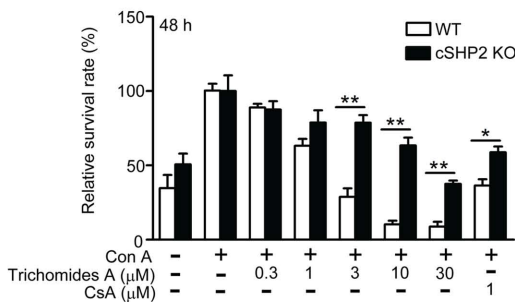
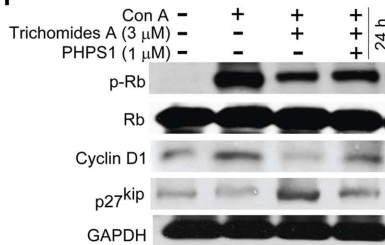
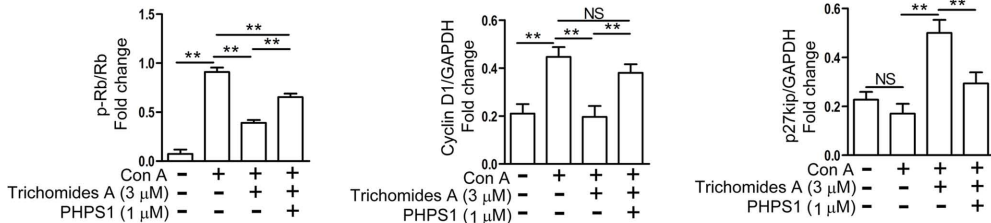
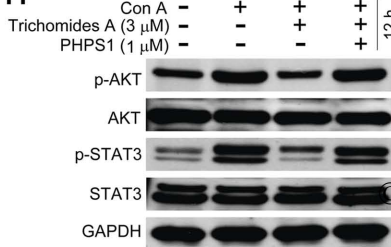
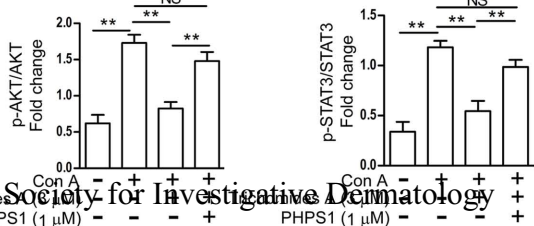


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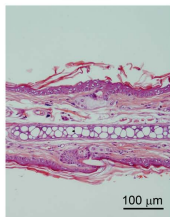
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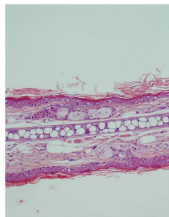
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Sensitization (SEN)

SEN: 5% PCI-CS

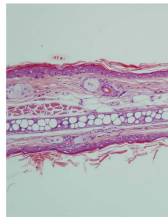
Solvent-control



Elicitation (ELI)

ELI: 0.5% PCI-CS

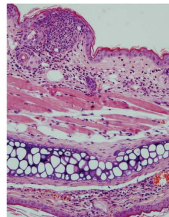
Solvent-control



SEN: 5% PCI-CS

ELI: 0.5% PCI-CS

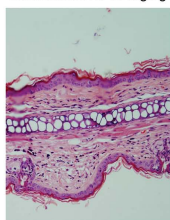
Solvent



SEN: 5% PCI-CS

ELI: 0.5% PCI-CS

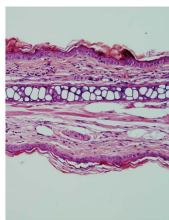
Trichomides A: 10 mg/kg



SEN: 5% PCI-CS

ELI: 0.5% PCI-CS

Trichomides A: 30 mg/kg

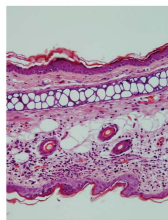


SEN: 5% PCI-CS

ELI: 0.5% PCI-CS

Trichomides A: 30 mg/kg

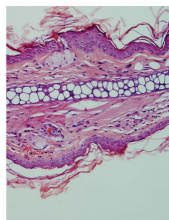
PHPS1: 1 mg/kg



SEN: 5% PCI-CS

ELI: 0.5% PCI-CS

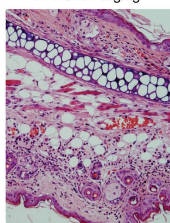
Dex: 5 mg/kg



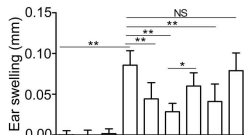
SEN: 5% PCI-CS

ELI: 0.5% PCI-CS

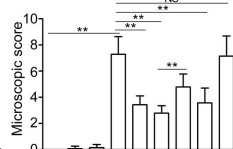
PHPS1: 1 mg/kg



b

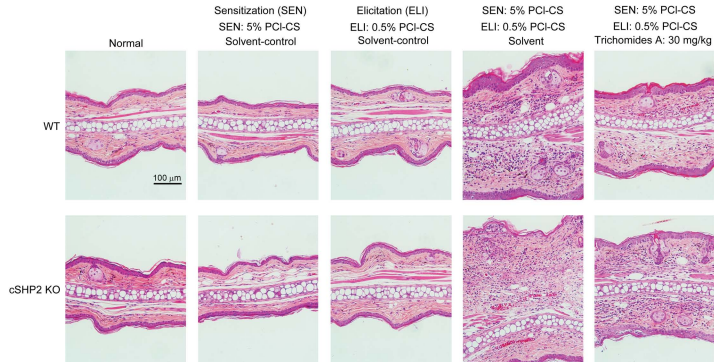
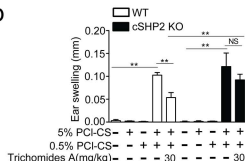
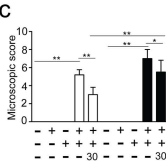
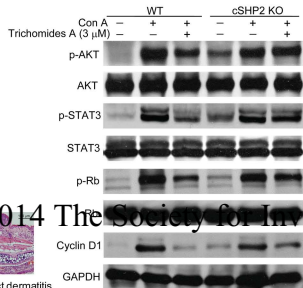


c



5% PCI-CS - + - + - + - + - +
 0.5% PCI-CS - - + + - - + + - -
 Trichomides A(mg/kg) - - - - 10 30 30 -
 PHPS1(mg/kg) - - - - - 1 - 1 -
 Dex(mg/kg) - - - - - - 5 -

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