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Neochromine S5 improves contact hypersensitivity through a selective effect on activated T lymphocytes

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ABSTRACT

Strategy on activated T cells is an effective treatment for T cell mediated diseases. By using a synthesized chromone derivative, we examined its effects on the activated T cells. This compound, (Z)-1,3-dihydroxy-9-methyl-13H-benzo[b]chromeno[3,2-f][1,4]oxazepin-13-one (neochromine S5), exhibited immunosuppressive activity in vitro and in vivo. Interestingly, neochromine S5 selectively inhibited proliferation and induced apoptosis in T lymphocytes activated by concanavalin A (Con A) in a dose-dependent manner but not in naïve T lymphocytes, distinct from quercetin. This compound triggered mitochondrial apoptotic pathway including cleavage of caspase 3, caspase 9 and PARP, downregulation of bcl-2 and release of cytochrome c in activated T cells, but did not affect ER stress or Fas signals. In addition, neochromine S5 downregulated the expression of CD25 and CD69 and the production of inflammatory cytokines, including TNFα, IFNγ and IL-2, improved ear swelling in mice with contact hypersensitivity, reduced $CD4^+$ T cells infiltration, and increased apoptosis of isolated T lymphocytes from peripheral lymph nodes. Moreover, neochromine S5 showed no effect on the weight of mice and their immune organs, while dexamethasone caused a significant weight loss. Taken together, our results suggest that neochromine S5 exerts a unique anti-inflammatory activity mainly through a selective effect on activated T cells, which is different from the current immunosuppressant, dexamethasone.

1. Introduction

During immune response, T lymphocytes will be activated and develop effector functions that help to kill the pathogens. However, the effector functions are also harmful to healthy tissue. To limit the damage, the host will evolve some mechanisms to shut down immune responses, including causing cell inactivation and inducing apoptosis of activated cells [1–3]. As to the mechanisms that participate in the progress of apoptosis, there include the direct activation of caspase cascade (mitochondrial pathway) [4], the activation of the Fas death receptor/Fas ligand (FasL) complex (death receptors) [5,6], and the ER stress-mediated pathway [7]. The activated T cells express several cell surface markers such as CD25 and CD69, and produce specific cytokines such as TNFα, IFNγ, IL-2, and so on. In addition, a number of studies have illustrated that the increased expression of CD25, CD69 and production of inflammatory cytokines correlates with autoimmune and inflammatory diseases [8–11]. By means of such mechanisms, the apoptosis induction in such as activated T cells as well as the inhibition on inflammatory cytokines or T cell activation has been used as an effective strategy in the drug discovery for various immune diseases [12,13].

However, there is still lack of drugs selectively targeting the effector population such as activated T cells to avoid the injury of irrelevant cells. In previous studies, we have reported that several herbal extracts showed a selective inhibition on activated T lymphocytes [12–14]. These are quite different from the commonly used immunosuppressants, which have severe side effects primarily owing to their poor selectivity, including glucocorticoids, cyclophosphamide and even cyclosporine A [15–17].

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In the present study, we studied their effect on activated and non-activated T cells and found that (Z)-1,3-dihydroxy-9-methyl-13H-benzo[b]chromeno[3,2-f][1,4]oxazepin-13-oneF (neochromine S5), a synthesized chromone derivative, inhibited the proliferation and increased apoptosis of activated T cells, but had little effect on non-activated cells. In contact hypersensitivity, neochromine S5 prevented ear swelling and inhibited $CD4^+$ T cells infiltration. Such selective effect of neochromine S5 was further confirmed in a murine model of T cell-mediated contact hypersensitivity.

2. Materials and methods

2.1. Synthesis of neochromine S5

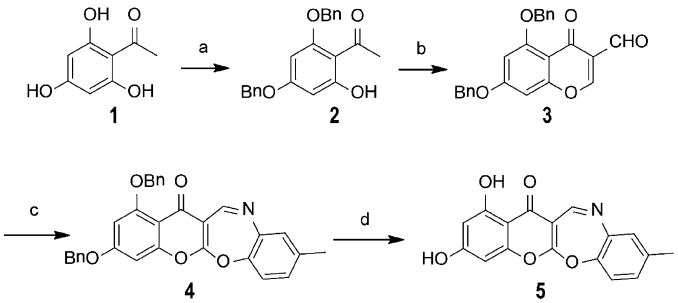
All reagents and solvents for the synthesis of neochromine S5 were commercially available and used without further purification. Melting points were determined on a Taike X-4 digital micromelting point apparatus and uncorrected. ^1H and ^{13}C NMR spectra were taken on a Bruker DPX-300 spectrometer, using TMS as an internal standard (chemical shifts in δ). ESI-HR-MS were obtained on Esquire 4000 mass spectrometer.

To a solution of 1-(2,4,6-trihydroxyphenyl)ethanone (**1**) (60 g, 0.36 mol) in HMPA (300 mL) was added K_2CO_3 (148 g, 1.07 mol) and BnCl (86.3 mL, 0.75 mol), and the suspension was stirred at 90 °C for 3 h. The solid was filtered, and the filtrate was poured into ice-water. The pH of the solution was adjusted to 2 by adding diluted hydrochloric acid. The resulting solid was filtered and recrystallized in CH_2Cl_2/MeOH to give 1-(2,4-bis(benzyloxy)-6-hydroxyphenyl)ethanone (**2**). Yield: 75%; ^1H NMR ($CDCl_3$, 300 MHz) δ: 2.56 (3H, s) 5.06 (4H, s), 6.11 (1H, s), 6.17 (1H, s), 7.41 (10H, m), 14.04 (1H, s).

To a solution of **2** (15.0 g, 43 mmol) in DMF (100 mL) was added $POCl_3$ (19.5 mL, 213 mmol) dropwise at 0 °C and the solution was stirred at room temperature for 12 h. The reaction mixture was poured into ice water, and resulting solid was dissolved in CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified through chromatography eluting with CH_2Cl_2/EtOAc (25/1) to give 5,7-bis(benzyloxy)-4-oxo-4H-chromene-3-carbaldehyde (**3**). Yield: 70%; ^1H NMR ($CDCl_3$, 300 MHz) δ: 5.11 (2H, s), 5.22 (2H, s), 6.56 (1H, d, J = 2.1 Hz), 6.58 (1H, d, J = 2.1 Hz), 7.42 (8H, m), 7.59 (2H, d, J = 7.5 Hz), 8.32 (1H, s), 10.38 (1H, s); ESI-MS m/z: 387 $[M+H]^+$.

To a solution of **3** (386 mg, 1 mmol) in toluene (10 mL) was added *o*-aminophenol (1 mmol) and *p*TSA (cat. amount), and the reaction mixture was stirred at 80 °C for 3 h and concentrated in vacuo. The residue was dispersed in xylene (10 mL) and DDQ (271 mg, 1.2 mmol) was added. The suspension was stirred at 130 °C for 3 h. The reaction mixture was cooled and poured into cold petroleum ether. The resulting solid was filtered and purified through chromatography to yield 1,3-bis(benzyloxy)-9-methyl-13H-benzo[b]chromeno[3,2-f][1,4]oxazepin-13-one (**4**).

To a solution compound **4** (0.5 mmol) in anhydrous CH_2Cl_2 at −78 °C was added a solution of BBr_3 in CH_2Cl_2 (1.1 mL, 1 M), and the suspension was stirred at room temperature for 8 h. The reaction was quenched by the addition of water (1 mL), and the resulting solid was filtered, washed with water and recrystallized in acetone/CH_2Cl_2 to afford (Z)-1,3-dihydroxy-9-methyl-13H-benzo[b]chromeno[3,2-f][1,4]oxazepin-13-one (**5**), which was named as neochromine S5. Yield: 52% (3 steps); mp 289–291 °C; ^1H NMR (C_5D_5N, 300 MHz) δ: 2.34 (3H, s), 6.69 (1H, s), 6.76 (1H, s), 7.15 (1H, d, J = 7.7 Hz), 7.55 (1H, d, J = 7.7 Hz), 7.70 (1H, s), 9.04 (1H, s), 13.34 (1H, br); ^{13}C NMR (C_5D_5N, 75 MHz) δ: 22.41, 96.68, 102.35, 106.61, 111.83, 113.90, 121.60, 128.10, 135.94, 143.43, 159.30, 161.14, 164.81, 167.89, 179.46; HRMS-ESI (m/z): calcd for $C_{17}H_{12}NO_5$ $[M+H]^+$: 310.0715 found: 310.0709.



Reagents and conditions: (a) BnCl, K_2CO_3, HMPA, 90 °C, 3 h; (b) $POCl_3$, DMF, 0 °C to rt, 12 h; (c) (i) *o*-aminophenol, *p*-TsOH, toluene, 80 °C, 3 h; (ii) DDQ, xylene, 130 °C, 3 h; (d) BBr_3, CH_2Cl_2, −78 °C to room temperature, 8 h.

2.2. Animals and cells

Female BALB/c mice (6–8 weeks of age, 18–22 g) were obtained from the Experimental Animal Center of Yangzhou University (Yangzhou, Jiangsu, China). They were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12 h light–dark cycle. Animal welfare and experiments were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used. Jurkat cell was purchased from the Shanghai Institute of Cell Biology (Shanghai, China).

2.3. Reagents and antibodies

CFSE cell proliferation kit was from Thermo Fisher Scientific Inc. (Waltham, MA). Annexin V-FITC/propidiumiodide (AV/PI) assay kit for flow cytometry was from Jingmei Biotech Co., Ltd. (Shenzhen, China). Cyclosporin A (CsA), thapsigargin (TG), concanavalin A (Con A), dimethylsulfoxide (DMSO), 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), 2,4,6-trinitrobenzene-sulphonic acid (TNBS) and quercetin were obtained from Sigma (St. Louis, MO). Picryl chloride (PCl) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Mouse anti-CD25-APC, mouse anti-CD69-FITC, mouse anti-CD44-FITC, mouse anti-CD62L-PE and mouse anti-CD4-FITC were from ebioscience Inc. (San Diego, CA). Mitochondrial membrane potential ($\Delta\Psi$m) assay kit with JC-1 was from Beyotime Institute of Biotechnology (Shanghai, China). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) Kit was from Calbiochem (La Jolla, CA). TNFα, IFNγ, and IL-2 ELISA kits were from Dakewe Biotech Co., Ltd. (Beijing, China). Dexamethasone (DEX) was from Xianju Pharmaceutical Co., Ltd. (Zhejiang, China). Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). PCl was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Glycylglycin and all other chemicals were obtained from Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China). The antibodies used in this study were as follows: anti-α-tubulin, anti-GADD153, anti-p-PERK (Santa Cruz Biotechnology, Santa Cruz, CA), anti-XBP-1, anti-PARP, anti-cleaved caspase 3, anti-cleaved caspase 9, anti-Bad, anti-Bcl-2 and anti-COX-IV (Cell Signaling Technology, Boston, MA), anti-cytochrome *c* (BD Pharmigen, San Diego, CA), peroxidase-labeled anti-mouse and anti-rabbit antibody (KPL, Gaithersburg, MD).

2.4. T cell purification and culture

T cells were isolated from lymph nodes and spleen of BALB/c mice and purified using Dynal® mouse T cell Negative Isolation Kit (Thermo Fisher Scientific Inc., Waltham, MA) via standard procedure, and the purity was higher than 97%. All cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% (v/v) humidified CO_2 incubator.

2.5. Cell activation and proliferation

T cells isolated from BALB/c mice were stimulated by Con A (5 μg/ml) for 24 h, as activated T cells, and those without Con A were indicated as non-activated T cells or naïve T cells. Anti-CD25-APC and anti-CD69-FITC were used to test the level of T cells activation by flow cytometric analysis. Proliferation of T cells was detected according to standard procedures using CFSE cell proliferation kit.

2.6. MTT assay

Purified mouse T cells were cultured in 96-well plates in RPMI 1640 medium (0.2 ml). After stimulated with Con A, the cells were treated with or without neochromine S5 for 24 h, then the cell viability was assessed by MTT assay. MTT (4 mg/ml in PBS, 20 μl per well) was added to each well. After 4 h of additional incubation, remove the culture media, 200 μl DMSO was added to dissolve the crystals. The absorption values at 570 nm were determined.

2.7. Cell apoptosis assay

Cell apoptosis was determined using Annexin V/PI staining, JC-1 staining and TUNEL assay. For Annexin V/PI staining, cells were incubated with Annexin V-FITC and PI for 20 min at room temperature in dark, and then tested through flow cytometric analysis. For JC-1 staining, cells were detected according to standard procedures using mitochondrial membrane potential ($\Delta\Psi$m) assay kit with JC-1 and samples were analyzed by flow cytometer. For TUNEL assay, the cells were detected according to standard procedures using TUNEL Kit and samples were analyzed by flow cytometer.

2.8. T cell subsets analysis

Central memory T cells (CD44hiCD62Lhi) and naïve T cells (CD44loCD62Lhi) were distinguished with anti-CD44 and anti-CD62L staining and analyzed by flow cytometer.

2.9. Western blotting

Cells were harvested and lysed with RIPA buffer for 30 min on ice. The protein concentration was determined with the BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA). An equal amount of protein was subjected to electrophoresis on 10% SDS–PAGE and transferred by electroblotting onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA). After blocking with 5% non-fat milk for 1 h at room temperature, the membrane was incubated with primary antibody overnight at 4 °C, and then incubated with secondary antibody for 1.5 h at room temperature. Detection was performed using a 20× LumiGLO Reagent and 20× Peroxide kit (Cell Signaling Technology, Boston, MA).

2.10. Real-time PCR

Total RNA was extracted from the cells with trizol reagent. The cDNA synthesis reaction was performed in a reaction mixture including 1 μg of total RNA, oligo (dT) 18 primers and M-MLV reverse transcriptase. PCR primer sequences were as follows: *β-actin*, forward 5′-CAACGAGCGGTTCCGATG and reverse 5′-GCCACAGGATTCCATACCCA; *Tnfα*, forward 5′-TTCTGTCTACT-GAACTTCGGGGTGATCGGTCC and reverse 5′–GTATGAGATAGCAA-ATCGGCTGACGGTGTGGG; *Ifnγ*, forward 5′-ATGAACGCTACA-CACTGCATC and reverse 5′-CCATCCTTTTGCCAGTTCCTC; *Il-2*, forward 5′-CTACAGCGGAAGCACAGC and reverse 5′-TCCTCA-GAAAGTCCACCA. Real-time PCR was performed as described previously [18]. Briefly, the cDNA samples were performed with Bio-Rad CFX Connect real-time system using IQ SYBR Green supermix (Bio-Rad Laboratories, Inc., Hercules, CA), and threshold cycle numbers were obtained using Bio-Rad CFX Manager software. Conditions for amplification were 1 cycle of 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s.

2.11. Cytokine analysis by ELISA assay

TNFα, IFNγ, and IL-2 in the supernatant were measured with ELISA kit, according to the manufacturer's instruction.

2.12. PCl-induced contact hypersensitivity

The PCl-induced contact hypersensitivity was measured according to the method described by Fei et al. [19]. BALB/c mice were sensitized by applying 100 μl of 1% PCl in ethanol on the abdomen. Neochromine S5 (3 or 10 mg/kg) and DEX (5 mg/kg) were orally administered for 6 days after sensitization. Control animals with contact hypersensitivity were given water instead of drugs. After 5 days, they were challenged with 30 μl of 1% PCl in olive oil on the right ear. 24 h later, the left and right ears thickness were measured with an engineer's micrometer (Mitutoyo Co., Tokyo, Japan). Ear swelling was worked out according to the difference between the right and left ears in thickness. The mice spleen index and thymus index were calculated as follows:

$$\text{Spleen index} = \frac{\text{spleen weight(mg)}}{\text{body weight(g)}} \times 10$$

$$\text{Thymus index} = \frac{\text{thymus weight(mg)}}{\text{body weight(g)}} \times 10$$

$$\text{Lymph node index} = \frac{\text{lymph node weight(mg)}}{\text{body weight(g)}} \times 10$$

2.13. Hapten-specific T cell proliferation

The detection procedure was performed as described previously [19]. Splenocytes, isolated from BALB/c mice 5 days after PCl sensitization, were treated with 1.0 mM TNBS (water-soluble analog of PCl) and 25 mg/ml mitomycin C, then incubated at 37 °C for 30 min. After being washed 3 times in HBSS (containing 0.6% glycylglycin), the cells were used as stimulator cells (trinitrophenylated splenocytes). Meanwhile, lymphocytes were obtained from the above described sensitized mice after the adherent cells were removed, and used as responder cells. The 4×10^5 stimulator cells and 2×10^5 responder cells were co-cultured for 72 h. Then cell proliferation was examined by MTT assay. The stimulation index (SI) was calculated as follows:

$$\text{Stimulation index} = \frac{\text{OD}_{\text{stimulated cells}}}{\text{OD}_{\text{nonstimulated cells}}} \times 10$$

2.14. Statistical analysis

Results were expressed as means ± SEM of three independent experiments with each experiment including triplicate sets in vitro, or of eight animals per group in vivo. Student's *t* test was used to test the difference between two groups. One-way ANOVA and post hoc tests were applied when there were more than two groups in the independent variable. $p < 0.05$ was considered to be significant.

3. Results

3.1. Neochromine S5 inhibited proliferation and induced apoptosis in Con A-activated T cells

The chemical structure and the ^{1}H NMR spectrum of neochromine S5 was shown in Fig. 1A. T cells from BALB/c mice were activated with Con A (5 μg/ml) and subsequently incubated

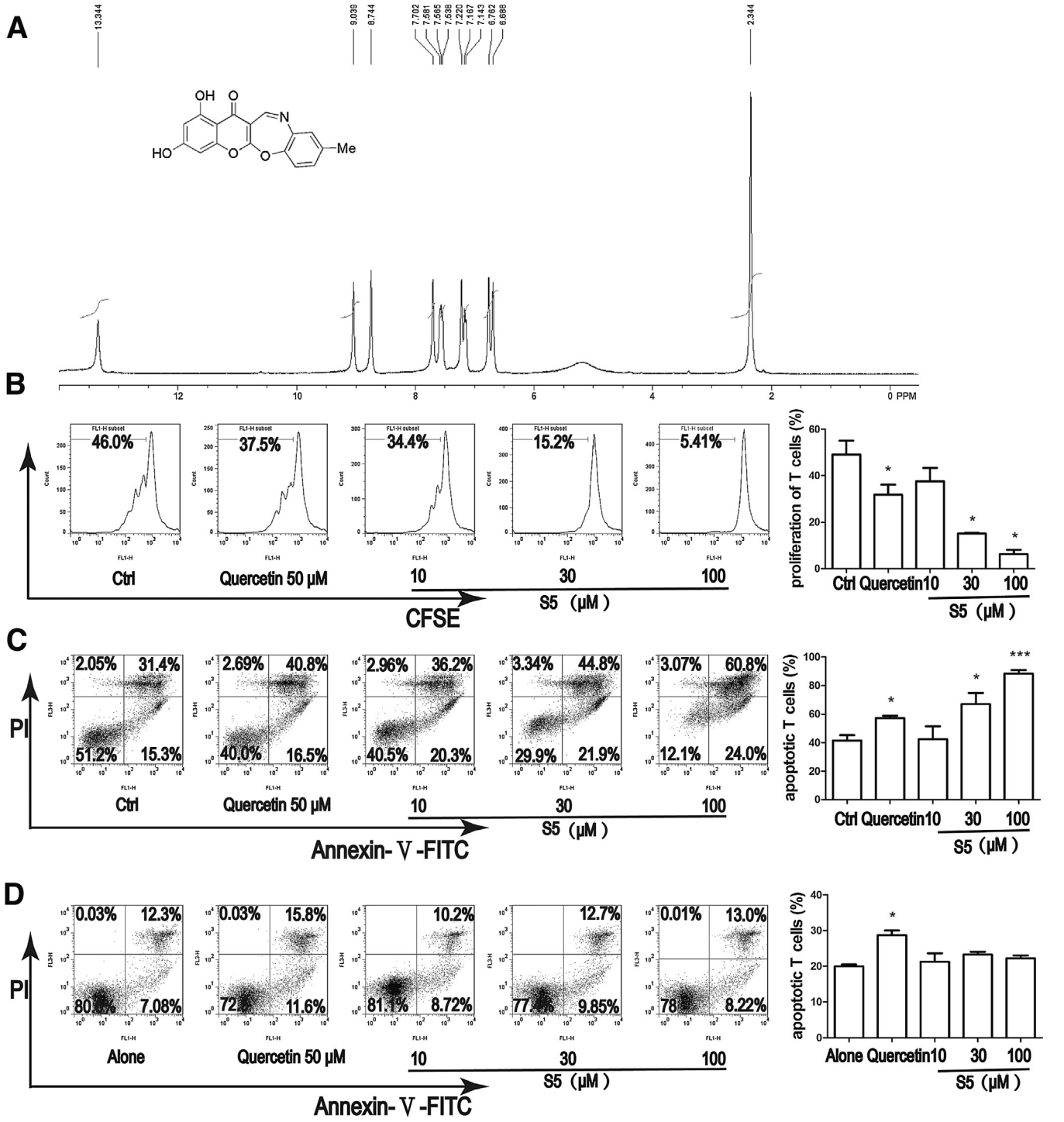


Fig. 1. Effects of neochromine S5 on proliferation and apoptosis of Con A-activated T cells. (A) The chemical structural formula and the ^{1}H NMR spectrum of neochromine S5. (B) T lymphocytes isolated from BALB/c mice were labeled with CFSE, and then incubated with Con A and various concentrations of neochromine S5 for 72 h. The proliferation rate was examined by flow cytometry. (C) T lymphocytes from BALB/c mice were incubated with Con A for 24 h, and were treated with various concentrations of neochromine S5 for the subsequent 24 h. (D) T lymphocytes from BALB/c mice were treated with various concentrations of neochromine S5 for 24 h without activation. In C and D, cells were co-stained with Annexin V/PI to determine apoptosis by flow cytometry. Data (B–D) shown here are one of three independent experiments. Values are shown as mean ± SEM of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs controls.

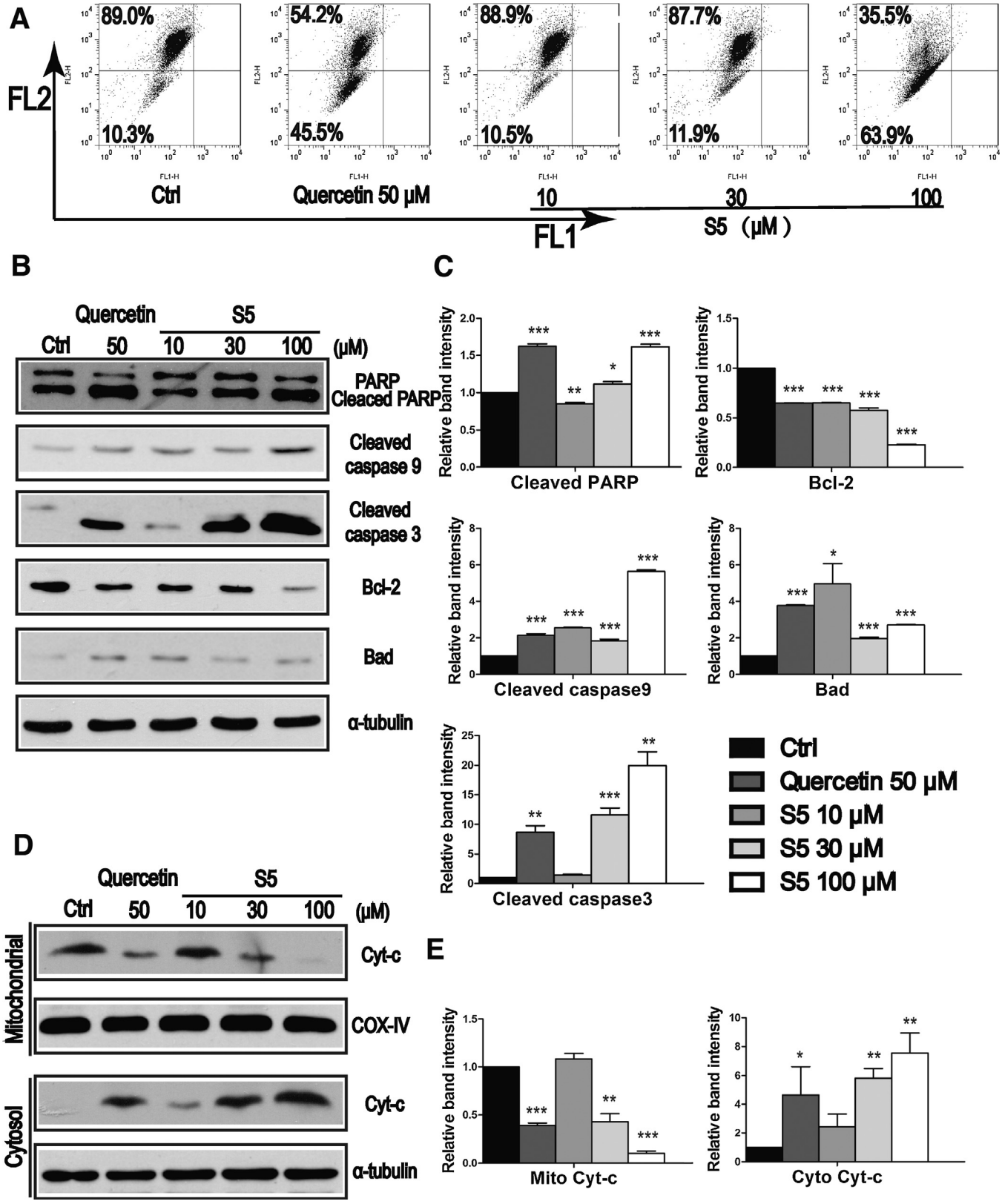


Fig. 2. Effects of neochromine S5 on mitochondrial apoptotic pathway in Con A-activated T cells. T lymphocytes from BALB/c mice were activated with Con A and incubated with neochromine S5 as mentioned above. (A) $\Delta\Psi$m was measured by JC-1 staining. (B) Protein levels of PARP, Cleaved caspase 3, Cleaved caspase 9, Bad and Bcl-2 were analyzed by western blotting. (D) Cytochrome *c* of mitochondria and cytoplasm was detected by western blotting. Data shown here are one of three independent experiments. (C, E) Relative band intensity was determined via Image J software. Values are means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs controls.

with various concentrations of neochromine S5 (10, 30 and 100 μM). Then, the proliferation rate was examined by CFSE staining. Neochromine S5 dose-dependently inhibited the proliferation of Con A-activated T cells (Fig. 1B). In addition, Annexin V/PI staining indicated that neochromine S5 induced apoptosis in activated but not non-activated T cells (Fig. 1C and D). Quercetin was used as a positive control, which induced apoptosis both in activated and non-activated T cells.

3.2. Neochromine S5 triggered mitochondrial apoptotic pathway but not ER stress or Fas-dependent signals in Con A-activated T cells

To investigate the molecular mechanism of neochromine S5 on apoptosis of activated T cells, we determined effects of neochromine S5 on mitochondrial signals. T cells from BALB/c mice were stimulated with Con A and incubated with various concentrations of neochromine S5 (10, 30 and 100 μM). JC-1 staining was used to determine the $\Delta\Psi$m. Neochromine S5 caused collapsing of the $\Delta\Psi$m in activated T cells (Fig. 2A) and increased PARP cleavage, Bad expression, caspase 3 and caspase 9 activation and reduced Bcl-2 expression (Fig. 2B and C). In addition, neochromine S5 significantly induced a release of cytochrome *c* from mitochondria to cytosol in a dose-dependent manner (Fig. 2D and E).

Then, we assayed the intrinsic ER stress and the death receptor-mediated pathway. p-PERK, GADD153 and XBP-1 expressions were analyzed by western blotting, which are the major molecules involved in ER-stress (Fig. 3A). As the result, neochromine S5 had no effect on p-PERK, GADD153 and XBP-1 whereas TG (as a positive control) increased ER stress significantly. To test the death receptors pathway, Jurkat cells and FADD$^{-/-}$ Jurkat cells were activated by PHA (0.5 μg/ml) and incubated with various concentrations of neochromine S5 (10, 30 and 100 μM) or quercetin. Apoptosis was confirmed by TUNEL assay (Fig. 3B). The apoptotic rate induced by quercetin was greatly decreased in FADD$^{-/-}$ Jurkat cells as compared with that in wild type Jurkat cells. However, neochromine S5 induced apoptosis at the same rate in both wild type and FADD$^{-/-}$ Jurkat cells.

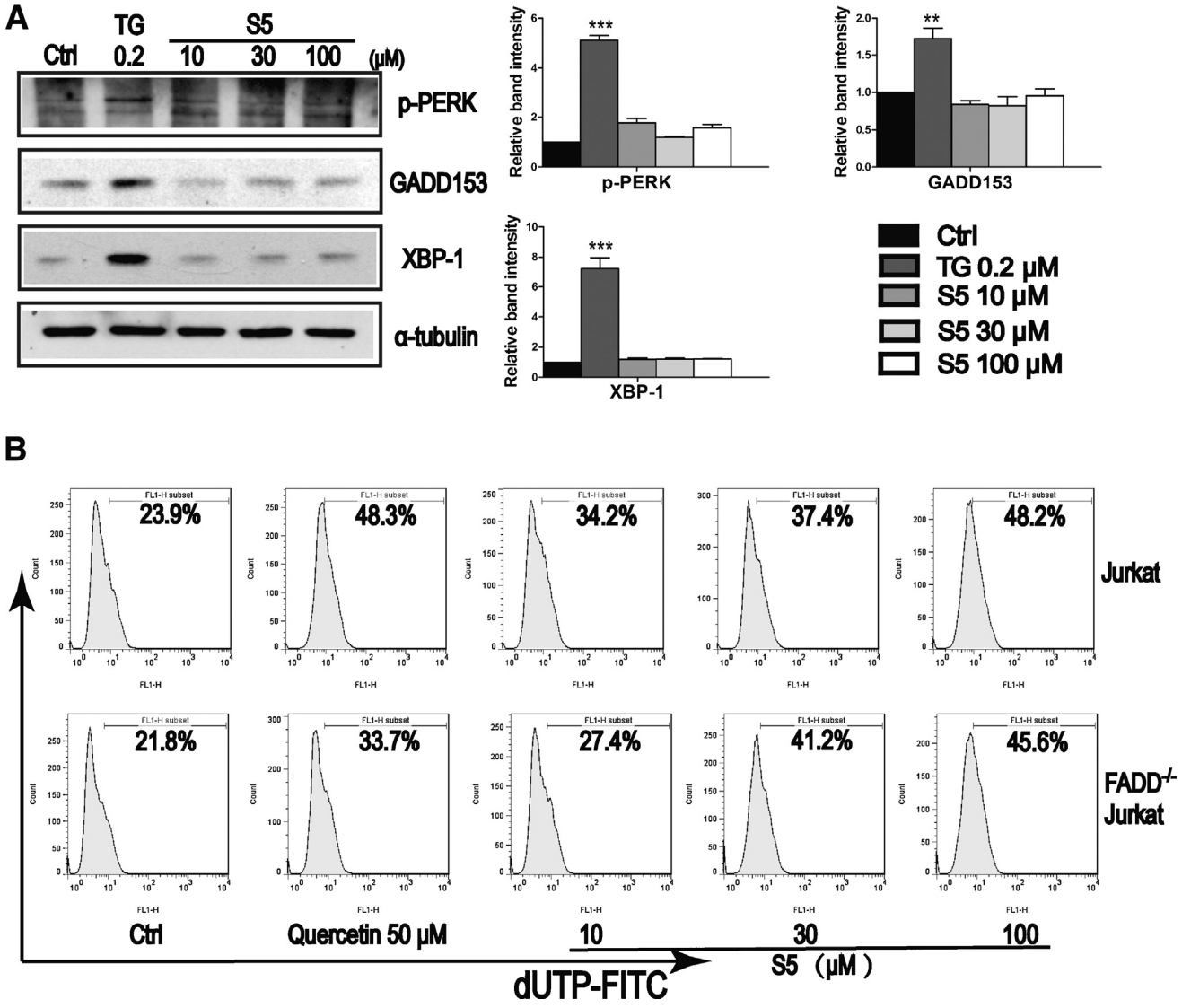


Fig. 3. Effects of neochromine S5 on ER stress and death receptor signals in Con A-activated T cells. (A) T lymphocytes from BALB/c mice were treated as above. Protein levels of GADD153, p-PERK and XBP-1 were analyzed by western blotting as shown in the left panel, and relative band intensity was indicated in the right panel. Values are means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs controls. (B) Jurkat and FADD$^{-/-}$ Jurkat cells were activated with PHA for 24 h, treated with neochromine S5 for another 24 h, and then stained with dUTP-FITC by flow cytometry assay. Data shown here are one of three independent experiments.

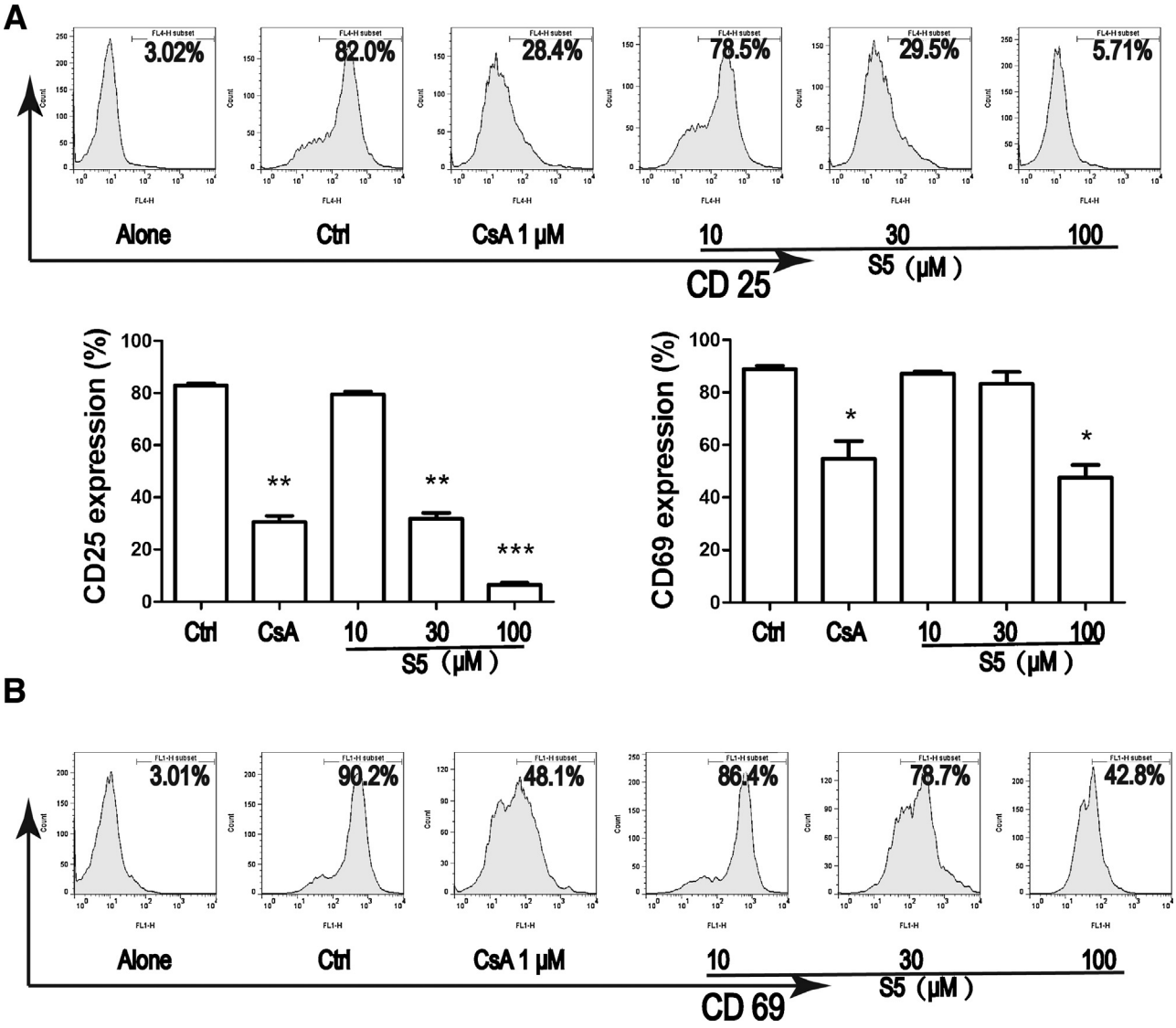


Fig. 4. Effects of neochromine S5 on cell surface CD25 and CD69 expressions in Con A-activated T cells. T lymphocytes were incubated with Con A and neochromine S5 as mentioned above, and surface (A) CD25 and (B) CD69 expression was analyzed by flow cytometry. Data shown here are one of three independent experiments. Values are means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs controls.

3.3. Neochromine S5 inhibited CD25 and CD69 expression and prevented the cytokines prolife in Con A-activated T cells

CD25 and CD69 have been served as markers of activated T lymphocytes [20]. They can be triggered by several agents interacting with the T cell receptor (TCR)/CD3 complex [21]. The surface expressions of CD25 and CD69 were up-regulated in Con A-activated T cells, and were down-regulated by neochromine S5 treatment in a dose-dependent manner (Fig. 4A and B). CsA, a well-known immunosuppressant, was used as a positive control, which also showed a significant inhibitory effect on both CD25 and CD69 expressions.

Furthermore, neochromine S5 dose-dependently inhibited the expression of pro-inflammatory cytokines, TNFα, IFNγ and IL-2, in activated T cells in both protein and mRNA levels (Fig. 5A and B).

3.4. Neochromine S5 ameliorated ear swelling, prevented CD4+ T cells infiltration, and induced apoptosis of T lymphocytes in mice with contact hypersensitivity

Contact hypersensitivity as an animal model of T cell mediated allergic contact dermatitis has been studied extensively. In PCl-induced contact hypersensitivity, oral administration of neochromine S5 inhibited ear swelling significantly (Fig. 6A). In this case, the body weight and immune organ index were not influenced by the neochromine S5 treatment, while DEX, the positive control, greatly caused weight loss and reduced the weights of thymus, lymph node and spleen (Fig. 6B). As the improvement of the hypersensitivity, histopathologic injuries, severe inflammatory cell infiltration, vascular congestion, and moderate edema in the dermis and subcutaneous tissue were mainly lowered by neochromine S5 treatment, where 10 mg/kg of neochromine S5 showed an improvement to a similar extent with 5 mg/kg of DEX (Fig. 6C). Neochromine S5 also dose-dependently prevented infiltration of $CD4^+$ T cells into ear (Fig. 6D) and increased the percentages of apoptotic T cells (Fig. 6E). In addition, neochromine S5 decreased the percentages of central memory T cells but not naïve T cells (Fig. 6F).

3.5. Neochromine S5 inhibited CD69 and inflammatory cytokine expression in hapten-specifically activated T cells isolated from PCl-sensitized mice

To confirm the effect of neochromine S5 on the activated T cells, we did an ex vivo experiment, where T cells were isolated from

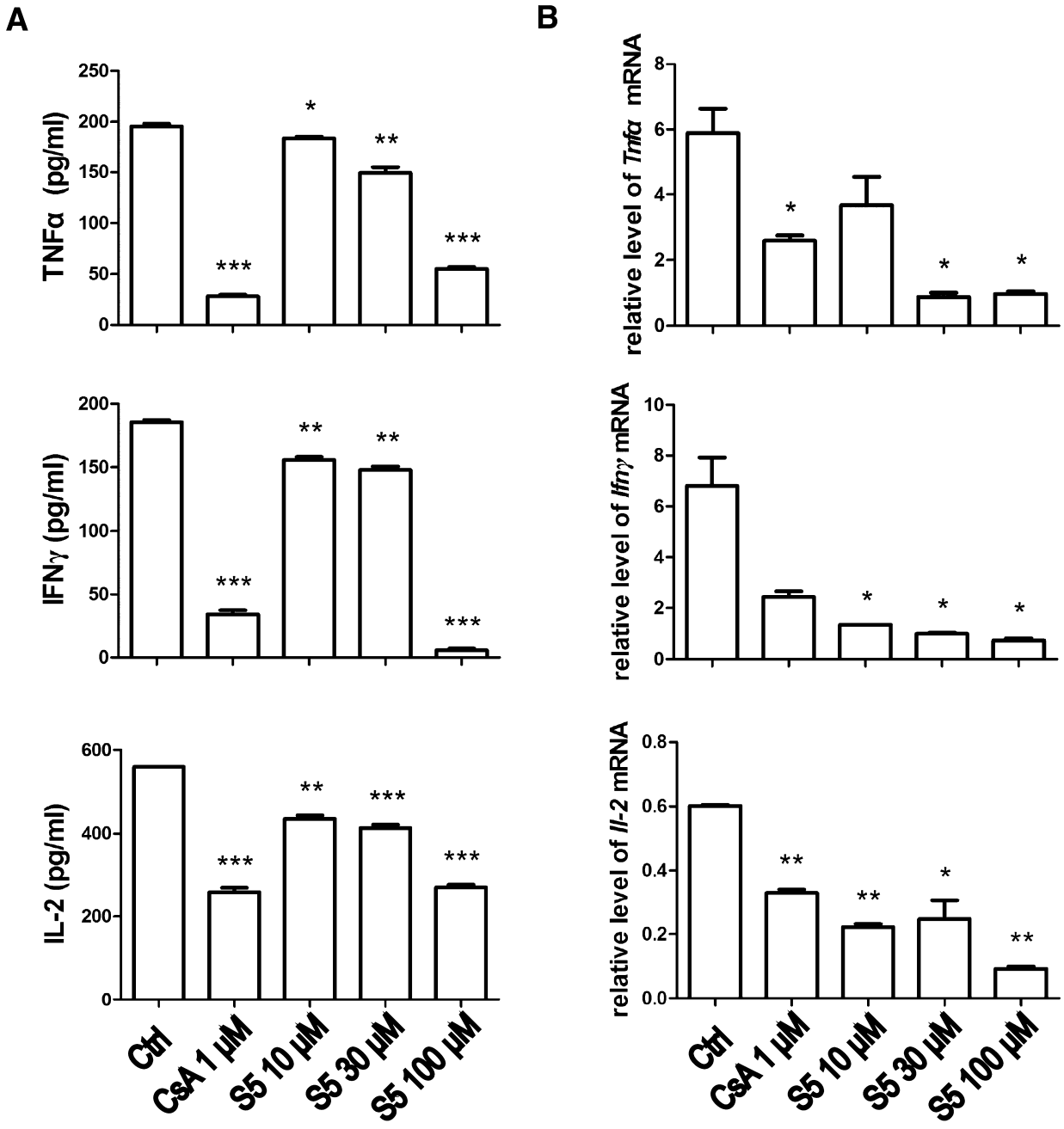


Fig. 5. Effect of neochromine S5 on inflammatory cytokines in Con A-activated T lymphocytes. T lymphocytes isolated from BALB/c mice were treated with Con A and neochromine S5 as above. (A) The content of Inflammatory cytokines in supernatant was measured by ELISA. (B) The mRNA expression was detected with real-time PCR. Data are shown as means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs controls.

PCl-sensitized mice and triggered with hapten TNBS or trinitrophenylated splenocytes. As the results, the treatment of neochromine S5 concentration-dependently reduced CD69 expression in activated T lymphocytes after 12 h (Fig. 7A), and mRNA expressions of *Tnfα*, *Ifnγ* and *Il-2* after 6 h (Fig. 7B). Furthermore, the hapten-specific T cell proliferation was dose-dependently decreased after 72 h treatment of neochromine S5 (Fig. 7C).

4. Discussion

Activated T cell is the potential target for treatment of autoimmune diseases and we have focused our researches on finding compounds with immunosuppressive effect selectively on activated T cells [12–14,19,22–24]. In this study, we chose neochromine S5, a synthesized chromone derivative, to examine its effective properties against activated T cells. The results indicated that, compared with CsA and DEX, neochromine S5 showed high immunosuppressive activity through a selective targeting on activated T cells with low side effects in vitro and in vivo.

Firstly, we found that neochromine S5 suppressed proliferation and induced apoptosis in Con A-activated T cells in a concentration-dependent manner using MTT, CFSE and Annexin V/PI assay (Fig. 1B and C). It should be noted that neochromine S5 had little effect on non-activated T cells at the work concentrations mentioned above (Fig. 1D). In comparison, the chromone compound quercetin also inhibited the proliferation of activated T cells and caused apoptosis of both activated and non-activated

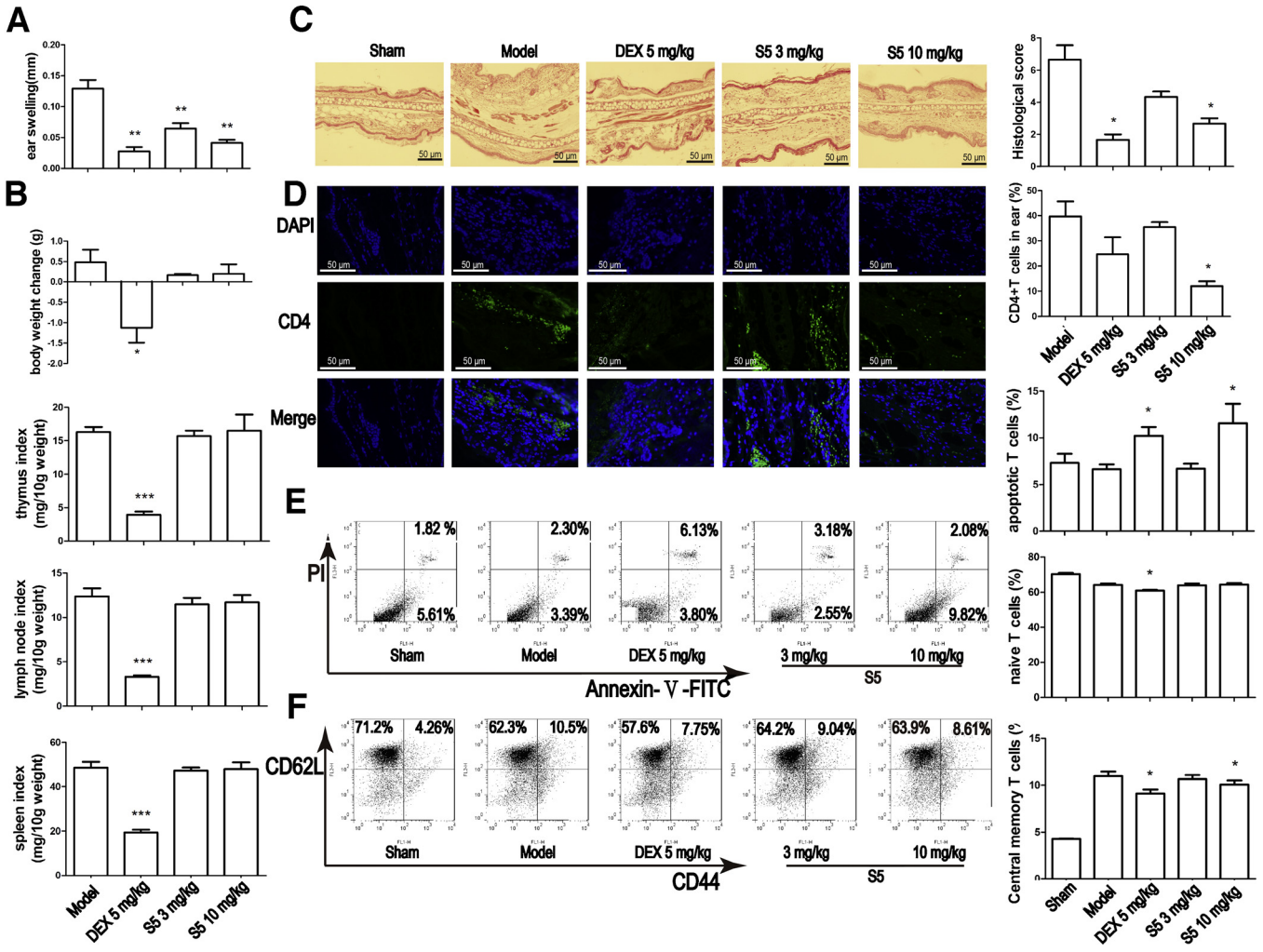


Fig. 6. Effect of neochromine S5 on PCl-induced contact hypersensitivity in mice. Mice were sensitized by PCl on the abdomen and drugs were given orally at different dosages per day after the first sensitization. After 5 days treatment, mice were challenged with PCl on the right ear. 24 h later, the left and right ears thickness were measured with an engineer's micrometer and tissues were harvested. (A) Ear swelling. (B) Body weight, thymus index, lymph node index and spleen index. (C) Representative photographs of HE-stained ear sections and histological score. (D) Immunofluorescence of $CD4^+$ T cells in ear sections labeled with mouse anti-CD4-FITC and DAPI. Cell number was counted using image J software and showed in the right. (E) T lymphocytes were isolated from peripheral lymph nodes at the end of the experiments, and stained with Annexin V/PI for flow cytometry assay. (F) T lymphocytes were isolated from peripheral lymph nodes at the end of the experiments, and stained with anti-CD44/anti-CD62L for flow cytometry assay. The column charts about percentage of apoptosis T cells, naïve T cells, and central memory T cells were shown in the right. Values are means ± SEM of eight animals per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs controls.

T cells (Fig. 1). These findings suggest that neochromine S5 may have a unique mechanism underlying its selective effect on activated T cells.

To understand the effect of neochromine S5 on apoptotic processes of activated T cells, we further found the collapsing of $\Delta\Psi$m (Fig. 2A), increases in PARP cleavage, Bad expression, caspase 3 and caspase 9 activation and reduction in Bcl-2 expression in the neochromine S5-treated activated T cells (Fig. 2B and C). In addition, a significantly increased release of cytochrome *c* from mitochondria to cytosol was also observed dose-dependently (Fig. 2D and E). These results indicate that neochromine S5 induced apoptosis of activated T cells through a mitochondrial pathway.

Intrinsic mitochondria-dependent pathway, extrinsic death receptor-dependent pathway and the intrinsic ER stress-mediated pathway work together to regulate the function of T lymphocytes [7]. Although Fas ligand (FasL), and Fas-mediated signaling play a role in the induction of lymphocyte apoptosis [25], neochromine S5 was still capable of inducing apoptosis in activated $FADD^{-/-}$ Jurkat cells which had deficiency in the Fas pathway. Besides neochromine S5 failed to induce the expression of ER stress-related proteins PERK, XBP-1 and GADD153 in activated T cells (Fig. 3). These results indicated neither Fas-dependent pathway nor ER stress-mediated pathway was involved in neochromine S5 induced apoptosis. Therefore, the activation of intrinsic mitochondrial pathway is predominantly involved in the effect of neochromine S5 against activated T cells.

Additionally, CD25, CD69 and inflammatory cytokines are closely correlated with development of autoimmune diseases [8–11]. Besides of the inducing apoptosis in activated T cells, neochromine S5 also showed inhibition on cell surface markers and inflammatory cytokines production (Figs. 4 and 5).

Contact hypersensitivity, an animal model of allergic contact dermatitis, is recognized as a classical example of T cell-mediated delayed type immune response to cutaneous sensitization and subsequent challenge with haptens [26–29]. The pathogenesis of contact hypersensitivity can be characterized as delayed-type hypersensitivity (DTH) which is typically used for evaluation of in vivo immunocompetency since it entirely depends on effects of T cells. In the model, naïve T cells were primed and differentiate into central memory T cells and effector T cells when the antigen-presenting cells present antigenic peptides to them [30]. Specific for, T cells in inflammatory ears were T_E, and regional lymph nodes

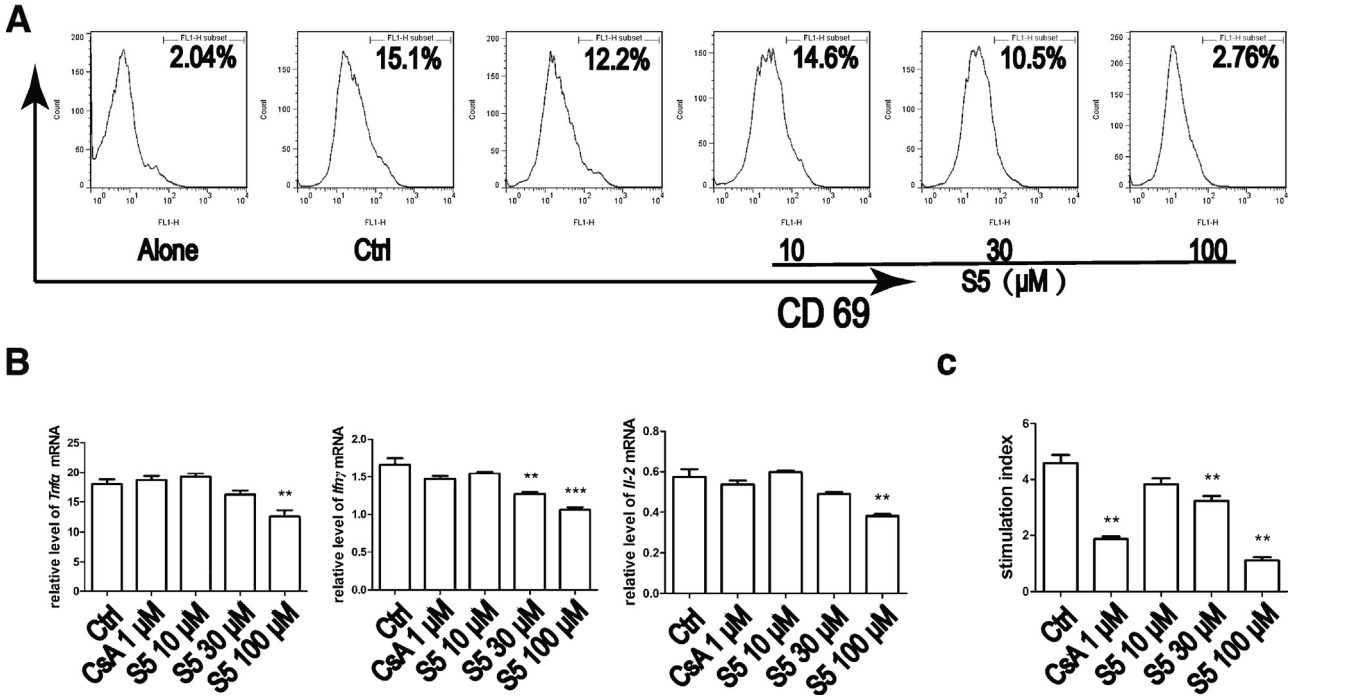


Fig. 7. Effects of neochromine S5 on activation and proliferation of hapten-specifically activated T cells ex vivo. T cells isolated from mice after 5-day sensitization were triggered with TNBS (1.0 mM) in the presence of neochromine S5. (A) After incubation for 12 h, CD69 expression was determined with flow cytometry. Data shown here are one of three independent experiments. (B) T cells were harvested for mRNA detection with real-time PCR at 6 h after incubation. (C) Splenocytes isolated from PCl-sensitized mice after removing the adherent cells were co-cultured with trinitrophenylated splenocytes in the presence of neochromine S5 for 72 h at 37 °C. The cell proliferation was assessed by MTT. For other details, see the Section 2. Data are shown as means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs controls.

were naïve T cells and central memory T cells [31]. In our experiments, oral administration of neochromine S5 significantly inhibited the ear swelling in response to PCl, prevented infiltration of $CD4^+$ T cells (effector T cells) in the edematous sections, induced T lymphocytes apoptosis, decreased the percentage of central memory T cells in draining lymph nodes, and showed no effect in naïve T cells (Fig. 6). Neochromine S5 also greatly blocked the proliferation of hapten-specifically activated T cells (Fig. 7), which were usually seen in the skin challenged by the relevant haptens [32–34]. However, neochromine S5 had no influence on the migration of T cells (data was not shown here). Thus, neochromine S5 maybe induced apoptosis of central memory T cells and effector T cells to suppressed contact hypersensitivity in mice.

Inflammatory factors released from macrophages also contribute to the response in contact hypersensitivity, so we examined effect of neochromine S5 in macrophages. And results showed neochromine S5 had low inhibition on IL-1β and IL-6 levels in LPS-primed bone marrow–derived macrophage (BMDM) (data was not shown).

It was noteworthy that neochromine S5 did not induce body weight loss and immune organ atrophy, perhaps due to neochromine S5 showing no effects on naïve T lymphocytes. However, DEX-treated mice were greatly sacrificed with these side effects in contact hypersensitivity. These results revealed that neochromine S5 had potential for the treatment of T cell-mediated disorders because of its high selectivity and low toxicity.

In conclusion, neochromine S5 inhibited proliferation and increased apoptosis of activated T cells, and it was efficacious in T cell-mediated DTH. Unlike the classic immunosuppressants, CsA and DEX, as well as a flavone compound quercetin, neochromine S5 showed low toxicity owing to its selectivity on activated T cells, not naïve T cells. Therefore, this compound might provide a lead compound from new chromone derivatives for developing novel immunosuppressants distinct from the present approaches.

Conflict of interest

Q. Xu and X. Wu are currently exploring the commercial implications of these findings and there is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bcp.2014.08.032.

References

[1] Strasser A, Pellegrini M. T-lymphocyte death during shutdown of an immune response. Trends Immunol 2004;25:610–5.
[2] Panneerselvam P, Singh LP, Selvarajan V, Chng WJ, Ng SB, Tan NS, et al. T-cell death following immune activation is mediated by mitochondria-localized SARM. Cell Death Differ 2013;20:478–89.
[3] Vergani D, Choudhuri K, Bogdanos DP, Mieli-Vergani G. Pathogenesis of autoimmune hepatitis. Clin Liver Dis 2002;6:727–37.

[4] Granado-Serrano AB, Martin MA, Bravo L, Goya L, Ramos S. Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). J Nutr 2006;136:2715–21.
[5] Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res 2009;11:206.
[6] Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 2001;93:1714–23.
[7] Opferman JT, Korsmeyer SJ. Apoptosis in the development and maintenance of the immune system. Nat Immunol 2003;4:410–5.
[8] Marzio R, Mauel J, Betz-Corradin S. CD69 and regulation of the immune function. Immunopharmacol Immunotoxicol 1999;21:565–82.
[9] Gleisner MA, Reyes P, Alfaro J, Solanes P, Simon V, Crisostomo N, et al. Dendritic and stromal cells from the spleen of lupic mice present phenotypic and functional abnormalities. Mol Immunol 2013;54:423–34.
[10] Streitz M, Fuhrmann S, Thomas D, Cheek E, Nomura L, Maecker H, et al. The phenotypic distribution and functional profile of tuberculin-specific CD4 T-cells characterizes different stages of TB infection. Cytometry B: Clin Cytom 2012;82:360–8.
[11] Barnaba V, Benvenuto R, Buttinelli C, Salvetti M, Toma L, Fiori P. Phenotypic and functional characterization of cloned T-lymphocytes from cerebrospinal fluid of patients with multiple sclerosis. Riv Neurol 1990;60:183–5.
[12] Shen Y, Luo Q, Xu H, Gong F, Zhou X, Sun Y, et al. Mitochondria-dependent apoptosis of activated T lymphocytes induced by astin C, a plant cyclopeptide, for preventing murine experimental colitis. Biochem Pharmacol 2011;82:260–8.
[13] Sun Y, Qin Y, Gong FY, Wu XF, Hua ZC, Chen T, et al. Selective triggering of apoptosis of concanavalin A-activated T cells by fraxinellone for the treatment of T-cell-dependent hepatitis in mice. Biochem Pharmacol 2009;77:1717–24.
[14] Feng LL, Wu XF, Liu HL, Guo WJ, Luo Q, Tao FF, et al. Vaticaffinol, a resveratrol tetramer, exerts more preferable immunosuppressive activity than its precursor in vitro and in vivo through multiple aspects against activated T lymphocytes. Toxicol Appl Pharmacol 2013;267:167–73.
[15] Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. Nat Rev Immunol 2004;4:24–34.
[16] Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351:2715–29.
[17] Kahan BD. Individuality: the barrier to optimal immunosuppression. Nat Rev Immunol 2003;3:831–8.
[18] Wang L, Shen Y, Song R, Sun Y, Xu J, Xu Q. An anticancer effect of curcumin mediated by down-regulating phosphatase of regenerating liver-3 expression on highly metastatic melanoma cells. Mol Pharmacol 2009;76:1238–45.
[19] Fei M, Wu X, Xu Q. Astilbin inhibits contact hypersensitivity through negative cytokine regulation distinct from cyclosporin A. J Allergy Clin Immunol 2005;116:1350–6.
[20] Morris JC, Waldmann TA. Advances in interleukin 2 receptor targeted treatment. Ann Rheum Dis 2000;59(Suppl. 1):i109–14.
[21] Zhou J, Stanners J, Kabouridis P, Han H, Tsoukas CD. Inhibition of TCR/CD3-mediated signaling by a mutant of the hematopoietically expressed G16 GTP-binding protein. Eur J Immunol 1998;28:1645–55.
[22] Wu XF, Xu R, Ouyang ZJ, Qian C, Shen Y, Wu XD, et al. Beauvericin ameliorates experimental colitis by inhibiting activated T cells via downregulation of the PI3K/Akt signaling pathway. PLoS ONE 2013;8:e83013.
[23] Wu X, Fan J, Ouyang Z, Ning R, Guo W, Shen Y, et al. Tupistra chinensis extract attenuates murine fulminant hepatitis with multiple targets against activated T lymphocytes. J Pharm Pharmacol 2014;66:453–65.
[24] Guo W, Liu W, Hong S, Liu H, Qian C, Shen Y, et al. Mitochondria-dependent apoptosis of con A-activated T lymphocytes induced by asiatic acid for preventing murine fulminant hepatitis. PLoS ONE 2012;7:e46018.
[25] Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992;356:314–7.
[26] Eisen HN, Orris L, Belman S. Elicitation of delayed allergic skin reactions with haptens; the dependence of elicitation on hapten combination with protein. J Exp Med 1952;95:473–87.
[27] Flax MH, Caulfield JB. Cellular and vascular components of allergic contact dermatitis. Am J Pathol 1963;43:1031–53.
[28] Kaplan DH, Igyarto BZ, Gaspari AA. Early immune events in the induction of allergic contact dermatitis. Nat Rev Immunol 2012;12:114–24.
[29] Kimber I, Maxwell G, Gilmour N, Dearman RJ, Friedmann PS, Martin SF. Allergic contact dermatitis: a commentary on the relationship between T lymphocytes and skin sensitising potency. Toxicology 2012;291:18–24.
[30] Riemann H, Schwarz T, Grabbe S. Pathomechanisms of the elicitation phase of allergic contact dermatitis. J Dtsch Dermatol Ges 2003;1:613–9.
[31] Ueyama A, Imura K, Kasai-Yamamoto E, Tai N, Nagira M, Shichijo M, et al. Kv1.3 blockers ameliorate allergic contact dermatitis by preferentially suppressing effector memory T cells in a rat model. Clin Exp Dermatol 2013;38:897–903.
[32] Yamamoto H, Nonaka M, Katz DH. Suppression of hapten-specific delayed-type hypersensitivity responses in mice by idiotype-specific suppressor T cells after administration of anti-idiotypic antibodies. J Exp Med 1979;150:818–29.
[33] Cavani A, Albanesi C, Traidl C, Sebastiani S, Girolomoni G. Effector and regulatory T cells in allergic contact dermatitis. Trends Immunol 2001;22:118–20.
[34] Grabbe S, Schwarz T. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. Immunol Today 1998;19:37–44.