

Verbascoside Alleviates Atopic Dermatitis-Like Symptoms in Mice via Its Potent Anti-Inflammatory Effect

Yongxi Li Haiyang Yu Yan Jin Min Li Caijie Qu

Qingdao Municipal Hospital, Qingdao, China

Keywords

Atopic dermatitis · DNCB · Verbascoside · Th2 · Inflammation

Abstract

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by the interplay between multiple genetic and environmental factors. The pathogenesis of AD remains incompletely understood. Treatment with topical steroids for chronic AD symptoms has severe side effects and so a new treatment is required. Verbascoside is a hydrophilic phenylethanoid glycoside with antioxidant, anti-inflammatory properties. **Methods:** Verbascoside was evaluated in AD-like lesions induced by the repetitive and alternative application of 2,4-dinitrochlorobenzene (DNCB) in BALB/c mice. Overall symptomatic score and serological and molecular changes of the skin lesions were investigated. **Results:** Verbascoside relieved the overall AD-like symptoms such as scratching behavior and skin lesion severity. At whole-body level, verbascoside significantly reduced DNCB-induced IgE and Th2 cytokines in the peripheral blood. At the skin lesion site, verbascoside also inhibited DNCB-induced production of proinflammatory cytokine TNF- α , IL-6, and IL-4 mRNA. In a human monocyte THP-1 model, verbascoside

could suppress DNCB-induced upregulation of CD86 and CD54 at the cell surface, the secretion of the proinflammatory cytokines TNF- α and IL-6, and the activation of NF κ B signaling in a dose-dependent manner. **Conclusion:** Our results demonstrate that verbascoside could be a potential therapeutic agent for the treatment of AD. © 2018 S. Karger AG, Basel

Introduction

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease, and is characterized by complex pathophysiology and a wide spectrum of clinical phenotypes [1, 2]. AD usually presents during early infancy and childhood, but it can also persist into or even start in adulthood. It affects 11–30% of children and 2–10% of adults, depending on the population studied [1, 3]. It is often manifested as erythematous, thickened, epidermal lesions that vary in their intensity and the body surface area that is affected [2, 4]. It is a major skin disorder that imposes a considerable burden on patients' quality of life, health outcomes, and work productivity [5].

Edited by: Holger Garn, Marburg.

Early models of etiology attributed the symptoms of AD to cutaneous inflammation at the lesion sites, but recent studies have established that it is driven by both terminal keratinocyte differentiation defects and strong type 2 immune responses [6, 7]. Several observations suggest that AD is the cutaneous manifestation of a systemic disorder that also gives rise to asthma, food allergy, and allergic rhinitis [8, 9]. These conditions are all characterized by elevated serum IgE levels and peripheral eosinophilia [10, 11]. The major elements involved in the immune dysregulation are Langerhans cells, inflammatory dendritic epidermal cells, monocytes, macrophages, lymphocytes, mast cells, and keratinocytes. The interactions between different cell compartments lead to a predominance of Th2 over Th1 cells through an intricate cascade of cytokine signaling. For instance, the Th2 cytokines IL-4, IL-5, IL-10, and IL-13 are increased in the skin and blood, and there is a corresponding decrease in Th1 cytokines, mainly IFN- γ and IL-2 [12, 13]. In turn, cutaneous inflammation and immune dysregulation affect epidermal structure and function. The crosstalk between keratinocytes and immune cells is also crucial in the regulation of itch.

Although many immunopathological features of AD have recently been elucidated, it is worth noting that the fundamental etiology of these abnormalities remains unclear. AD cannot be cured at present, and the aim of management is to alleviate symptoms and achieve long-term disease control with multistep approaches [14–16]. Topical steroid therapy remains the first-line treatment for AD. However, this treatment for chronic AD is associated with severe side effects, including atrophy, acne, cataracts, growth retardation, and red, burning skin. A new therapeutic agent is required to tackle the challenge, and natural products have become attractive candidates.

Phenylethanoid glycosides are naturally existing, water-soluble compounds with remarkable biological properties that are widely distributed in the plant kingdom [17]. Structurally, they are characterized by cinnamic acid (C₆–C₃) and hydroxyphenylethyl (C₆–C₂) moieties that are attached to a β -glucopyranose (apiose, galactose, rhamnose, xylose, etc.) via a glycosidic bond [18]. Verbascoside is a phenylethanoid glycoside that was first isolated from mullein (the leaves of *Verbascum thapsus*), and later also found in several other plant species. Traditionally, plants with high concentrations of verbascoside have been used in folk medicine to treat inflammation and microbial infections. Verbascoside appears to be involved in the inhibition of type I allergies. It exhibits profound anti-inflammatory activities towards keratinocytes that are

stimulated by bacterial lipopolysaccharides (LPS) and IFN- γ [19]. The anti-inflammatory and anti-irritant activities of verbascoside from *Kigelia africana* have been attributed to its ability to inhibit inducible nitric oxide synthase (iNOS) and NO release from macrophages as stimulated by LPS [20]. Verbascoside has been reported to exhibit antiproliferative activities towards some tumor cells in vitro [21], induce human gastric carcinoma cell differentiation and apoptosis by telomere-telomerase-cell cycle-dependent modulation [22], and repair DNA damage caused by oxidative stress [23].

In this study, we aimed to characterize the therapeutic effect of the topical application of verbascoside on AD-like skin lesions. We used a well-established murine AD model in which BALB/c mice were challenged by 2,4-dinitrochlorobenzene (DNCB). Our results demonstrated that verbascoside reduced the incidence of DNCB-induced scratching and also dermatitis severity. It attenuated the DNCB-induced elevation of IgE and Th2 cytokines (IL-4 and IL-13) in the blood. It inhibited the DNCB-induced expression of proinflammatory cytokine (TNF- α , IL-6, and IL-4) mRNA in skin lesions. Verbascoside can dose-dependently antagonize the upregulation of the costimulatory molecules CD86 and CD54 and secretion of the proinflammatory cytokines TNF- α and IL-6 in THP-1 cells upon DNCB stimulation. It can potentially block the activation of the NF κ B signaling pathway under the same conditions. Our study provides a mechanistic understanding on the regulatory function of verbascoside in a DNCB-induced AD model, and supports its therapeutic potential for AD treatment.

Methods

Reagents

Verbascoside ($\geq 99\%$ HPLC, #V4015) and DNCB ($\geq 99\%$, #237329) were purchased from Sigma, anti-human CD54 (clone; 6.5B5) and its mouse IgG isotype control from DAKO (Glostrup, Denmark), anti-human CD86 (clone; Fun-1) and its mouse IgG isotype control from BD Pharmingen (San Diego, CA, USA), and p65, phosphorylated (p)-p65 (pS536), I κ B α , and p-I κ B α (pS32) antibodies were from Cell Signaling. The mouse filaggrin ELISA kit (LS-F9021) was from LSBio.

Cell Culture

THP-1 cells were purchased from ATCC. Cells were cultured in RPMI 1640 medium (GIBCO BRL, Grand Island, NY, USA) containing 1% (v/v) 100 U/mL penicillin G, and 100 mg/mL streptomycin (GIBCO), 10% (v/v) heat-inactivated fetal bovine serum medium (Hyclone, USA) and 2 mM L-glutamine (GIBCO). All the cells were then maintained in 25-cm² culture flasks at 37 °C in humidified cell incubator supplemented with 5% CO₂.

Mouse Strain

All experimental protocols involving the use of live animals were approved by the Institutional Animal Care and Use Committee. The 6- to 8-week-old male and female BALB/c mice were obtained from the Animal Center and housed with 5 mice per cage in a room maintained at $23 \pm 3^\circ\text{C}$ and a relative humidity of $55 \pm 10\%$ throughout the study. Animal treatment and maintenance complied with guidelines issued by the ethics committee for animal welfare.

DNCB-Induced AD Model

To induce AD-like symptoms, DNCB (Sigma-Aldrich, MO, USA) was used. Briefly, the dorsal hair of the mice was removed using an electronic clipper 2 days before DNCB treatment. An application of 200 μL of 1% DNCB (in acetone:olive oil = 4:1) was made to the shaved dorsal skin for sensitization. The repeated challenge was performed on the same site with 0.2% DNCB once every 3 days for about 2 weeks. The mice were divided into 4 groups ($n = 6$ per group): (1) vehicle-treated controls, (2) DNCB-treated only, (3) 1% verbascoside (in acetone:olive oil 4:1)-treated only, and (4) DNCB + 1% verbascoside-treated group.

Evaluation of Scratching Behavior in Mice with AD-Like Skin Lesions

Scratching behavior was observed after the completion of all treatments. The mice were placed in cages and allowed to acclimatize for 15 min, and the number of scratching behaviors in the next 10 min was counted. This was repeated 6 times (60 min in total). Scratching behavior was defined in this experiment as movement with the hind paws. Scratching of the nose, ears, and dorsal skin with the hind paws was recorded, while licking of the belly and dorsal skin during grooming was ignored. All evaluations were performed in a blinded manner.

Evaluation of the Severity of AD-Like Skin Lesions

The severity of the dermatitis in the dorsal skin of DNCB-induced AD mice was evaluated on day 18. The severity of 4 symptoms (erythema/hemorrhage, edema, excoriation/erosion, and scarring/dryness) was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). Dermatitis score was defined as the sum of these individual scores in accordance with the method of Matsuda et al. [24].

Hematoxylin and Eosin Staining and Measurement of Skin Thickness

Histological sections (4 μm) were cut from paraffin-embedded formalin-fixed skin tissues harvested from the AD lesions. Sections were stained with H&E, and skin thickness was determined with ImageJ software by measuring the distance from the top of the granular layer to the junction between the dermis and the subcutaneous fat.

Measurement of Cytokine Levels in Serum by ELISA

Whole blood was collected from mice in each of the treatment groups on the last day of the study (day 19). The serum was then collected by centrifugation for 20 min at 12,000 rpm and stored at -80°C until further use. The level of serum IgE was measured with ELISA kits: total IgE ELISA (ab157718, Abcam, Cambridge, MA, USA) or customized dinitrophenol (DNP)-specific IgE ELISA (D8406, Sigma, USA). The levels of serum cytokines were deter-

mined with ELISA kits for IL-4, IL-13, TNF- α (R&D Systems, Minneapolis, MN, USA), and IL-6 (Raybiotech) following the manufacturer's instructions.

RNA Extraction and Real-Time PCR Analysis

Total RNA in skin tissue was isolated using Trizol[®] reagent according to the protocol from the manufacturer on day 45. The equal amount of RNA was reversely transcribed into cDNA using Prime Script[™] RT reagent kit with gDNA Eraser[®]. Quantitative real-time PCR was performed using Stratagene M $\times$ 3000 P (Agilent Technologies Inc., Waldbronn, Germany) with SYBR[®] premix Ex Taq[™] and specific primers of TNF- α , IL-4, IL-6, and GAPDH. The amplification program consisted of 1 cycle of 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 31 s, and the last cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Fluorescence intensity was measured in real time using the optical module. Melt curves were used to determine product specificity. Results were normalized to the level of *gapdh* gene expression. Primer sequences were as listed below:

TNF- α

5'-TACTGAACTTCGGGGTGATTGGTCC-3'
5'-CAGCCTTGTCCTTGAAGAGAACC-3'

IL-6

5'-CCGGAGAGGAGACTTCACAG-3'
5'-GGAAATTGGGGTAGGAAGGA-3'

IL-4

5'-AGATGGATGTGCCAAACGTCCTCA-3'
5'-AATATGCGAAGCACCTTGGAAGCC-3'

GAPDH

5'-CACCACCCTGTTGCTGTAGCC-3'
5'-ACCACAGTCCATGCCATCAC-3'

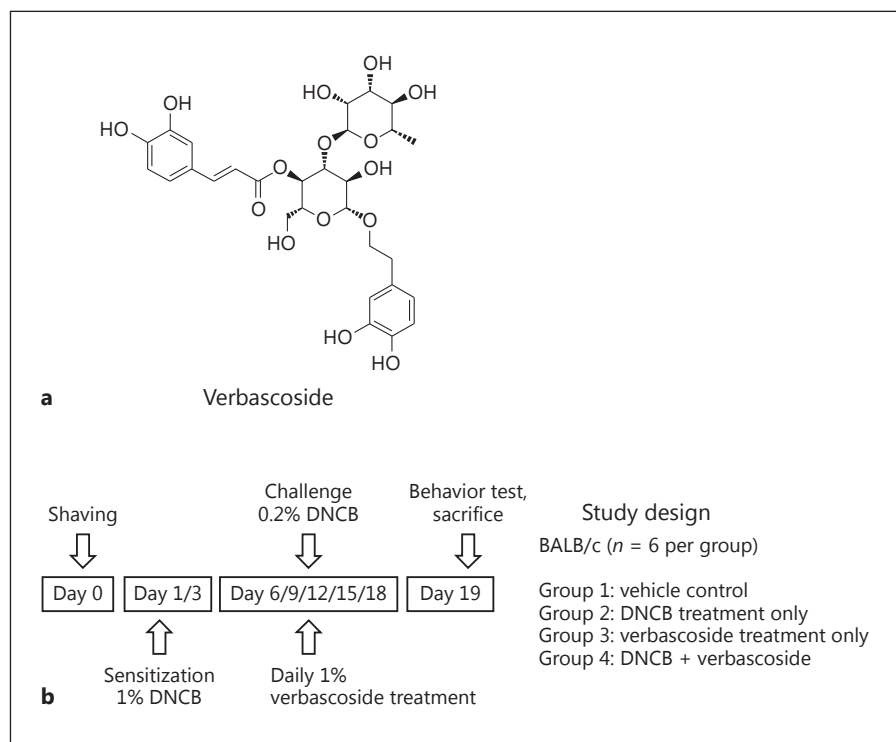
Flow Cytometry

THP-1 cells were stimulated with DMSO (vehicle control), DNCB (5 $\mu\text{g}/\text{mL}$), or DNCB plus verbascoside (5 or 10 μM) at the same time for 24 h. An equal amount of cells was stained with antibodies specific for human CD86 and CD54 for 30 min, followed by FITC-conjugated secondary antibody incubation in a staining buffer (PBS containing 1% FBS and 0.01% sodium azide) for 30 min on ice, and analyzed by fluorescence-activated cell sorting (FACS) on a FACScan analyzer, using Cell-Quest software (both from BD Biosciences, Franklin Lakes, NJ, USA).

Functional Study of ex vivo Differentiated Th2 Cells

Naïve CD4⁺ T cells were harvested from the mouse spleens and differentiated into Th2 CD4⁺ cells ex vivo using the CellXVivo Mouse Th2 cell differentiation kit (R&D Systems). On day 6 of differentiation, they were activated with anti-mouse CD3 and anti-mouse CD28 (provided in the kit). The activated Th2 cells were then treated with different concentrations of verbascoside or PBS as indicated. After treatment for 24 h, the cell culture supernatant was collected, and the secretion of cytokines IL4 and IL5 was determined by ELISA.

Fig. 1. a Chemical structure of verbascoside. **b** Experiment design. The shaved dorsal skin was applied with 200 μ L of 1% DNCB (in acetone:olive oil [4:1]) for sensitization. The repeated challenge was performed at the same site with 0.2% DNCB once every 3 days for about 2 weeks.



Western Blotting Analysis
Lysate from the THP-1 cells after treatment was prepared by incubating the cells in lysis buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol [v/v]) at 4 °C for 30 min. Protein concentration was determined using the BCA protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysate (40 μ g) was resolved in 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Temecula, CA, USA). After being blocked with 10% milk in TBST at room temperature for 1 h, the membrane was then probed with primary antibodies against p65, p-p65 (S536), I κ B α , and p-I κ B α (S32) proteins at 4 °C for 12 h. Protein bands were quantified by densitometric analysis with ImageJ.

Statistical Analysis
All data were presented as mean $\pm$ SD and were analyzed by paired and unpaired two-tailed Student *t* tests. Differences between groups were considered statistically significant when *p* < 0.05.

Results

Verbascoside Ameliorated DNCB-Induced Scratching and Dermatitis Severity

Verbascoside (Fig. 1a) is one of the naturally occurring water-soluble phenylethanoid glycosides that are widely distributed in the plant kingdom, most of which are isolated from medicinal plants. It possesses pharmacologi-

cally beneficial activities for human health, including antioxidant, anti-inflammatory, and antineoplastic properties, in addition to numerous wound-healing and neuroprotective properties. Here, we evaluated its function in the widely accepted murine AD model induced by DNCB. The design of the experiment is shown in Figure 1b. Briefly, BALB/c mice were randomly divided into 4 groups (*n* = 6 per group) including a vehicle control group, a DNCB model group, a verbascoside only group, and a DNCB plus verbascoside treatment group. The animals' backs were shaved on day 0, sensitized with 1% DNCB on days 1 and 3. The DNCB model group was challenged with 0.2% DNCB every 3 days from day 6 to day 18. The vehicle control group was stimulated with vehicle only in the same manner. For the verbascoside only group and the DNCB plus verbascoside treatment group, the animals were treated with 1% verbascoside daily on days 6–18 (Fig. 1b).

The overall disease progression and manifestation were assessed by evaluating the scratching behavior and the severity of the skin lesions in this DNCB-induced AD-like model. Compared to the vehicle controls where scratching was barely seen, the DNCB model group showed scratching behavior approximately 12 times within the 60-min observation period (Fig. 2a). The ver-

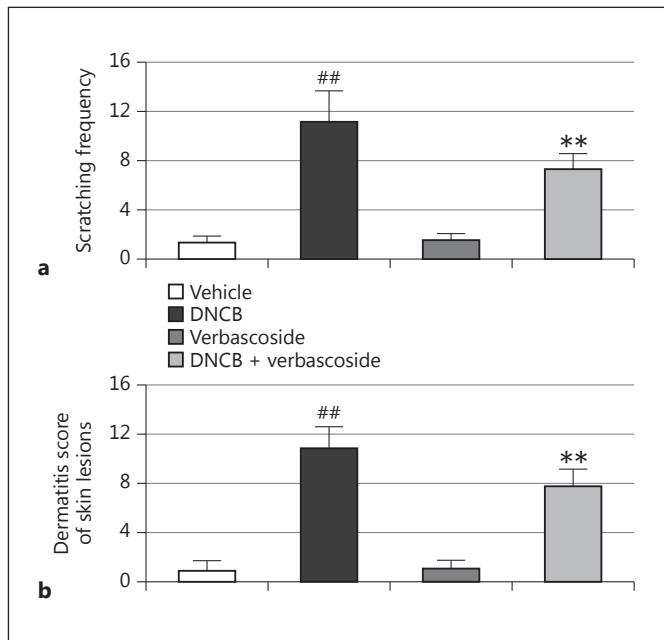


Fig. 2. Effects of verbascoside on DNCB-induced AD-like symptoms in mice. **a** Scratching behavior was assessed on the last day of the study (day 19). Briefly, the number of scratching behaviors over 10 min was recorded; this was repeated 6 times (i.e., for 60 min in total). **b** The severity of clinical symptoms (erythema/hemorrhage, edema, excoriation/erosion, and scarring/dryness) of the AD-like skin lesions was evaluated macroscopically as the dermatitis score. The data are presented as mean $\pm$ SD ($n = 6$). ## $p < 0.01$, significantly different from control group by paired t test; ** $p < 0.01$, significantly different from DNCB treated group by paired t test.

basco-side only group was similar to the vehicle controls, suggesting that it had no irritating effect. The DNCB plus verbascoside treatment group showed a significantly reduced scratching frequency of about 7.5 times/h (Fig. 2a). A similar pattern was observed when scoring the dorsal skin lesions, where we could clearly see that vehicle controls and verbascoside only groups had a very low severity score of 1.5 (Fig. 2b). The DNCB model group's score was 10.5, and the DNCB plus verbascoside treatment group's score was significantly reduced at around 7. In addition, we also performed a histological analysis on skin samples harvested on the last day of the study. As shown in online Supplementary Figure S1A (see www.karger.com/doi/10.1159/000486958 for all online suppl. material), DNCB alone induced cutaneous hyperplasia, which is one of the key features of AD-like lesions. Consistent with the dermatitis score, verbascoside significantly reduced skin hyperplasia caused by DNCB. We quantified the average skin thickness for different treatment

groups (online suppl. Fig. S1B). Verbasco-side only treatment decreased dorsal skin thickness induced by DNCB but it had no effect on skin thickness or organizational structure (online suppl. Fig. S1A, B). At the molecular level, filaggrin is essential for the regulation of epidermal homeostasis and is strongly implicated in AD pathogenesis. DNCB-induced lesions showed a markedly reduced level of filaggrin, implying a perturbed barrier function in our AD-like model (online suppl. Fig. S1C). Interestingly, verbascoside treatment restored the filaggrin level at the AD lesion site, which was in line with the improvement at a physiological level. Taken together, these results indicate that our DNCB-induced AD model was successful, with verbascoside treatment markedly reducing the overall scratching behavior incidence as well as the severity of the skin lesions.

Verbasco-side Suppressed DNCB-Induced Elevation of IgE and the Th2 Cytokines IL-4 and IL-13 in the Blood

Despite the high heterogeneity of AD, recent research has indicated that IgE and the Th2 cytokines play a fundamental role in AD pathogenesis [25–27]. To gain an overall profile of serum IgE and Th2 cytokines, we next examined the IgE, IL-4, and IL-13 levels by ELISA in the different groups of the murine AD-like model. Whole blood was collected at the end of experiment (day 19) and sera were prepared according to ELISA protocol. The IgE level in the peripheral blood was at basal low level in the vehicle and verbascoside only groups, i.e., about 2.5 ng/mL (Fig. 3a). However, DNCB stimulation readily elevated IgE to about 12 ng/mL. Strikingly, the DNCB plus verbascoside treatment group showed an approximately 50% reduction in IgE compared to the DNCB model group. We also sought to measure DNP-specific IgE that was directly involved in DNCB-mediated dermatitis pathogenesis. As shown in online supplementary Figure S2, DNCB stimulation significantly increased DNP-specific IgE in the serum. In line with the total IgE result, verbascoside treatment markedly reduced the serum level of DNP-specific IgE in DNCB-challenged mice. The Th2 cytokines IL-4 and IL-13 were induced by DNCB treatment to about 4–5 times the vehicle group levels of 90 and 400 pg/mL, respectively (Fig. 3b). The verbascoside only group showed levels of IL-4 and IL-13 similar to those in the vehicle control group. These 2 cytokines were reduced by one-third in the DNCB plus verbascoside treatment group when compared to the DNCB model group. These ELISA results indicated that verbascoside treatment was able to significantly suppress the DNCB-mediated induction of IgE and the Th2 cytokines.

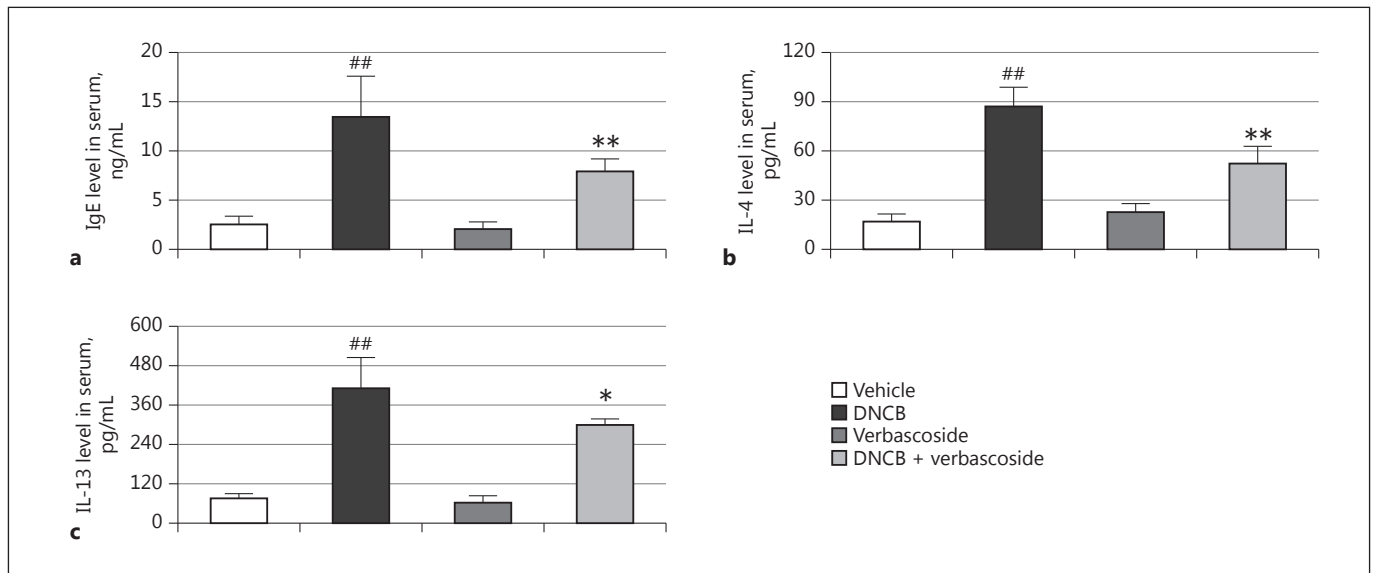


Fig. 3. Effects of verbascoside on the DNCB-induced increase in serum IgE and Th2 cytokines in mice. **a** Sample blood was collected on the last day of the study (day 19). The level of serum IgE was measured with ELISA. **b** Th2 cytokines (IL-4 and IL-13) in

serum were measured with ELISA. The data are presented as mean $\pm$ SD ($n = 6$). ^{##} $p < 0.01$, significantly different from control group by paired t test; ^{*} $p < 0.05$, ^{**} $p < 0.01$, significantly different from DNCB treated group by paired t test.

Verbasco-side Inhibited DNCB-Induced Expression of Proinflammatory Cytokine TNF- α , IL-6, and IL-4 mRNA in Skin Lesions

AD is a multifactorial disorder associated with Th2-polarized CD4 T cells that can produce excess amounts of IL-6, IL-5, IL-4, and IL-13. TNF- α is an important proinflammatory cytokine and is involved in the immunopathology of AD [28]. IL-6 is secreted by activated T cells and macrophages. It regulates the immune response, inflammation and pathogen responses, bone metabolism, and hematopoiesis [9, 25, 29]. To investigate the effect of verbascoside on the skin lesions induced by DNCB, we next measured the local mRNA level in the lesion tissue by real-time PCR. The DNCB stimulation group showed enhanced levels of TNF- α and IL-6, to about 6 times the level in the vehicle control group which was normalized as 1 (Fig. 4a). The verbascoside only group showed levels of TNF- α and IL-6 similar to those in the vehicle controls. The DNCB plus verbascoside treatment group showed significantly decreased levels of TNF- α and IL-6, approximately 60–70% of the DNCB model group levels. We also measured the IL-4 level in the same experiment. IL-4 is a key cytokine to promote Th2 lineage differentiation and induce B cell class switching to IgE. A similar pattern was observed to the results with TNF- α and IL-6. IL-4 mRNA in DNCB model group tissues were induced to

about 4 times the basal level observed in the vehicle group (Fig. 4b). DNCB plus verbascoside treatment showed a significant decrease (50%) in the IL-4 level compared to the DNCB model group. These results were consistent with the previous ELISA data about the overall serum IL-4 level, and suggested that verbascoside was able to locally suppress the immune response elicited by DNCB in the skin tissue.

Verbasco-side Antagonized DNCB-Induced T Cell Costimulatory Factors CD86 and CD54, Proinflammatory Cytokines, and NF κ B Pathway Activation in THP-1 Cells

To understand the molecular mechanism of the verbascoside effect and extend the murine model results to a human cell system, we next used a widely accepted human monocyte cell line, THP-1, to investigate the detailed function of verbascoside on critical costimulatory signaling for the activation of T cells such as CD86 and CD54 and proinflammatory cytokines such as TNF- α and IL-6. THP-1 is a human leukemia monocytic cell line extensively used to study monocyte/macrophage functions, mechanisms, and signaling pathways [30]. Macrophages have been shown to play an important role in AD immunopathogenesis [9, 25]. CD86 is an important costimulatory molecule on antigen-presenting cells, and helps with

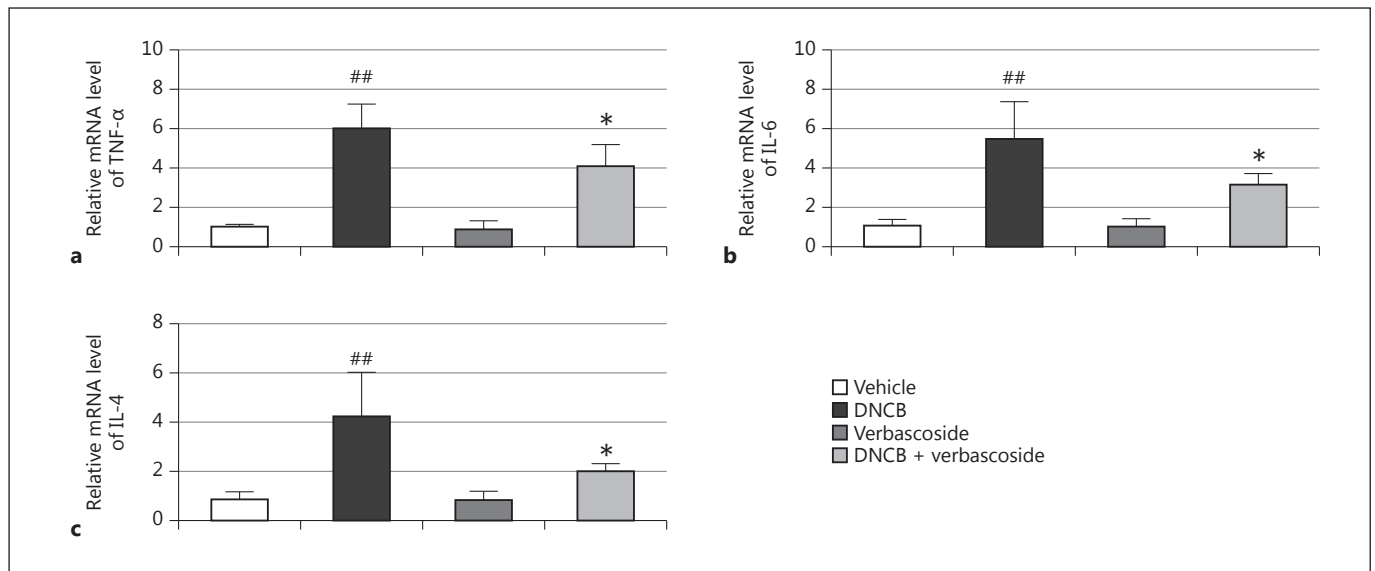


Fig. 4. Effects of verbascoside on the expression of cytokines in the skin of DNCB-treated mice. The dorsal skin was collected on the last day of the study (day 19). Total RNA was extracted from skin tissue with Trizol reagent. The proinflammatory cytokines TNF- α (**a**) and IL-6 (**b**) were determined by real-time PCR. **c** IL-4 expres-

sion level was also measured by real-time PCR. The data are presented as mean $\pm$ SD ($n = 6$). ^{##} $p < 0.01$, significantly different from control group; ^{*} $p < 0.05$, significantly different from DNCB-treated group as analyzed with the paired t test.

prime T cell differentiation and activation. CD54 is a cell adhesion molecule, expressed on macrophages, lymphocytes, and endothelial cells, which promotes stable interaction and facilitates leukocyte endothelial transmigration. CD86 and CD54 expression on THP-1 cells was used in an in vitro skin sensitization assay to monitor the activity of sensitizer such as DNCB [31, 32]. CD86 and CD54 levels on the THP-1 cell surface were induced by 5- μ M DNCB stimulation to about 3 times that of the DMSO-treated control group (Fig. 5a). The DNCB plus verbascoside treatment showed a dose-dependent suppression of both CD86 and CD54, the 5- μ M verbascoside-treated sample with a 30% decrease and 10- μ M of verbascoside causing a more significant reduction of 50% compared to DNCB stimulation group. Isotype controls for anti-CD86 and anti-CD54 were also tested on DNCB-stimulated cells to confirm the specificity of target detection in our assay (online suppl. Fig. 4). Similarly, the levels of TNF- α and IL-6 secreted by THP-1 cells upon treatment with DNCB were 25- and 16-fold the basal levels, respectively (Fig. 5b). The TNF- α level was reduced to 15- and 11-fold that of the basal level by the 5- μ M and 10- μ M verbascoside treatment, respectively. The IL-6 level was decreased by 12- and 8-fold the basal level by the 5- μ M and 10- μ M verbascoside treatment, respectively, suggest-

ing a strong dose-dependent response. To confirm that verbascoside did not affect THP-1 viability, we also measured cell death with TUNEL assay. The DNCB and verbascoside treatments did not affect the percentage of dead cells (online suppl. Fig. S3).

We next investigated the molecular signaling mechanism of verbascoside treatment by Western blotting. As previously reported, verbascoside can inhibit activation of the NF κ B pathway [17, 33], a master transcription factor regulating the downstream response of many proinflammatory stimuli [34]. Here, we also tested whether verbascoside attenuated the DNCB-induced inflammatory response via the modulation of NF κ B activation. DNCB-treated samples showed strong activation of p65 and I κ B α compared to the DMSO-treated controls (Fig. 5c, lane 2 vs. lane 1). The 5- μ M verbascoside treatment significantly downregulated the phosphorylation of p65 and I κ B α . The 10- μ M verbascoside treatment almost completely abolished the activation of the NF κ B components, with phosphorylation of p65 and I κ B α being reduced to a level similar to that in the DMSO-treated controls. We also quantified the activation of I κ B α and p65 with densitometry. Verbasco-side showed a dose-dependent suppression of DNCB-induced I κ B α and p65 phosphorylation (Fig. 5d). This result revealed that verbasco-

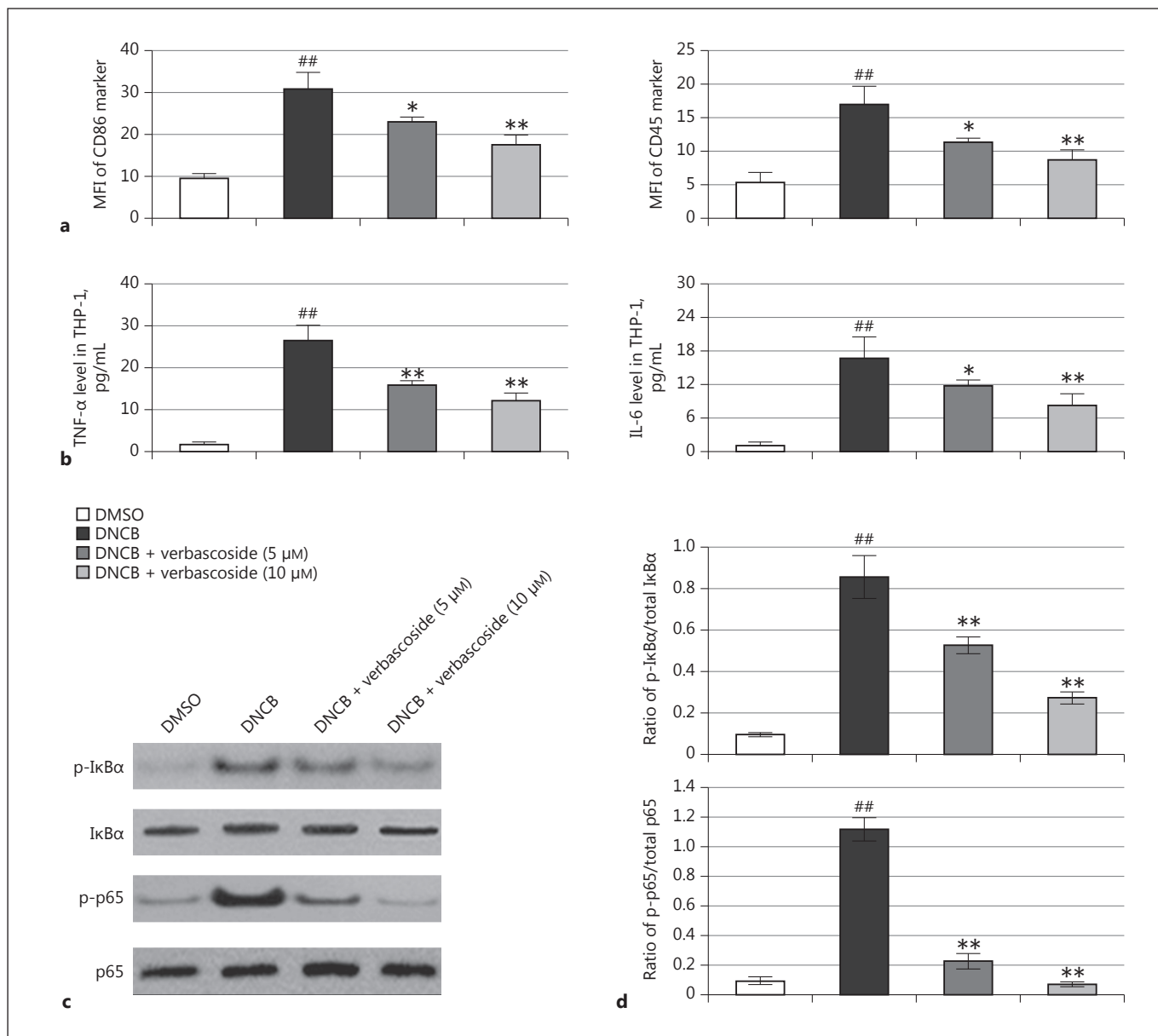


Fig. 5. Effects of verbascoside on the biological response of THP-1 to DNCB. **a** THP-1 cells were treated with the indicated chemicals for 24 h before harvesting. Cell surface markers CD86 and CD45 were measured by flow cytometry. The mean fluorescence intensity (MFI) was used as indicator of surface marker expression. **b** The cell supernatants were also collected for the measurement of TNF-α and IL-6 production. **c** The cell lysate of THP-1 after treatment was subjected to SDS-PAGE to determine the levels of total

p65, phosphorylated (p)-p65 (S536), total IκBα, and p-IκBα (S32). **d** Densitometry analysis of Western blot was performed on THP-1 cells. The values represent the ratio of p-IκBα/total IκBα or p-p65/total p65. Data are representatives of at least three independent experiments. ^{##} $p < 0.01$, significantly different from DMSO treatment; ^{*} $p < 0.05$, ^{**} $p < 0.01$, significantly different from DNCB treatment as analyzed with unpaired t test.

side was able to potently suppress the DNCB-induced activation of NFκB signaling pathways; this served as part of the underlying mechanism for the previous results obtained in THP-1 (Fig. 5a, b). In addition, we also tested

the effect of verbascoside in vitro on differentiated Th2 cells. We measured the production of Th2 cytokines IL-4 and IL-5 as a functional readout. As shown in online supplementary Figure 5, activated Th2 CD4⁺ cells produced

higher levels of IL-4 and IL-5. As expected, verbascoside treatment directly decreased IL-4 and IL-5 production in Th2 cells with a dose-dependent effect. Taken together, verbascoside may exert pleiotropic immunomodulatory effects on the different immune cells involved in AD pathogenesis.

Discussion

AD is the most common chronic inflammatory skin disease, and it often precedes asthma and allergic disorders. It is driven by both terminal keratinocyte differentiation defects and a strong type 2 immune response. AD pathology is characterized with skin barrier abnormalities and immune dysregulation, at local and systemic levels and with high heterogeneity [1, 2, 4]. It imposes considerable burdens upon the quality of life, health outcomes, and work productivity of sufferers, given that the standard steroid treatment has intrinsic side effects and that AD is often associated with microbial organism infection [10, 15]. Verbascoside is a naturally occurring, water-soluble phenylethanoid glycoside, which is hydrophilic in nature and possesses pharmacologically beneficial activities for human health, including antioxidant, anti-inflammatory, and antineoplastic properties, in addition to numerous wound-healing and neuroprotective properties [17, 18, 35].

In this study, we tested the effect of verbascoside in an DNCB-induced AD-like murine model. Our results demonstrated that verbascoside could ameliorate the overall manifestation of AD-like symptoms such as scratching behavior and skin lesion severity. At the whole-body level, verbascoside significantly suppressed DNCB-induced IgE and Th2 cytokines in the peripheral blood. At local skin lesion sites, verbascoside inhibited DNCB-induced proinflammatory cytokines like TNF- α and IL-6 as well as IL-4 mRNA production. Using a human monocyte model with THP-1, we revealed that verbascoside could suppress the upregulation of CD86 and CD54 at the cell surface, and abolish the DNCB-induced proinflammatory cytokines TNF- α and IL-6 as well as the activation of the NF κ B pathway (which served as a mechanism of its anti-inflammatory effect in the AD-like lesions).

As mentioned previously, AD is a highly heterogeneous and complex disease characterized by abnormal immunological and inflammatory pathways, and involves a defective skin barrier, exposure to environmental agents, and neuropsychological factors [2]. AD patients often develop evidence of allergic IgE-mediated sensitiza-

tion. Acute AD skin lesions exhibit Th2-dominant inflammation characterized by dermal infiltration of CD4+ T cells and eosinophils, with the deposition of eosinophil products and the increased skin expression of Th2 cytokines. Subsequently, the chronic phase demonstrates a local Th1 IFN- γ response and tissue remodeling, with increased deposition of collagen and dermal thickening [6, 12]. In the DNCB-induced murine AD-like model adopted in our study, we clearly observed the upregulation of serum IgE and Th2 cytokines such as IL-4 and IL-13 (Fig. 3), suggesting that the model recapitulated the manifestation of AD. The downregulation of IgE and IL-4/IL-13 serum levels by verbascoside treatment correlated well with ameliorated AD-like lesion severity and scratching behavior (Fig. 2). The level of proinflammatory cytokines at the lesion was consistently suppressed by verbascoside treatment in AD. Our result is supported by the report that an IL-4-deficient mouse model showed inhibitory effects in DNCB-induced dermatitis [36] versus transgenic mice with overexpression of IL-4 developing spontaneous pruritus and chronic dermatitis at the age of 4 months [37]. An earlier report was mainly focused on the anti-inflammatory effect of verbascoside being due to the inhibition of histamine and arachidonic acid release from mast cell [35]. Our study is the first to point out the suppressive role of verbascoside on IgE and the type 2 immune response in AD model.

It has been described that epidermal cytokines such as IL-1 β , TNF- α , and IL-6 play a crucial role in the induction and elicitation phases of allergic contact dermatitis upon exposure to sensitizers [15, 25]. Following antigen uptake, and in the presence of a danger signal generated by microbial antigens that include normal flora and/or mechanical injury (with its release of mediators such as IL-1 and TNF- α), immature dendritic cells acquire the phenotype of functional antigen-presenting cells. They can induce the expression of major histocompatibility complex class II molecules and key costimulatory molecules such as CD80 and CD86 [38]. In our study, we measured TNF- α in the overall body serum and locally at the lesion site. We chose THP-1, a widely used monocyte and macrophage model, to investigate the molecular mechanism of the effect of verbascoside on a DNCB-induced allergic immune response. Verbascoside has shown a potent anti-inflammatory effect in the same cell line. It can inhibit LPS- and IFN- γ -induced activation of iNOS and the production of free radical species [33]. Besides its effect in macrophages and leukocytes, it has also been reported to inhibit TNF- α - and IFN- γ -induced NF κ B activation, ERK phosphorylation, and subsequent

AP-1 activation in human keratinocytes [19]. The suppression of these proinflammatory pathways by verbascoside leads to a more potent inhibition of the chemokine IL-8, downstream recruitment of granulocytes to the inflammatory loci, and cell proliferation [19, 39], all characteristics of inflamed tissues [12]. Our finding of the inhibitory effect of verbascoside on the activation of the NF κ B pathway in THP-1 cells is consistent with these reports. Specifically, 40 μ M of verbascoside has been shown to strongly inhibit NO, TNF- α , and IL-12 production in response to LPS and IFN- γ [40]; in our study, the concentrations of verbascoside were much lower, to achieve the suppressive effect in both the animal model

and the cell line model, further supporting its therapeutic application for AD treatment.

In conclusion, we demonstrate, for the first time, that verbascoside has a potent anti-inflammatory effect in a DNCB-induced AD model. As an antioxidant, anti-inflammatory, water-soluble natural compound, verbascoside robustly suppressed the Th2 immune response and relieved AD symptoms without severe side effects. Verbascoside is a promising therapeutic agent, especially for patients with chronic AD, since this involves systemic dysregulation of the immune response, and the current standard treatment of prolonged topical steroid administration is frequently associated with severe side effects.

References

- 1 Bieber T: Atopic dermatitis. *Ann Dermatol* 2010;22:125–137.
- 2 Weidinger S, Novak N: Atopic dermatitis. *Lancet* 2016;387:1109–1122.
- 3 Shaw TE, Currie GP, Koudelka CW, Simpson EL: Eczema prevalence in the United States: data from the 2003 National Survey of Children's Health. *J Invest Dermatol* 2011;131:67–73.
- 4 Bieber T, D'Erme AM, Akdis CA, Traidl-Hoffmann C, Lauener R, Schappi G, Schmid-Grendelmeier P: Clinical phenotypes and endophenotypes of atopic dermatitis: where are we, and where should we go? *J Allergy Clin Immunol* 2017;139:S58–S64.
- 5 Senra MS, Wollenberg A: Psychodermatological aspects of atopic dermatitis. *Br J Dermatol* 2014;170(suppl 1):38–43.
- 6 Roesner LM, Werfel T, Heratizadeh A: The adaptive immune system in atopic dermatitis and implications on therapy. *Expert Rev Clin Immunol* 2016;12:787–796.
- 7 Schmid-Grendelmeier P, Ballmer-Weber BK: Atopic dermatitis – current insights into pathophysiology and management. *Ther Umsch* 2010;67:175–185.
- 8 Leung DY, Bieber T: Atopic dermatitis. *Lancet* 2003;361:151–160.
- 9 Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA: New insights into atopic dermatitis. *J Clin Invest* 2004;113:651–657.
- 10 Biedermann T, Skabytska Y, Kaesler S, Volz T: Regulation of T cell immunity in atopic dermatitis by microbes: the yin and yang of cutaneous inflammation. *Front Immunol* 2015;6:353.
- 11 Brunner PM, Silverberg JI, Guttman-Yassky E, Paller AS, Kabashima K, Amagai M, Luger TA, Deleuran M, Werfel T, Eyerich K, Stingl G; Councilors of the International Eczema Council: Increasing comorbidities suggest that atopic dermatitis is a systemic disorder. *J Invest Dermatol* 2017;137:18–25.
- 12 Novak N, Bieber T, Leung DY: Immune mechanisms leading to atopic dermatitis. *J Allergy Clin Immunol* 2003;112:S128–S139.
- 13 Werfel T, Allam JP, Biedermann T, Eyerich K, Gilles S, Guttman-Yassky E: Cellular and molecular immunologic mechanisms in patients with atopic dermatitis. *J Allergy Clin Immunol* 2016;138:336–349.
- 14 Brunner PM, Guttman-Yassky E, Leung DY: The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies. *J Allergy Clin Immunol* 2017;139:S65–S76.
- 15 Heratizadeh A, Werfel T: Anti-inflammatory therapies in atopic dermatitis. *Allergy* 2016;71:1666–1675.
- 16 Werfel T, Biedermann T: Current novel approaches in systemic therapy of atopic dermatitis: specific inhibition of cutaneous Th2 polarized inflammation and itch. *Curr Opin Allergy Clin Immunol* 2015;15:446–452.
- 17 Alipieva K, Korkina L, Orhan IE, Georgiev MI: Verbascoside – a review of its occurrence, (bio)synthesis and pharmacological significance. *Biotechnol Adv* 2014;32:1065–1076.
- 18 Jimenez C, Riguera R: Phenylethanoid glycosides in plants: structure and biological activity. *Nat Prod Rep* 1994;11:591–606.
- 19 Georgiev M, Pastore S, Lulli D, Alipieva K, Kostyuk V, Potapovich A, Panetta M, Korkina L: Verbascum xanthophoeniceum-derived phenylethanoid glycosides are potent inhibitors of inflammatory chemokines in dormant and interferon-gamma-stimulated human keratinocytes. *J Ethnopharmacol* 2012;144:754–760.
- 20 Picerno P, Autore G, Marzocco S, Meloni M, Sanogo R, Aquino RP: Anti-inflammatory activity of verminoside from *Kigelia africana* and evaluation of cutaneous irritation in cell cultures and reconstituted human epidermis. *J Nat Prod* 2005;68:1610–1614.
- 21 Wartenberg M, Budde P, De Marees M, Grunheck F, Tsang SY, Huang Y, Chen ZY, Hescheler J, Sauer H: Inhibition of tumor-induced angiogenesis and matrix-metalloproteinase expression in confrontation cultures of embryoid bodies and tumor spheroids by plant ingredients used in traditional Chinese medicine. *Lab Invest* 2003;83:87–98.
- 22 Zhang F, Jia Z, Deng Z, Wei Y, Zheng R, Yu L: In vitro modulation of telomerase activity, telomere length and cell cycle in MKN45 cells by verbascoside. *Planta Med* 2002;68:115–118.
- 23 Li YM, Han ZH, Jiang SH, Jiang Y, Yao SD, Zhu DY: Fast repairing of oxidized OH radical adducts of DAMP and DGMP by phenylpropanoid glycosides from *Scrophularia ningpoensis* Hemsl. *Acta Pharmacol Sin* 2000;21:1125–1128.
- 24 Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, Matsumoto M, Ushio H, Saito S, Askenase PW, Ra C: Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/NGA mice. *Int Immunol* 1997;9:461–466.
- 25 Galli SJ, Tsai M, Piliponsky AM: The development of allergic inflammation. *Nature* 2008;454:445–454.
- 26 Boguniewicz M, Leung DY: Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* 2011;242:233–246.
- 27 Liu FT, Goodarzi H, Chen HY: IgE, mast cells, and eosinophils in atopic dermatitis. *Clin Rev Allergy Immunol* 2011;41:298–310.
- 28 Danso MO, van Drongelen V, Mulder A, van Esch J, Scott H, van Smeden J, El Ghalbzouri A, Bouwstra JA: TNF- α and Th2 cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents. *J Invest Dermatol* 2014;134:1941–1950.

- 29 Toshitani A, Ansel JC, Chan SC, Li SH, Hanifin JM: Increased interleukin 6 production by T cells derived from patients with atopic dermatitis. *J Invest Dermatol* 1993;100:299–304.
- 30 Auwerx J: The human leukemia cell line, THP-1: a multifaceted model for the study of monocyte-macrophage differentiation. *Experientia* 1991;47:22–31.
- 31 Ashikaga T, Hoya M, Itagaki H, Katsumura Y, Aiba S: Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive end points for contact sensitizers. *Toxicol In Vitro* 2002;16:711–716.
- 32 Sakaguchi H, Ashikaga T, Miyazawa M, Kosaka N, Ito Y, Yoneyama K, Sono S, Itagaki H, Toyoda H, Suzuki H: The relationship between CD86/CD54 expression and THP-1 cell viability in an in vitro skin sensitization test – human cell line activation test (H-CLAT). *Cell Biol Toxicol* 2009;25:109–126.
- 33 Speranza L, Franceschelli S, Pesce M, Reale M, Menghini L, Vinciguerra I, De Lutiis MA, Felaco M, Grilli A: Anti-inflammatory effects in THP-1 cells treated with verbascoside. *Phytother Res* 2010;24:1398–1404.
- 34 Tak PP, Firestein GS: NF- κ B: a key role in inflammatory diseases. *J Clin Invest* 2001;107:7–11.
- 35 Lee JH, Lee JY, Kang HS, Jeong CH, Moon H, Whang WK, Kim CJ, Sim SS: The effect of acetoside on histamine release and arachidonic acid release in RBL-2H3 mast cells. *Arch Pharm Res* 2006;29:508–513.
- 36 Traidl C, Jugert F, Krieg T, Merk H, Hunzelmann N: Inhibition of allergic contact dermatitis to DNCB but not to oxazolone in interleukin-4-deficient mice. *J Invest Dermatol* 1999;112:476–482.
- 37 Chan LS, Robinson N, Xu L: Expression of interleukin-4 in the epidermis of transgenic mice results in a pruritic inflammatory skin disease: an experimental animal model to study atopic dermatitis. *J Invest Dermatol* 2001;117:977–983.
- 38 de Vries IJ, Langeveld-Wildschut EG, van Reijssen FC, Dubois GR, van den Hoek JA, Bihari IC, van Wichen D, de Weger RA, Knol EF, Thepen T, Bruijnzeel-Koomen CA: Adhesion molecule expression on skin endothelia in atopic dermatitis: effects of TNF- α and IL-4. *J Allergy Clin Immunol* 1998;102:461–468.
- 39 Potapovich AI, Lulli D, Fidanza P, Kostyuk VA, De Luca C, Pastore S, Korkina LG: Plant polyphenols differentially modulate inflammatory responses of human keratinocytes by interfering with activation of transcription factors NF κ B and AHR and EGFR-ERK pathway. *Toxicol Appl Pharmacol* 2011;255:138–149.
- 40 Rao YK, Fang SH, Hsieh SC, Yeh TH, Tzeng YM: The constituents of *Anisomeles indica* and their anti-inflammatory activities. *J Ethnopharmacol* 2009;121:292–296.

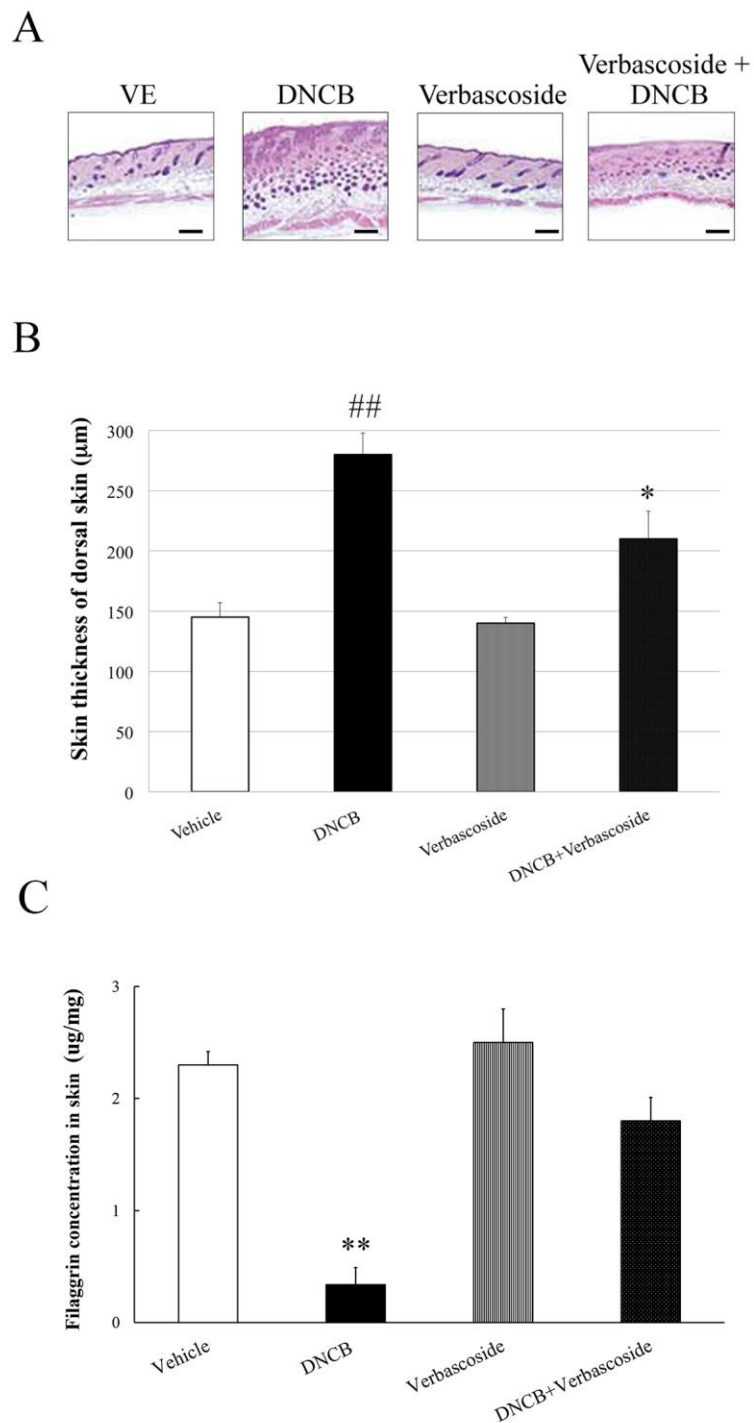


Figure S1. Effect of verbascoside on dorsal skin in animal dermatitis model. A) Representative examples of skin sections (4 μm) stained with hematoxylin–eosin. Skin samples were harvested from dorsal skins of mice from different treatment groups on the last day of the study. Scale bar = 100 μm. B) Histological analysis of

skin thicknesses of the mice from different experimental groups. The data are presented as the mean $\pm$ SD (n =6). ## p<0.01, significantly different from control group by paired t-test; * p<0.05, significantly different from DNCB treated group by paired t-test. C) Skin samples were lysed and total proteins were extracted. Filaggrin protein in the lysate was measured by ELISA. The expression level was normalized to total protein. The data are presented as the mean $\pm$ SD (n =6). ** p<0.01, significantly different from vehicle group.

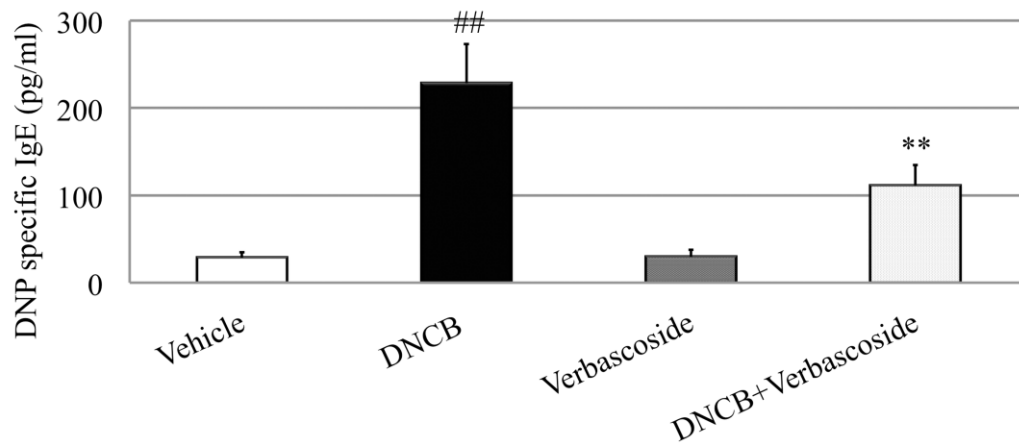


Figure S2. Effect of verbascoside on DNCB induced increase of DNP-specific IgE in serum. Sample blood was collected on the last day of the study (day 19). The level of serum DNP-specific IgE was measured with ELISA. The data are presented as the mean $\pm$ SD (n =6). ## p<0.01, significantly different from control group; ** p<0.01, significantly different from DNCB treated group.

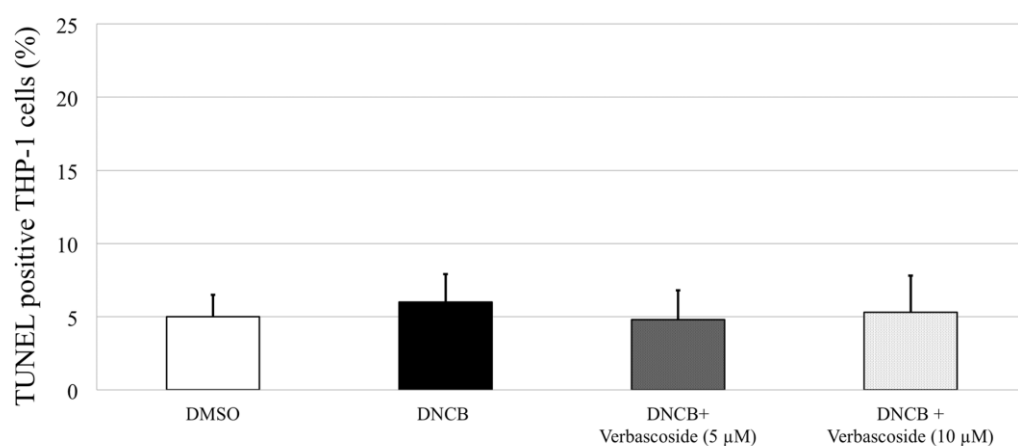


Figure S3. Effects of DNCB or verbascoside treatments on THP-1 cells viability.

THP-1 cells were harvested with exposure to the indicated chemicals for 24 h. Cell viability was determined by TUNEL assay. Positively stained cells in the assay were counted as dead cells.

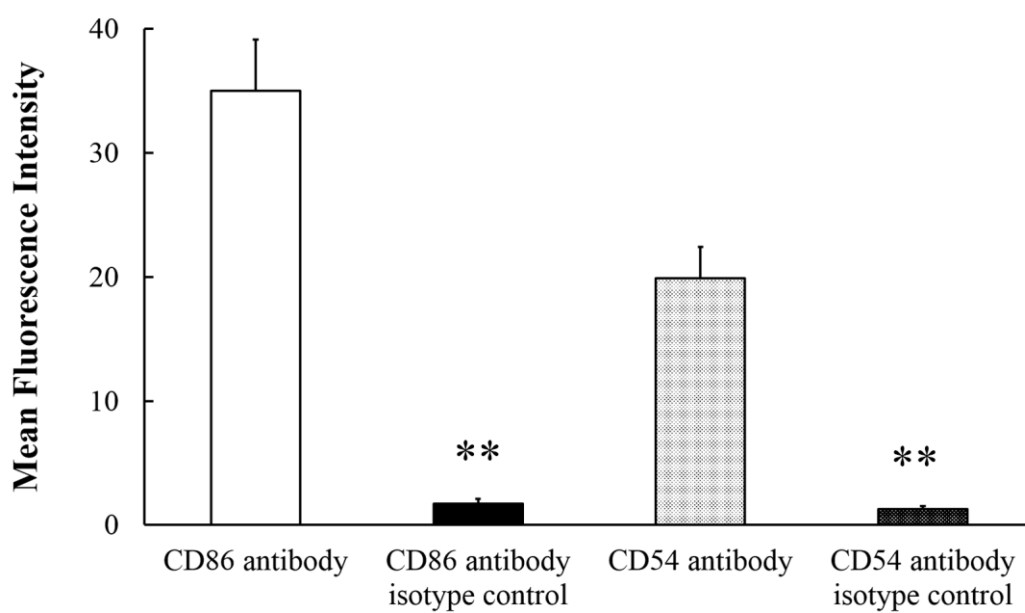


Figure S4. Isotype control antibodies show no reactivity on THP-1 cells.

THP-1 cells treated with DNCB for 24h were harvested. The isotype control antibodies for CD86 and CD54 were tested for non-specific binding on these cells by flow cytometry. The MFI was used as indicator of surface marker expression. **

$p < 0.01$, significantly different from the respective positive group as analysed with unpaired t-test.

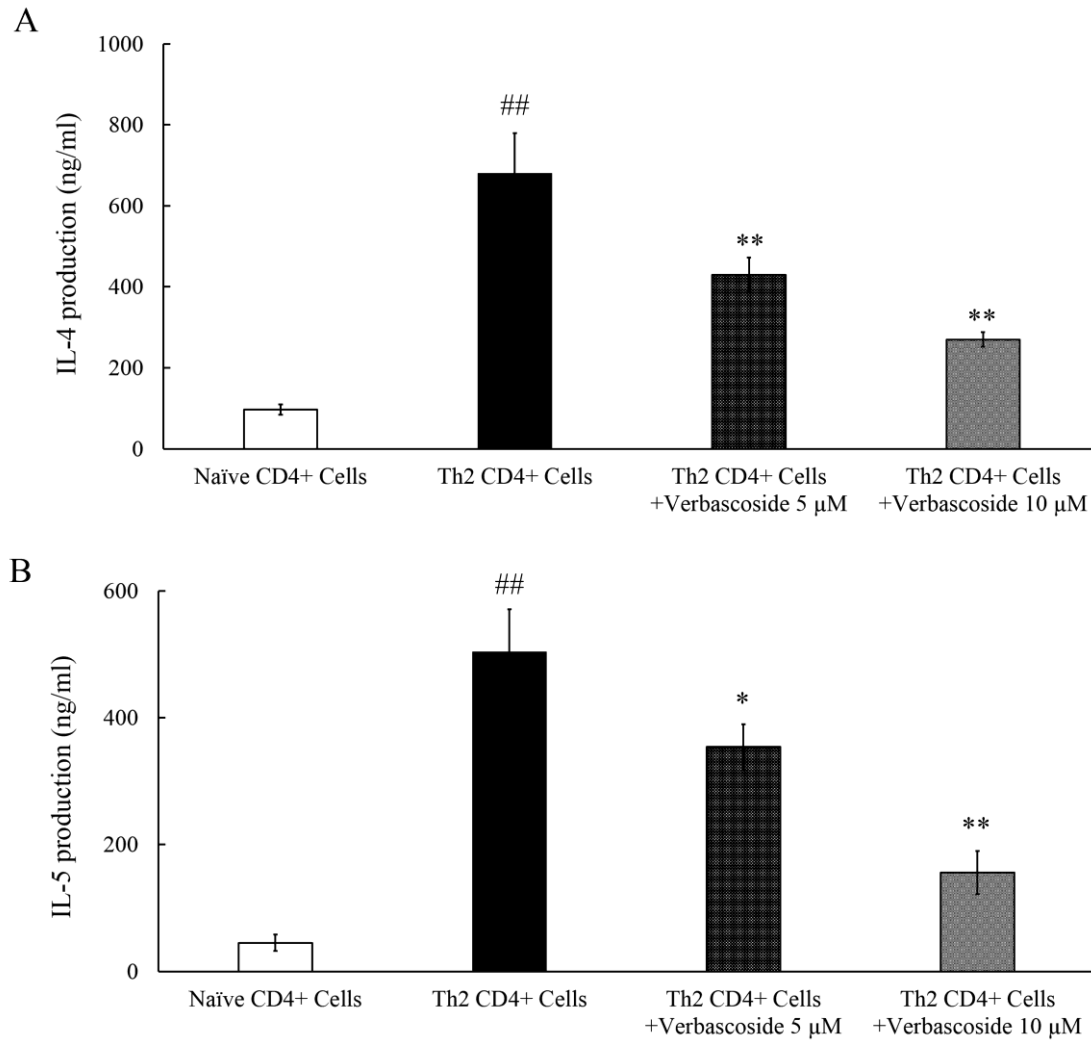


Figure S5. Effects of verbascoside on Th2 CD4+ cells. Naïve CD4+ Cells were first differentiated into Th2 CD4+ cells in culture. The activated Th2 cells were then treated with PBS, 5 µM or 10 µM verbascoside for 24 h and the cell culture media were harvested. IL-4 and IL-5 levels in the media were quantified by ELISA. The data are presented as the mean $\pm$ SD (n=4). ## $p < 0.01$, significantly different from naïve T cells group; * $p < 0.05$, ** $p < 0.01$, significantly different from Th2 cells control group treated with PBS as analysed with unpaired t-test.