

3,3'-Diindolylmethane alleviates acute atopic dermatitis by regulating T cell differentiation in a mouse model

Xianxian Wu^{a,1}, Jinxuan Liu^{a,1}, Chaoqin Chen^a, Zhen Huang^a, Yuhui Zang^a, Jiangning Chen^a, Lei Dong^a, Junfeng Zhang^{a,b,*}, Zhi Ding^{a,c,*}

^a State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Xianlin Avenue 163, Nanjing, 210023, Jiangsu, China

^b Collaborative Innovation Center of Chemistry for Life Sciences, Nanjing University, Hankou Road 22, Nanjing, 210093, Jiangsu, China

^c Changzhou High-Tech Research Institute of Nanjing University, Changzhou, 213164, China

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ABSTRACT

Atopic dermatitis is a severe, chronic relapsing inflammatory disease of the skin with family clustering. It is characterized into acute phase, which is dominated by T helper 2-type immune responses, and chronic phase, which is dominated by T helper 1-type immune responses. Studies have shown that 3,3'-diindolylmethane not only has antitumor effects but also can relieve symptoms of inflammatory diseases by inhibiting the nuclear factor- κ B signaling pathway and regulating T cell differentiation. To study the effect of 3,3'-diindolylmethane on atopic dermatitis and the underlying mechanism, a mouse model of acute atopic dermatitis was established using 2,4-dinitrofluorobenzene. After intraperitoneal injection of 3,3'-diindolylmethane, skin erythema and edema in mice were significantly alleviated. Furthermore, 3,3'-diindolylmethane reduced immune activation, probably by inhibiting the secretion of thymic stromal lymphopoietin by keratinocytes. 3,3'-Diindolylmethane also promoted the differentiation of regulatory T cells and inhibited the activation of T helper 2 and T helper 17 cells to reduce atopic dermatitis-related immune responses. However, it showed no significant effect on the differentiation of T helper 1 cells. These results indicate that 3,3'-diindolylmethane has a significant inhibitory effect on T helper 2 cells in the acute phase of atopic dermatitis. Our findings may provide not only more insights into the pathological mechanism of AD, but also a new candidate medicine for it.

1. Introduction

Atopic dermatitis (AD) is a serious, chronic relapsing inflammatory skin disorder involving severe pruritus, dryness, and clinical manifestations such as erythematous edema and lichenification of skin lesions (Peters and Peters, 2019). Epidemiological surveys have shown that approximately 5% of adults and 18% of children worldwide are AD patients, and the incidence of AD is increasing year by year (Nutten, 2015). The pathogenesis of AD is extremely complicated; it is related to genetic factors, skin barrier destruction, immune system disorders and environmental changes (Nutten, 2015). At present, the treatment of AD employs traditional and novel methods. Traditional medications used are glucocorticoids (Ingawale and Mandlik, 2020), cyclosporine A (Carreras et al., 2020), and topical calcineurin inhibitors (TCIs), which includes topical tacrolimus ointment (Protopic®) (Nakahara et al., 2018) and pimecrolimus cream (Elidel®) (Jensen et al., 2013). These

medicines have good anti-inflammatory effects, but their long-term use may lead to many side effects, such as skin atrophy, and nephrotoxicity (Kusari et al., 2019). Novel medications include topical PDE4 inhibitors: Crisaborole (Eucrisa™) (Zebda and Paller, 2018) and systemic biological treatment: dupilumab (Dupixent®) (Seegraber et al., 2018). They are promising drugs with favorable results in clinical trials, however, with nearly unaffordable medication cost (Kusari et al., 2019). Novel, safe and cheap treatments are still urgently needed.

Different from allergic contact dermatitis, the initial stage of AD is an inflammatory skin disease mainly caused by Th2 immune responses (Owen et al., 2018). When external allergens/antigens are taken up by Langerhans cells (LCs) in the epidermis, the activated LCs migrate to the draining lymph nodes, promoting the polarization of naïve T cells to Th2 cells, with increased IL-4, IL-5, IL-10, and IL-13 production (Chu et al., 2011; D'Auria et al., 2016). In the acute phase of AD, keratinocytes secrete thymic stromal lymphopoietin (TSLP), which activates LCs and

* Corresponding authors at: Xianlin Avenue 163, Nanjing, 210023, Jiangsu, China.

E-mail addresses: jfzhang@nju.edu.cn (J. Zhang), dingzhi@nju.edu.cn (Z. Ding).

¹ Contribute equally to the manuscript.

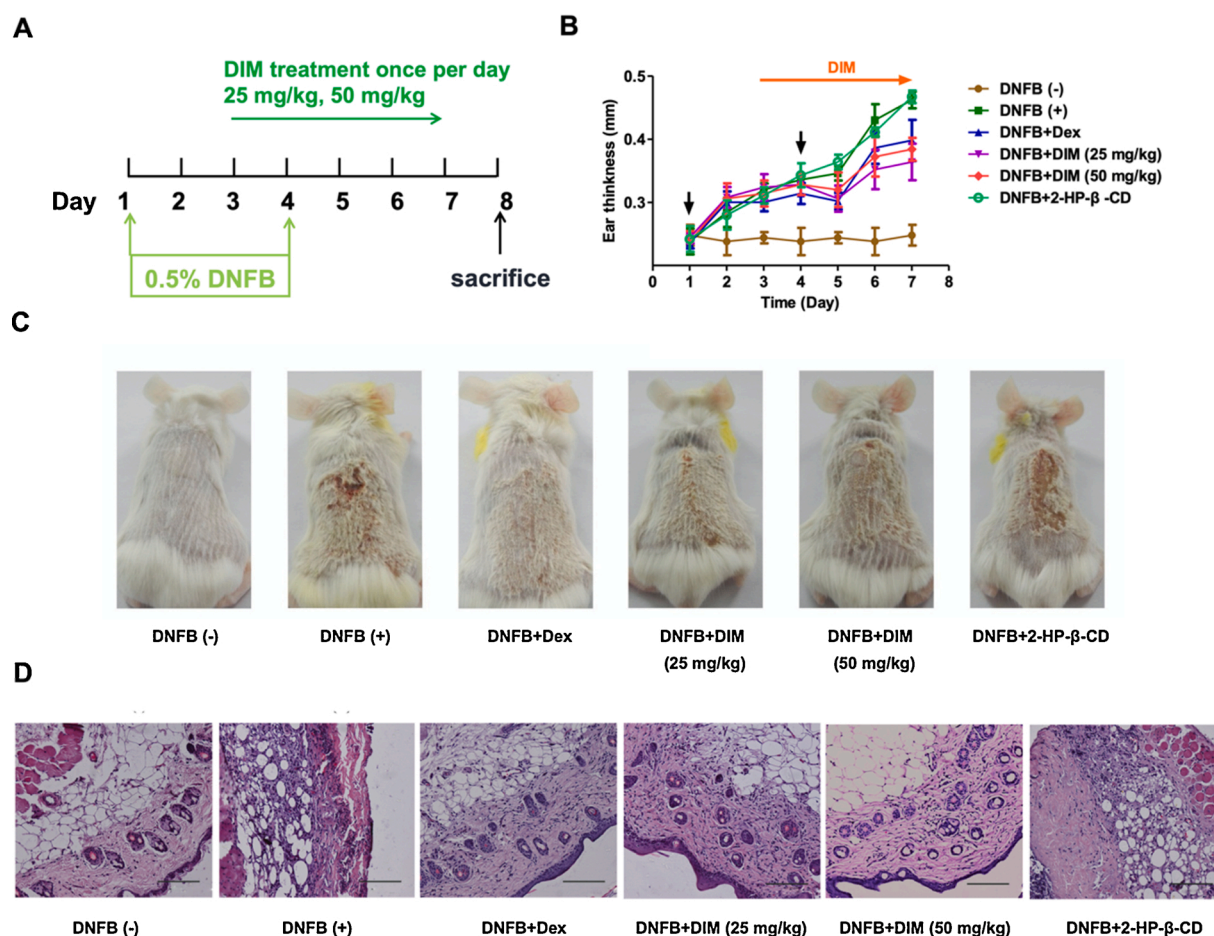


Fig. 1. Therapeutic effects of DIM on the AD mouse model. (A) The protocol for induction and treatment of AD in BALB/c mice. (B) The thickness of mouse right ears was measured every day during the whole process ($n = 6$). Data are shown as mean $\pm$ SD values. Student's t -test, *: $p < 0.05$; **: $p < 0.01$. (C) The representative images of the mice from control, AD, Dex-treated, DIM-treated, and solution-treated control groups were obtained on day 8 ($n = 3$). (D) Pathological tissue sections were detected on day 8 ($n = 3$); black arrows indicate the dermis. Scale bar = 100 μ m.

dermal dendritic cells, and aggravates the Th2-type response (Asahina and Maeda, 2017). The aggregation of FcεRI on inflammatory dendritic epidermal cells promotes the production of IL-12 and IL-18, thereby inducing a transition to a Th1-polarized environment, which is accompanied by the secretion of IFN- γ by Th1 cells and transition to the chronic phase of AD. The dermis layer is thickened, and the skin gradually shows lichenification (Lee et al., 2015; Peters and Peters, 2019; Shin and Greer, 2015). AD was previously considered a Th2/Th1 biphasic T cell-mediated immune disorder. With the deepening of research, the concepts of AD progresses have evolved to a Th17/Th22/Treg multiple-cytokine axes disorder (Malik et al., 2017). IL-17A is a pro-inflammatory cytokine and promotes the production of IL-8, TNF- α , and TSLP (Klonowska et al., 2018). It was also reported to infiltrate AD lesions, modify epithelial cells to produce inflammatory cytokines, and down-regulate the expression of epidermal barrier genes such as filaggrin (Malik et al., 2017). In addition, Treg cells have been reported to attenuate skin inflammation in AD mouse models (Malhotra et al., 2018; Shin et al., 2018; Van Gool et al., 2019). A recent study showed that introducing the same specific mutations in human FoxP3 locus will lead to an uncontrolled Th2 response and cause obvious skin and lung inflammation (Van Gool et al., 2019).

3,3'-Diindolylmethane (DIM) is a natural compound formed during the autolysis of glucuronides in *Brassica* species, which include broccoli, cauliflower, cabbage and so on. It is currently commercialized as an immune enhancing dietary supplement. In recent studies, DIM was found to reduce lipopolysaccharide (LPS)-induced inflammatory responses by inhibiting the NF- κ B signaling pathway in cardiomyocytes

(Luo et al., 2018). It has also been reported that DIM can inhibit colitis in mouse models by binding the aryl hydrocarbon receptor (AhR), followed by inhibition of Th2/Th17 differentiation and induction of Treg differentiation (Huang et al., 2013a). DIM has been studied in preclinical trials to treat hepatitis (Tomar et al., 2015), intestinal radiation injury (Lu et al., 2019), dermatological conditions (Hood and Miller, 2018) etc., and in clinical trials for prostate cancer (NCT00450229, phase II) and precancerous conditions (NCT00462813, phase III), but its efficacy against AD and the underlying mechanisms remain poorly understood. Therefore, in this study, we evaluated the therapeutic effect and studied the anti-inflammatory mechanism of DIM in a mouse model of AD.

2. Materials and methods

2.1. Materials

2, 4-Dinitrofluorobenzene (DNFB) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DIM (purity: >99%) and dexamethasone (Dex) were obtained from Shanghai Future Biochemical Reagent Industry Co., Ltd. (Shanghai, China). Cell Counting Kit-8 (CCK-8) was obtained from Dongren Chemical Technology (Kyushu Island, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from 4A Biotech Co. Ltd. (Beijing, China). Poly (I: C), Dulbecco's modified Eagle's medium (DMEM), and RPMI 1640 were supplied by Invitrogen (Carlsbad, CA, USA). Taq enzyme, deoxy-ribonucleotide triphosphate (dNTP), Evagreen, and 10 $\times$ buffer (Mg $^{2+}$ plus) were obtained from TaKaRa (Tokyo, Japan). CD4 T cell magnetic beads, anti-

CD3/CD28 antibody, and anti-IFN- γ and anti-IL-12 antibodies were obtained from BD Biosciences (San Jose, CA, USA). The HaCaT cell line was purchased from the Shanghai Library of Chinese Academy of Sciences (Shanghai, China). 2-Hydroxypropyl- β -cyclodextrin (2-HP- β -CD) and 6-diamidino-2-phenylindole (DAPI) were supplied by Sigma-Aldrich (Saint Louis, USA). Anti-mouse GATA3/estrogen receptor α (ER α)/forkhead box P3 (FoxP3) antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). NF- κ B p65 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 594-conjugated donkey anti-rat IgG (H+L) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (H+L) antibodies were supplied by Life Technologies (CA, USA). All other chemicals used were of analytical grade, and all solutions were prepared with Milli-Q water.

2.2. Animals and treatments

Thirty-six female BALB/c mice weighing 20 g (aged 6–8 weeks) were purchased from the experimental animal center of Nanjing Medical University and maintained under standardized specific pathogen-free conditions in the animal facility of the State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University. Mice were housed in standard cages and provided free access to food and water. The animals were maintained at a constant temperature (20–21 °C) and humidity (55 $\pm$ 5%), with a 12-h light/dark cycle. The animals were handled in accordance with the Helsinki Declaration of 1975 (as revised in 2008) concerning animal rights, and the protocols were approved by the Institutional Animal Care and Use Committee of Model Animal Research Center of Nanjing University. The mice were used for the induction of AD as previously described and the experimental procedure is shown schematically in Fig. 1A (Jin et al., 2009). Briefly, DNFB was diluted in a mixture of acetone and olive oil (3:1). One hundred microliters of 0.5% DNFB solution was applied to the shaved skin of the back of the mice and 20 μ L was applied on the right ear on day 1 and day 4. Being DIM is a poorly water-soluble compound, it was dispersed in 2-HP- β -CD to enhance its water-solubility as a hydrophilic formulation as previously described (Strickley, 2004). Two doses of DIM, 25 mg/kg or 50 mg/kg, based on previous studies, were administered to the mice via intraperitoneal injection once per day from day 3 to day 7 (Dong et al., 2010). All animals were sacrificed on day 8, and the treated skin samples were maintained at 4 °C for further analysis.

2.3. Histopathological examination

The mice were euthanized by cervical dislocation on the 8th day after treatment. Skin samples of the back were obtained, fixed in Bouin's buffer, paraffin-embedded, and sectioned. The sections were stained with hematoxylin-eosin (H&E) and analyzed using a microscope (Nikon TE2000-U; Tokyo, Japan).

2.4. Cytokine analysis

Approximately 100 mg of skin tissue from the treatment site was collected and rinsed in phosphate-buffered saline (PBS; at 1 mg/ μ L), precooled at 4 °C, and then homogenized on ice by using Lysing Matrix D tubes and Fast Prep-24 homogenizer (MP Biomedicals, Santa Ana, Canada). Supernatants were collected after centrifugation at 20879g for 15 min at 4 °C. The concentrations of IL-4, IL-5, IL-13, IL-17A, and IFN- γ in the supernatant were determined using ELISA kits according to the manufacturer's instructions.

2.5. mRNA quantification

The mRNA expression levels of ER α , GATA3, T-bet, ROR γ t, and FoxP3 in mouse skin tissue were evaluated with RT-PCR. Total RNA was extracted from naive T cells or the skin samples by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was

Table 1

Sequences of primers used for RT-PCR analysis of mouse ER α , GATA3, T-bet, ROR γ t and FoxP3 mRNAs.

Target	Forward primer (5'–3')	Reverse primer (5'–3')
T-bet	TGCCAGGGAACCGCTTATAT	GTTGGAAGCCCCCTTGTGT
GATA3	CGGGTTCGGATGTAAGTCG	GTACCATCTCGCCGCCAC
ER- α	GCGGCATACGGAAGACC	CAAGGACAAGGCAAGGGCTA
ROR γ t	CCAGGAGCAATGGAAGTCG	CCGTGTAGAGGGCAATCTCA
FoxP3	GCACCTTCTGCTGCCACTG	GGCCTTGCCCTTCTCATCC
β -actin	GGTGTGATGGTGGGAATGGG	ACGGTTGGCCTTAGGGTTACG

determined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). A PrimeScript RT Reagent Kit (Takara, Shiga, Japan) was used for cDNA synthesis from total RNA. RT-PCR was performed with LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN, USA) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The specificity of each reaction was verified by plotting a dissociation curve with continuous fluorescence measurements. CT values were determined using fixed threshold settings. The amount of mRNA relative to the internal control, β -actin, was calculated using the equation $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{mRNA} - \Delta CT_{\beta-actin}$). The primers used are shown in Table 1 (Huang et al., 2013a).

2.6. Immunofluorescent staining

ER- α and GATA3 expression levels in skin tissue were measured using immunofluorescence staining. The tissue sections were blocked with bovine serum albumin for 1 h and incubated with a rabbit anti-mouse ER- α or GATA3 antibody overnight at 4 °C. To detect infiltrating Treg cells in the skin tissue, the sections were stained with a rabbit anti-mouse CD4 antibody and a rat anti-mouse FoxP3 antibody under the same conditions. The secondary antibody, fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit or tetra-methylrhodamine-5-(and-6)-isothiocyanate (TRITC)-labeled goat anti-rat antibody (KPL, Gaithersburg, MD, USA), was then added and incubation was performed at 37 °C for 1 h. After the nuclei were stained with DAPI, the sections were imaged using a fluorescent microscope (Nikon Ti-C2, Tokyo, Japan).

2.7. Cell culture and extraction

Spleens from treated mice were extracted and single-cell suspensions were prepared. Naive CD4⁺ T cells were sorted using anti-mouse CD4 magnetic beads. Th2 cell differentiation was induced using 5 μ g/mL CD3 and CD28 antibodies, 20 ng/mL IL-2, 100 ng/mL IL-4, 10 μ g/mL anti-IFN- γ , and 10 μ g/mL anti-IL-12. The supernatant was collected after incubation with 4 μ g/mL DIM for 72 h. The levels of IL-4, IL-5, and IL-17A were measured with ELISA.

2.8. Cytotoxicity assay

To detect the toxicity of DIM to HaCaT cells, an immortal human keratinocyte cell line from adult human skin, the cells were exposed to 1, 2, 3, 4, 5, 10, and 20 μ g/mL DIM and cultured at 37 °C for 24 h. Then the cells (passage 55–60) were co-cultured with 10 μ L of CCK8 for 3 h. Cell viability was presented with optical absorbance at 540 nm.

2.9. HaCaT cell stimulation

Different concentrations of DIM (2, 3, 4, and 5 μ g/mL) were co-cultured with HaCaT cells (passages 55–60) for 1 h, and then 20 ng/mL TNF- α , 100 ng/mL IL-4 and 20 ng/mL poly (I: C) were added to stimulate cell secretion of TSLP. RNA was extracted after 6 h, and the culture supernatant was collected after 48 h and maintained at 4 °C for ELISA.

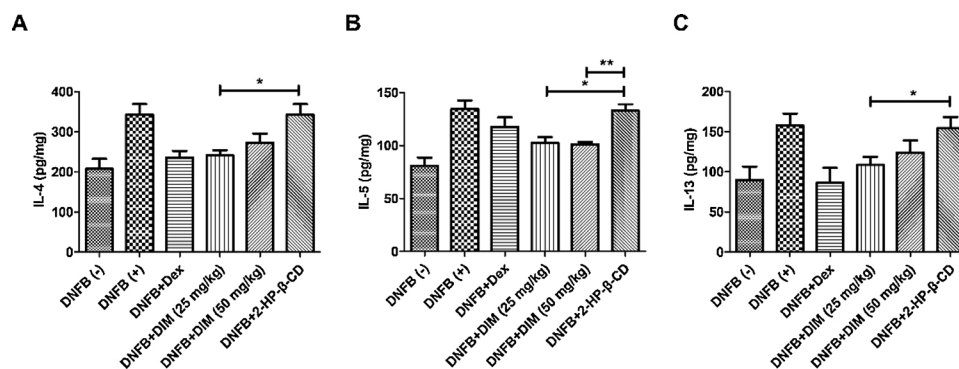


Fig. 2. The effects of DIM on Th2 cytokines in AD mice after 5 days. The levels of (A) IL-4, (B) IL-5, and (C) IL-13 in the skin tissue at the treated site were determined using ELISA. Data are shown as mean + SD values, $n = 6$; Student's t -test, *: $p < 0.05$; **: $p < 0.01$.

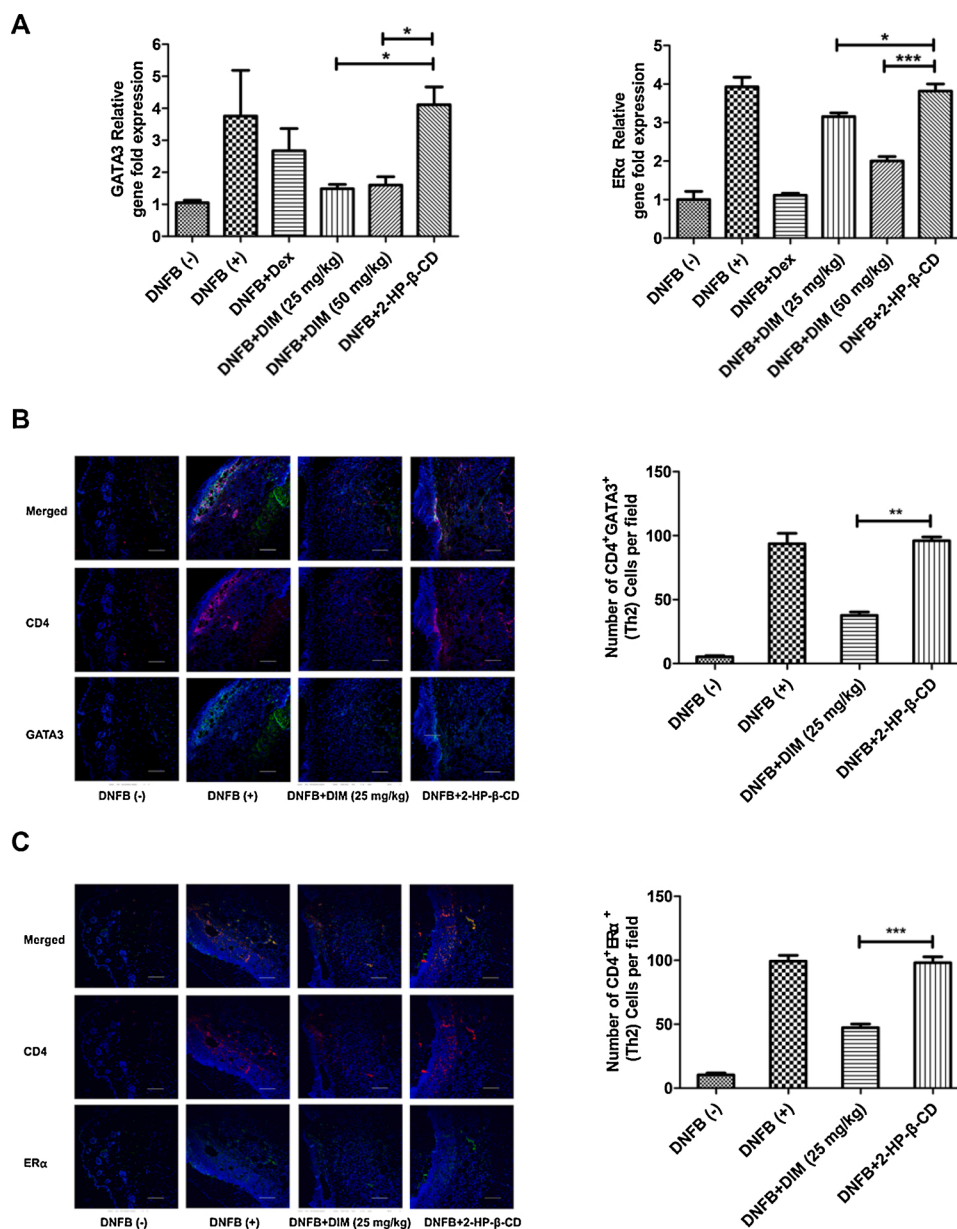


Fig. 3. The effects of DIM on Th2 cell-associated proteins in AD mice after 5 days. (A) mRNA levels of Th2 differentiation-related proteins, GATA3 and ERα, in skin tissue were measured using RT-PCR ($n = 6$). (B) & (C) Representative immunofluorescent staining images showing the expression of GATA3 and ERα in skin tissue. DAPI-labeled nuclei are stained blue, CD4 cells stained red, GATA3 or ERα stained green. Scale bar = 100 μ m ($n = 3$). Data shown as mean + SD values; Student's t -test, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

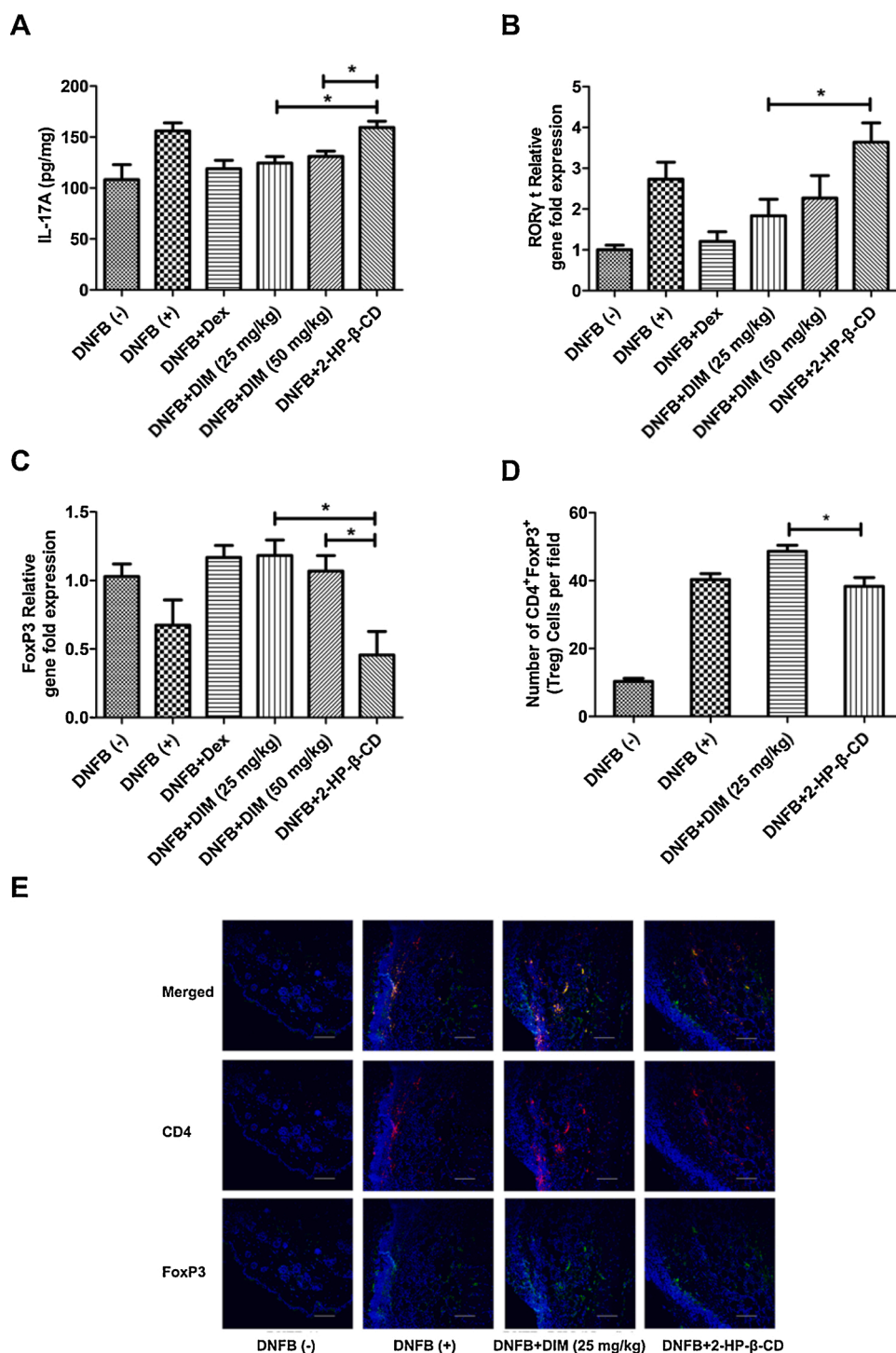


Fig. 4. The effects of DIM on Th1/Th17 cell infiltration in AD mice after 5 days. (A) The levels of IL-17A in the skin tissue were detected using ELISA. (B) mRNA levels of Th17 differentiation-related protein, RORγt, and (C) Treg differentiation-related protein, FoxP3, in skin tissue was measured using RT-PCR. (D) The numbers of CD4⁺FoxP3⁺ cells were counted. (E) Representative immunofluorescent staining images showing the expression of FoxP3 in skin tissue (n = 3). DAPI-labeled nuclei are stained blue, CD4⁺ cells stained red, and FoxP3⁺ Tregs stained green; scale bar = 100 μm (n = 6). Data shown as mean + SD values; Student's *t*-test, *: *p* < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.10. Statistical analysis

Data were analyzed using Prism 6.0 (GraphPad, CA, USA). Comparisons were performed using the Student's *t*-test where suited as indicated for the differences between experimental groups and control groups. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Therapeutic effects of DIM on AD

As shown in Fig. 1A, when DNFB was applied twice on the back and right ear of the mice with a 3-day interval, they developed AD phenotypes such as itching and erythema at the treated sites. Compared to that in the control group, the skin lesion condition alleviated after once per day treatment with 25 mg/kg or 50 mg/kg DIM for 5 days (Fig. 1C).

As shown in Fig. 1B, after the application of DNFB, the ears of the mice swelled up and thickened. Compared to the solution-treated control group and the untreated group, the thickness of the ear reduced

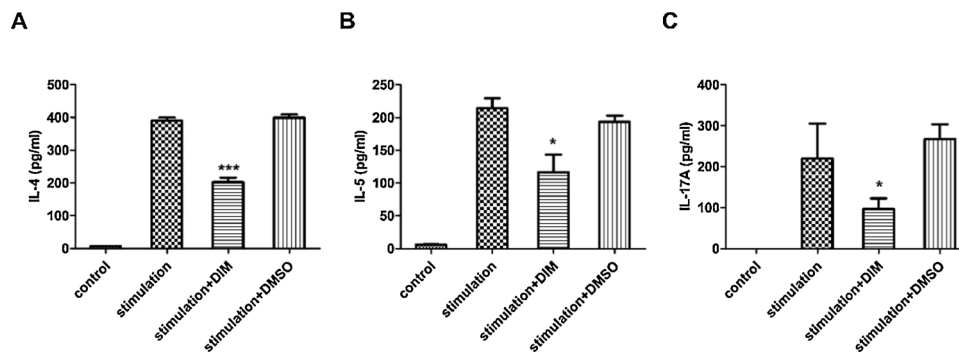


Fig. 5. The effect of DIM on the levels of Th2 differentiation-related cytokines *in vitro*. (A) Data from the control, AD, Dex-treated, DIM-treated, and solution-treated control groups. The levels of IL-4 were detected with ELISA after 72 h. (B) The levels of IL-5 after 72 h. (C) The levels of IL-17 after 72 h (n = 6). Data shown as mean + SD values; Student's *t*-test, *: *p* < 0.05; ***: *p* < 0.001.

approximately 0.1 mm after treatment with DIM or Dex, indicating that DIM alleviated skin swelling.

As can be seen from the pathological tissue sections of the mouse back (Fig. 1D), the structure of the normal skin was intact, while the skin of the animals in the DNFB group was severely damaged with compromised epidermal barrier. After treatment with DIM, compared to normal tissue, the dermis layer thickened, and the blood vessels showed increased nuclear density, probably due to angiectasis and increased inflammatory cell infiltration. DIM treatment can maintain the vascular morphology intact and the skin structure relatively complete, demonstrating its efficacy in the AD mouse model.

3.2. Effects of DIM on T cell differentiation in the skin lesions of AD mice

The levels of IL-4, IL-5 and IL-13 significantly increased after DNFB treatment. With 25 mg/kg DIM, the levels of the three cytokines significantly decreased compared to those in the DNFB group, while only IL-5 level significantly decreased in the 50 mg/kg DIM group (Fig. 2).

DIM treatment significantly inhibited the expression of GATA3 and ER α mRNA in the skin (Fig. 3A). In addition, the numbers of CD4⁺GATA3⁺ and CD4⁺ER α ⁺ T cells in the DIM-treated group, determined using immunofluorescent staining, were significantly lower than those in the untreated group (Fig. 3B & C). In our study, T-bet expression in skin tissues significantly increased after DNFB treatment for 4 days. Furthermore, IFN- γ concentration in the skin increased slightly but not significantly. The mice were in the Th2-biased inflammatory phase, and the secretion of IL-4, IL-5, and IL-13 could inhibit the production of IFN- γ . At the same time, it was found that DIM did not have a significant effect on T-bet expression and IFN- γ secretion, and thus had no effect on the differentiation of Th1 cells (data not shown).

Fig. 4A and B show that DIM can inhibit the expression of IL-17A and ROR γ t mRNA, thereby inhibiting the differentiation of Th17 cells. As shown in Fig. 4C, the expression of FoxP3 in the skin of mice with DNFB-induced AD was also inhibited, indicating that Treg cells were unable to exert normal immunomodulatory functions. After DIM treatment, the expression of FoxP3 in the skin increased significantly, suggesting that DIM can promote the differentiation of Treg cells in inflammation, thereby affording an anti-inflammatory effect. In addition, the effect of DIM on the differentiation of Treg cells was further confirmed by immunofluorescent staining. The number of CD4⁺FoxP3⁺ T cells in the skin tissue significantly increased after DIM treatment (Fig. 4D & E).

3.3. Effects of DIM on T cell differentiation *in vitro*

DIM treatment resulted in a significant inhibitory effect on Th2 differentiation *in vivo* in AD mice. The effects of DIM were further explored *in vitro* in naïve mouse CD4⁺ T cells. After inducing Th2 differentiation and co-cultured with 4 μ g/mL DIM for 72 h, the concentrations of IL-4 and IL-5 decreased significantly, indicating that DIM can inhibit the

differentiation of Th2 cells (Fig. 5A & B). In addition, the expression of IL-17A decreased after the addition of DIM (Fig. 5C). The inhibitory effects of DIM on Th2 and Th17 inflammatory responses were confirmed at the cellular level.

3.4. Effect of DIM on the secretion of TSLP in HaCaT cells and *in vivo*

The level of TSLP in skin tissue was determined after DIM treatment for 5 days. It was found that DIM inhibited the expression of TSLP in skin tissue (Fig. 6A).

To evaluate cytotoxicity, different concentrations of DIM (1–20 μ g/mL) were co-cultured with HaCaT cells for 24 h and the cell viability was measured (Fig. 6B). At DIM concentrations less than 4 μ g/mL, 100 % cell viability could be guaranteed. The levels of TSLP in the supernatant and its mRNA in the cells were examined after cell culture. As shown in Fig. 6C and D, poly (I: C) promoted the secretion of TSLP by HaCaT cells. When DIM was added, a significant decrease was detected in both protein and mRNA levels of TSLP.

In normal cells, inactivated NF- κ B dimers (p50 and p65) bind I κ B in the cytoplasm. After being stimulated, I κ B is degraded by ubiquitination. The NF- κ B dimers are translocated in the nucleus and bind DNA to perform transcriptional functions. After the addition of poly (I: C), p65, the NF- κ B subunit labeled with green fluorescence, increased in the nucleus (Fig. 6E). Treatment with DIM resulted in p65 being located more in the cytoplasm. Therefore, it was concluded that DIM can inhibit TSLP secretion and prevent p65 from entering the nucleus, thereby slowing down the progression of AD.

4. Discussion

In this study, the possible therapeutic effect of DIM on AD and the underlying mechanisms were studied in a mouse model of DNFB-induced AD and HaCaT cells. It was found that DIM reduced the concentrations of IL-4, IL-5, and IL-13, and suppressed the expression of ER α and GATA3. It also downregulated the expression of IL-17A and ROR γ t. Meanwhile, DIM upregulated the level of FoxP3 and promoted the differentiation of Treg cells, thereby exerting anti-inflammatory effects. These data indicate that DIM has anti-inflammatory and anti-AD activities. Subsequently, based on previous data in our lab (Huang et al., 2013a), we confirmed *in vitro* that DIM may regulate T cell differentiation by binding to AhR and the possible mechanisms are depicted in Fig. 7.

AhR is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) transcription factor family, which is essential for adaptive immune responses to xenobiotics (Hao and Whitelaw, 2013). A large amount of experimental data indicate that AhR ligands can induce or aggravate inflammatory diseases by stimulating the production of inflammatory factors. However, studies have shown that some ligands of AhR, such as DIM, have opposite effects on inflammatory responses when binding

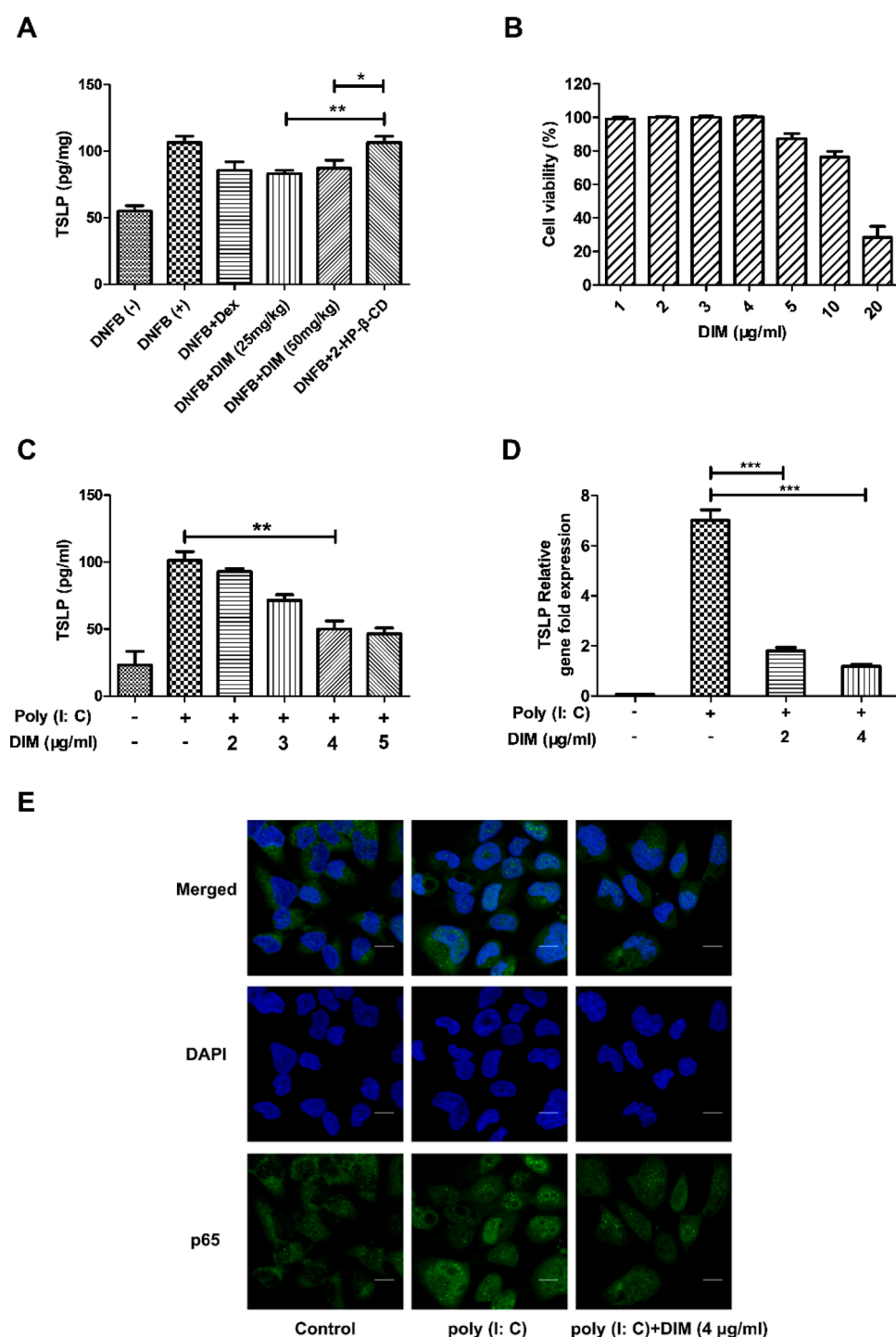


Fig. 6. The effects of DIM on TSLP secretion and p65 translocation in the skin of AD mice and HaCaT cells. (A) The expression of TSLP in skin tissue from the control, AD, Dex-treated, DIM-treated, and solution-treated control groups was detected with ELISA after 5 days ($n = 6$). (B) HaCaT cell viability was examined with CCK-8 after co-culture with different concentrations of DIM (1–20 μ g/mL) after 24 h ($n = 3$). (C) TSLP concentrations in the supernatant of poly (I: C)-stimulated HaCaT cells were measured with ELISA after co-culture with DIM for 48 h ($n = 3$). (D) The TSLP mRNA of poly (I: C)-stimulated HaCaT cells was measured with RT-PCR after co-culture with DIM for 6 h ($n = 3$). (E) The accumulation of p65 in the nucleus of HaCaT cells affected by DIM was examined with an immunofluorescence assay (representative images from three experiments. Scale bar = 20 μ m. Data shown as mean + SD values; Student's t -test, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

AhR (Dong et al., 2010; Rouse et al., 2014). DIM alleviated experimental arthritis and osteoclastogenesis by inhibiting the expression of receptor activator for nuclear factor κ B ligand (RANKL) (Dong et al., 2010). Similarly, in this study, DIM attenuates DNFB-induced AD by down-regulating Th2/Th17 and upregulating Treg cell-associated factors.

DIM is reported to exert its anti-inflammatory functions by binding AhR to regulate the differentiation of T cells (Gutierrez-Vazquez and Quintana, 2018) and inhibiting NF- κ B signaling pathway (Kim et al., 2014). In the acute phase of AD, abnormal keratinocytes overexpress TSLP and promote the differentiation of naive CD4⁺ T lymphocytes into Th2 cells (Wang et al., 2015). TSLP secretion can be induced by Poly (I: C), and DIM can suppress it via inhibition of the NF- κ B signaling

pathway (Kitagaki et al., 1997). NF- κ B is related to inflammatory diseases and plays multiple roles in transcription (Liu et al., 2017). When NF- κ B is translocated into the nucleus, a nuclear localization signal is observed, and it activates various inflammatory signaling pathways (Hung et al., 2018). In our study, DIM was found to reduce the expression of TSLP and block the translocation of p65 into the nucleus. Considering these findings and those reported by Pan et al., we speculate that DIM may reduce the expression of TSLP by hindering p65 from entering the nucleus, thereby preventing the activation of the NF- κ B signaling pathway and slowing down the progression of AD (Pan et al., 2018).

In previous studies conducted in our laboratory, it was found that 50

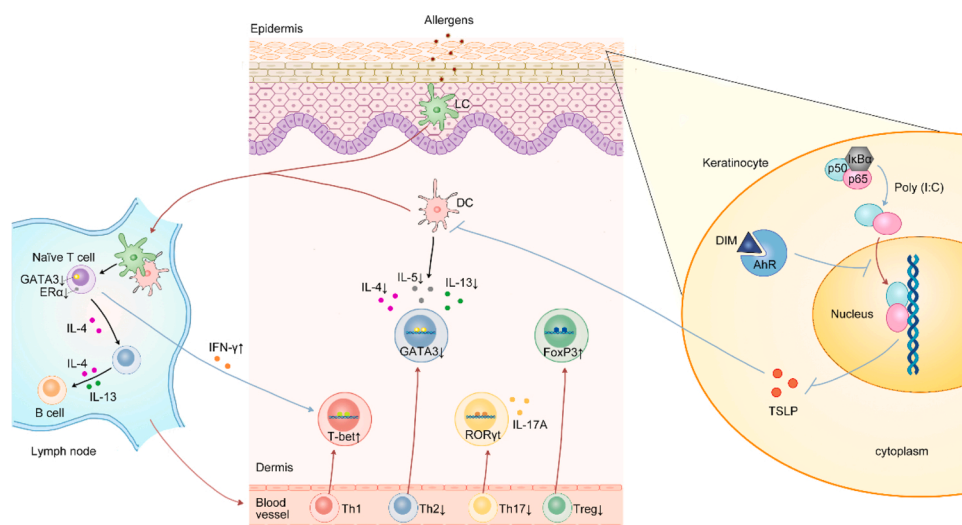


Fig. 7. The therapeutic mechanism of DIM on AD. When LCs are activated by external allergens in the skin, they migrate to the dermal lymph nodes and induce the differentiation of Th2 cells. At the same time, TSLP secreted by keratinocytes also activates LCs, aggravating the Th2-type response. DIM suppresses the Th2- and Th17-type responses, promotes Treg differentiation to alleviate inflammation, and decreases the expression of TSLP by inhibiting the NF- κ B signaling pathway. The brown line shows the cell migration pathway; the blue line indicates cell-cell interaction; and the black line indicates signal transduction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mg/kg DIM was more effective in treating colitis and arthritis in mice than was 10 mg/kg (Dong et al., 2010; Huang et al., 2013b). Therefore, we selected the doses of 25 and 50 mg/kg for AD treatment and found the efficacy of 25 mg/kg dose superior. In our study, DIM was dispersed in 2-HP- β -CD and administered *via* intraperitoneal injection for the convenience of operation and rapid drug absorption. However, a case report from Le et al. mentioned a severe drug reaction using DIM as a dietary supplement (Le et al., 2016). The dose-efficacy relationship of DIM on AD requires further investigation and clarification. As AD is usually accompanied by a compromised skin barrier, restoring the barrier function is considered beneficial in relieving the symptoms. DIM may be formulated into creams or lotions for topical application to further optimize the therapeutic efficacy and reduce potential systemic toxicity.

In conclusion, we have demonstrated the therapeutic effect of DIM against DNFB-induced AD in mice. This effect is exerted *via* the suppression of Th2-/Th17-type immune reactions, promotion of Treg differentiation, and inhibition of the NF- κ B signaling pathway. Therefore, DIM can be a potential candidate medicine for AD treatment.

CCRediT authorship contribution statement

Xianxian Wu: Methodology, Data curation, Writing - original draft. **Jinxuan Liu:** Methodology, Data curation, Writing - original draft. **Chaoqin Chen:** Visualization, Formal analysis. **Zhen Huang:** Conceptualization, Resources. **Yuhui Zang:** Conceptualization, Resources. **Jiangning Chen:** Conceptualization, Resources. **Lei Dong:** Conceptualization, Resources. **Junfeng Zhang:** Project administration, Supervision, Funding acquisition. **Zhi Ding:** Project administration, Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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References

- Asahina, R., Maeda, S., 2017. A review of the roles of keratinocyte-derived cytokines and chemokines in the pathogenesis of atopic dermatitis in humans and dogs. *Vet. Dermatol.* 28 (1), 16–e15. <https://doi.org/10.1111/vde.12351>.
- Carreras, J.J., Tapia-Ramirez, W.E., Sala, A., Guillot, A.J., Garrigues, T.M., Melero, A., 2020. Ultraflexible lipid vesicles allow topical absorption of cyclosporin A. *Drug Deliv. Transl. Res.* 10 (2), 486–497. <https://doi.org/10.1007/s13346-019-00693-4>.
- Chu, C.C., Di Meglio, P., Nestle, F.O., 2011. Harnessing dendritic cells in inflammatory skin diseases. *Semin. Immunol.* 23 (1), 28–41. <https://doi.org/10.1016/j.smim.2011.01.006>.
- D'Auria, E., Banderali, G., Barberi, S., Gualandri, L., Pietra, B., Riva, E., Cerri, A., 2016. Atopic dermatitis: recent insight on pathogenesis and novel therapeutic target. *Asian Pac. J. Allergy Immunol.* 34 (2), 98–108. <https://doi.org/10.12932/apo732.34.2.2016>.
- Dong, L., Xia, S., Gao, F., Zhang, D., Chen, J., Zhang, J., 2010. 3,3'-Diindolylmethane attenuates experimental arthritis and osteoclastogenesis. *Biochem. Pharmacol.* 79 (5), 715–721. <https://doi.org/10.1016/j.bcp.2009.10.010>.
- Gutierrez-Vazquez, C., Quintana, F.J., 2018. Regulation of the immune response by the aryl hydrocarbon receptor. *Immunity* 48 (1), 19–33. <https://doi.org/10.1016/j.immuni.2017.12.012>.
- Hao, N., Whitelaw, M.L., 2013. The emerging roles of AhR in physiology and immunity. *Biochem. Pharmacol.* 86 (5), 561–570. <https://doi.org/10.1016/j.bcp.2013.07.004>.
- Hood Jr., C.R., Miller, J.R., 2018. The ring Verruca plantaris in cantharidin use a case report. *J. Am. Podiatr. Med. Assoc.* 108 (2), 189–193. <https://doi.org/10.7547/16-115>.
- Huang, Z., Jiang, Y., Yang, Y., Shao, J., Sun, X., Chen, J., Dong, L., Zhang, J., 2013a. 3,3'-Diindolylmethane alleviates oxazolone-induced colitis through Th2/Th17 suppression and Treg induction. *Mol. Immunol.* 53 (4), 335–344. <https://doi.org/10.1016/j.molimm.2012.09.007>.
- Huang, Z., Jiang, Y., Yang, Y., Shao, J., Sun, X., Chen, J., Dong, L., Zhang, J., 2013b. 3,3'-Diindolylmethane alleviates oxazolone-induced colitis through Th2/Th17 suppression and Treg induction. *Mol. Immunol.* 53 (4), 335–344. <https://doi.org/10.1016/j.molimm.2012.09.007>.
- Hung, H.C., Feng, C.W., Lin, Y.Y., Chen, C.H., Tsui, K.H., Chen, W.F., Pan, C.Y., Sheu, J.H., Sung, C.S., Wen, Z.H., 2018. Nucleophosmin modulates the alleviation of atopic dermatitis caused by the marine-derived compound dihydroaustralsulfone alcohol. *Exp. Mol. Med.* 50 (2), e446. <https://doi.org/10.1038/emmm.2017.272>.
- Ingawale, D.K., Mandlik, S.K., 2020. New insights into the novel anti-inflammatory mode of action of glucocorticoids. *Immunopharmacol. Immunotoxicol.* 42 (2), 59–73. <https://doi.org/10.1080/08923973.2020.1728765>.
- Jensen, J.M., Weppner, M., Dahnhardt-Pfeiffer, S., Neumann, C., Brautigam, M., Schwarz, T., Folster-Holst, R., Proksch, E., 2013. Effects of pimecrolimus compared with triamcinolone acetonide cream on skin barrier structure in atopic dermatitis: a randomized, double-blind, right-left arm trial. *Acta Derm. Venereol.* 93 (5), 515–519. <https://doi.org/10.2340/00015555-1533>.
- Jin, H., He, R., Oyoshi, M., Geha, R.S., 2009. Animal models of atopic dermatitis. *J. Invest. Dermatol.* 129 (1), 31–40. <https://doi.org/10.1038/jid.2008.106>.
- Kim, H.W., Kim, J., Kim, J., Lee, S., Choi, B.R., Han, J.S., Lee, K.W., Lee, H.J., 2014. 3,3'-Diindolylmethane inhibits lipopolysaccharide-induced microglial hyperactivation and attenuates brain inflammation. *Toxicol. Sci.* 137 (1), 158–167. <https://doi.org/10.1093/toxsci/ktf240>.
- Kitagaki, H., Ono, N., Hayakawa, K., Kitazawa, T., Watanabe, K., Shiohara, T., 1997. Repeated elicitation of contact hypersensitivity induces a shift in cutaneous cytokine milieu from a T helper cell type 1 to a T helper cell type 2 profile. *J. Immunol.* (Baltim. Md.: 1950) 159 (5), 2484–2491.

- Klonowska, J., Glen, J., Nowicki, R.J., Trzeciak, M., 2018. New cytokines in the pathogenesis of atopic dermatitis-new therapeutic targets. *Int. J. Mol. Sci.* 19 (10) <https://doi.org/10.3390/ijms19103086>.
- Kusari, A., Han, A.M., Schairer, D., Eichenfield, L.F., 2019. Atopic dermatitis: new developments. *Dermatol. Clin.* 37 (1), 11–20. <https://doi.org/10.1016/j.det.2018.07.003>.
- Le, T.M., Sanders, C.J., van de Corput, L., van Erpecum, K.J., Rockmann, H., 2016. Drug rash with eosinophilia and systemic symptoms caused by the dietary supplement diindolylmethane. *J. Allergy Clin. Immunol. Pract.* 4 (1), 175–176. <https://doi.org/10.1016/j.jaip.2015.07.007>.
- Lee, J.H., Cho, D.H., Park, H.J., 2015. IL-18 and cutaneous inflammatory diseases. *Int. J. Mol. Sci.* 16 (12), 29357–29369. <https://doi.org/10.3390/ijms161226172>.
- Liu, T., Zhang, L., Joo, D., Sun, S.C., 2017. NF-kappaB signaling in inflammation. *Signal Transduct. Target. Ther.* 2 <https://doi.org/10.1038/sigtrans.2017.23>.
- Lu, L., Jiang, M., Zhu, C., He, J., Fan, S., 2019. Amelioration of whole abdominal irradiation-induced intestinal injury in mice with 3,3'-diindolylmethane (DIM). *Free Radic. Biol. Med.* 130, 244–255. <https://doi.org/10.1016/j.freeradbiomed.2018.10.410>.
- Luo, Q., Yang, A., Cao, Q., Guan, H., 2018. 3,3'-Diindolylmethane protects cardiomyocytes from LPS-induced inflammatory response and apoptosis. *BMC Pharmacol. Toxicol.* 19 (1), 71. <https://doi.org/10.1186/s40360-018-0262-x>.
- Malhotra, N., Leyva-Castillo, J.M., Jadhav, U., Barreiro, O., Kam, C., O'Neill, N.K., Meylan, F., Chambon, P., von Andrian, U.H., Siegel, R.M., Wang, E.C., Shivdasani, R., Geha, R.S., 2018. RORalpha-expressing T regulatory cells restrain allergic skin inflammation. *Sci. Immunol.* 3 (21) <https://doi.org/10.1126/sciimmunol.aao6923>.
- Malik, K., Heitmiller, K.D., Czarnowicki, T., 2017. An update on the pathophysiology of atopic dermatitis. *Dermatol. Clin.* 35 (3), 317–326. <https://doi.org/10.1016/j.det.2017.02.006>.
- Nakahara, T., Morimoto, H., Murakami, N., Furue, M., 2018. Mechanistic insights into topical tacrolimus for the treatment of atopic dermatitis. *Pediatr. Allergy Immunol.* 29 (3), 233–238. <https://doi.org/10.1111/pai.12842>.
- Nutten, S., 2015. Atopic dermatitis: global epidemiology and risk factors. *Ann. Nutr. Metab.* 66 (Suppl 1), 8–16. <https://doi.org/10.1159/000370220>.
- Owen, J.L., Vakharia, P.P., Silverberg, J.I., 2018. The role and diagnosis of allergic contact dermatitis in patients with atopic dermatitis. *Am. J. Clin. Dermatol.* 19 (3), 293–302. <https://doi.org/10.1007/s40257-017-0340-7>.
- Pan, Z., Zhou, Y., Luo, X., Ruan, Y., Zhou, L., Wang, Q., Yan, Y.J., Liu, Q., Chen, J., 2018. Against NF-kappaB/thymic stromal lymphopoietin signaling pathway, catechin alleviates the inflammation in allergic rhinitis. *Int. Immunopharmacol.* 61, 241–248. <https://doi.org/10.1016/j.intimp.2018.06.011>.
- Peters, N., Peters, A.T., 2019. Atopic dermatitis. *Allergy Asthma Proc.* 40 (6), 433–436. <https://doi.org/10.2500/aap.2019.40.4265>.
- Rouse, M., Rao, R., Nagarkatti, M., Nagarkatti, P.S., 2014. 3,3'-diindolylmethane ameliorates experimental autoimmune encephalomyelitis by promoting cell cycle arrest and apoptosis in activated T cells through microRNA signaling pathways. *J. Pharmacol. Exp. Ther.* 350 (2), 341–352. <https://doi.org/10.1124/jpet.114.214742>.
- Seegraber, M., Srour, J., Walter, A., Knop, M., Wollenberg, A., 2018. Dupilumab for treatment of atopic dermatitis. *Expert Rev. Clin. Pharmacol.* 11 (5), 467–474. <https://doi.org/10.1080/17512433.2018.1449642>.
- Shin, J.S., Greer, A.M., 2015. The role of Fc epsilon RI expressed in dendritic cells and monocytes. *Cell. Mol. Life Sci.* 72 (12), 2349–2360. <https://doi.org/10.1007/s00018-015-1870-x>.
- Shin, J.U., Kim, S.H., Noh, J.Y., Kim, J.H., Kim, H.R., Jeong, K.Y., Park, K.H., Lee, J., Chu, H., Lee, J.H., Yong, T.S., Park, J.W., Lee, K.H., 2018. Allergen-specific immunotherapy induces regulatory T cells in an atopic dermatitis mouse model. *Allergy* 73 (9), 1801–1811. <https://doi.org/10.1111/all.13465>.
- Strickley, R.G., 2004. Solubilizing excipients in oral and injectable formulations. *Pharm. Res.* 21 (2), 201–230. <https://doi.org/10.1023/b:pham.0000016235.32639.23>.
- Tomar, S., Nagarkatti, M., Nagarkatti, P.S., 2015. 3,3'-Diindolylmethane attenuates LPS-mediated acute liver failure by regulating miRNAs to target IRAK4 and suppress Toll-like receptor signalling. *Br. J. Pharmacol.* 172 (8), 2133–2147. <https://doi.org/10.1111/bph.13036>.
- Van Gool, F., Nguyen, M.L.T., Mumbach, M.R., Satpathy, A.T., Rosenthal, W.L., Giacometti, S., Le, D.T., Liu, W., Brusko, T.M., Anderson, M.S., Rudensky, A.Y., Marson, A., Chang, H.Y., Bluestone, J.A., 2019. A mutation in the transcription factor Foxp3 drives T helper 2 effector function in regulatory T cells. *Immunity* 50 (2), 362–377. <https://doi.org/10.1016/j.immuni.2018.12.016> e366.
- Wang, Q., Du, J., Zhu, J., Yang, X., Zhou, B., 2015. Thymic stromal lymphopoietin signaling in CD4(+) T cells is required for TH2 memory. *J. Allergy Clin. Immunol.* 135 (3), 781–791. <https://doi.org/10.1016/j.jaci.2014.09.015> e783.
- Zebda, R., Paller, A.S., 2018. Phosphodiesterase 4 inhibitors. *J. Am. Acad. Dermatol.* 78 (3 Suppl 1), S43–S52. <https://doi.org/10.1016/j.jaad.2017.11.056>.