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Silkworm dropping extract ameliorate trimellitic anhydride-induced allergic contact dermatitis by regulating Th1/Th2 immune response

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ABSTRACT

Allergic contact dermatitis (ACD) is an inflammatory skin disease caused by hapten-specific immune response. Silkworm droppings are known to exert beneficial effects during the treatment of inflammatory diseases. Here, we studied whether topical treatment and oral administration of silkworm dropping extract (SDE) ameliorate trimellitic anhydride (TMA)-induced ACD. In ACD mice model, SDE treatment significantly suppressed the increase in both ear thickness and serum IgE levels. Furthermore, IL-1 β and TNF- α levels were reduced by SDE. In allergic responses, SDE treatment significantly attenuated the production of the Th2-associated cytokine IL-4 in both ear tissue and draining lymph nodes. However, it increased the production of the Th1-mediated cytokine IL-12. Thus, these results showed that SDE attenuated TMA-induced ACD symptoms through regulation of Th1/Th2 immune response. Taken together, we suggest that SDE treatment might be a potential agent in the prevention or therapy of Th2-mediated inflammatory skin diseases such as ACD and atopic dermatitis.

Abbreviations: ACD: allergic contact dermatitis; AD: atopic dermatitis; APC: antigen presenting cells; CCL: chemokine (C-C motif) ligand; CCR: C-C chemokine receptor; Dex: dexamethasone; ELISA: enzyme-linked immunosorbent assay; IFN: interferon; Ig: immunoglobulin; IL: interleukin; OVA: ovalbumin; PS: prednisolone; SDE: silkworm dropping extract; Th: T helper; TMA: trimellitic anhydride; TNF: tumor necrosis factor

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Silkworm dropping extract; allergic contact dermatitis; Th1/Th2 immune balance; trimellitic anhydride; allergic inflammation

Allergic contact dermatitis (ACD), a form of delayed type hypersensitivity reaction, is one of the common inflammatory skin diseases [1,2]. Environmental and chemical antigens called haptens have been known to cause ACD. When haptens penetrate the stratum corneum, they stimulate an immune response by the innate immune system, which recognizes the hapten-protein conjugates [3]. Exposure to haptens causes the release of pro-inflammatory cytokines including interleukin (IL)-1 and tumor necrosis factor (TNF)- α by activated keratinocytes and Langerhans cells (LCs). These cytokines promote the maturation of LCs and stimulate their migration to the draining lymph nodes (dLNs) in the skin [4,5].

The allergen-specific CD4⁺ T helper cells play a critical role in the inflammatory reaction observed in allergic skin diseases [6]. In the trimellitic anhydride (TMA)-induced chronic ACD model, T helper (Th) cells infiltrated into the inflammatory site, and the production of Th2 cell-mediated

cytokines such as IL-4 and IL-13 increased by TMA treatment [7]. These cytokines promoted the differentiation of precursor naive CD4⁺ T cells into Th2 cells. Allergic Th2 immune responses are dominated by the autocrine signals of these cytokines [8,9]. To treat ACD caused by Th2 immune responses, effective agents and drugs such as anti-histamines, immune suppressors, steroids, and antagonists of Th2-related cytokines were developed [10–14]. However, long-term intake of these drugs has adverse side effects such as growth retardation, diabetes, hypertension, cataract, and osteoporosis [15,16]. Thus, in recent years, natural materials have continuously been explored as alternatives to anti-allergic medicines, owing to their safety and efficiency.

Jam-Bun, silkworm droppings or the excrements of the silkworm, includes all the constituents and metabolites of mulberry leaves because digested mulberry leaves are naturally fermented and metabolized by the enzymes or microbes in the silkworm intestine [17]. Silkworm droppings

contain various compounds such as lipids [17], lignans [18], chlorophyll [19], and flavonoids [20]. Among the compounds present in silkworm droppings, megastigmane sesquiterpenes were revealed to exert anti-inflammatory effects by increasing the expression of SIRT1 and heme oxygenase 1 in HEK239 cells [21]. However, the effect of silkworm droppings, especially in ACD, is highly unclear.

In the present study, we investigated the effects of silkworm dropping extract (SDE) on TMA-induced ACD mouse model. We evaluated whether SDE treatment inhibits the symptoms of TMA-induced ACD and regulates the production of pro-inflammatory and Th2-related cytokines in ear tissue and dLNs in TMA-induced ACD mouse model.

Materials and methods

Materials

TMA, isopropyl myristate, and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-4, IL-1 β , TNF- α , and immunoglobulin (Ig) E enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Biosciences (San Diego, CA, USA). T-PER[®] tissue protein extraction reagent was purchased from Thermo Fisher Scientific (Rockford, IL, USA).

Sample preparation

SDE was supplied by Health Recovery Food Co., Ltd. (Jangsu County, Korea). Briefly, the silkworm droppings obtained from fifth instar silkworms were dried under shade and immersed in water for 15 days. After complete drying, the droppings (20 kg) were mixed with ethanol (100 L) and extracted by ultrasonic waves at 20°C for 3 days. The supernatant of the extracted solution was collected by centrifugation, and the solvent was evaporated using a rotary evaporator. The concentrated extract was dried in a freeze dryer and stored at 4°C until use.

Animals

Animals were maintained under specific pathogen-free conditions following established guidelines, and the experimental protocol (permit number: KFRI-M-15,023) was approved by the Animal Care and Use Committee of Korea Food Research Institute. Six-week-old female BALB/c mice were purchased from OrientBio Inc., (Gyeonggi-do, Korea) and housed in an air-conditioned room (23°C $\pm$ 2°C) with a 12-h light/dark

cycle and treated according to the Korea Food Research Institute guidelines for animal care and use.

Induction of ACD and experimental schedule

We followed the methods of Jung, *et al* [22]. Briefly, 6-week-old mice were divided into four groups: naive, sham, SDE-treated, and dexamethasone (or prednisolone) groups. The mice were sensitized with 50 μ L of 5% TMA in mixed solvent (acetone/isopropyl myristate, 4:1. v/v) on their shaved right flank on Day 0, and 20 μ L of 2% TMA was applied to the surface of both ears on Days 5, 8, 11, 14, and 17. Animal experiments were independently managed according to the route of SDE administration: topical or oral. To investigate the effect of topical treatment of SDE, SDE (30 mg/mL) or dexamethasone (Dex, 20 μ g/mL) was dissolved in acetone, and 20 μ L of the samples was topically applied to both ears from Day 6 to 17 (Figure 1(a)). Meanwhile, to examine the effect of oral administration of SDE, SDE (200 mg/kg) or prednisolone (PS, 10 mg/kg) solved in phosphate-buffered saline was administered by oral gavage from Day 5 to 17 (Figure 1(b)). Ear thickness was measured at 24 h after TMA treatment by using a custom-built micrometer (Schering AG, Berlin, Germany). Mice were sacrificed on Day 18.

Preparation of ear tissue for ELISA

For cytokine measurement, the ear tissue of BALB/c mice was mechanically homogenized in 2 mL of T-PER tissue protein extraction reagent containing a protease inhibitor cocktail (Roche, Indianapolis, IN), and the samples were centrifuged at 25,000 $\times$ g for 30 min at 4°C. The concentration of total proteins in the supernatant was calculated using a protein assay kit (Bio-Rad Laboratories, USA).

Culture of dLNs

dLN cells from TMA-induced ACD mice were cultured for the analysis of immune response. Homogenized single dLN cells were collected and treated with red blood cell lysing buffer (Sigma-Aldrich). dLN cells were cultured with 2 μ g/mL concanavalin A (Sigma-Aldrich) in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells (1×10^6 cells/mL) were incubated for 48 h for cytokine analysis.

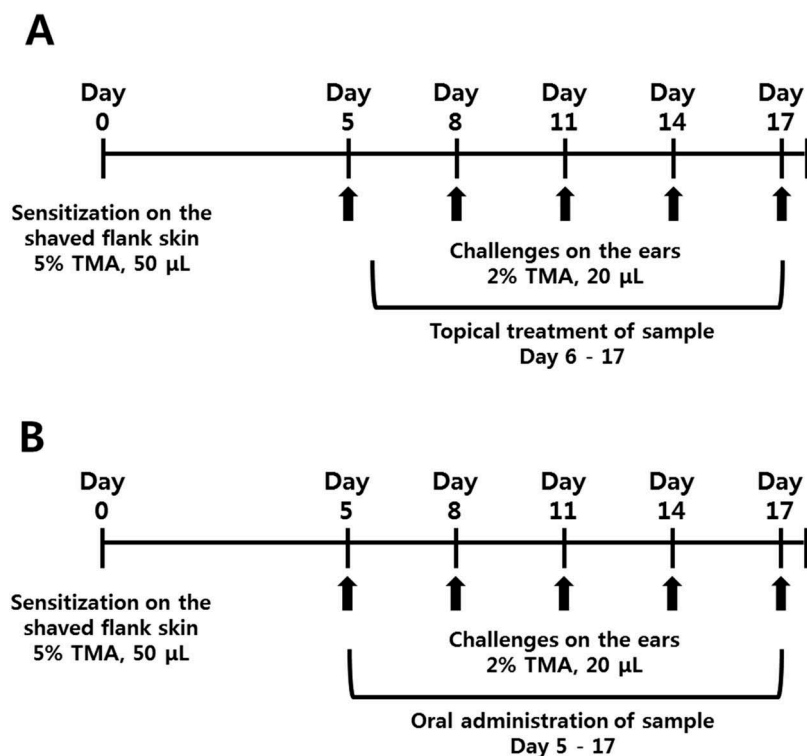


Figure 1. Experimental schedule of TMA treatment and ACD induction in ACD mouse model.

Experimental ACD was induced on the ears of BALB/c mice by sensitization with 5% TMA on Day 0 and treatment with 2% TMA on Days 5, 8, 11, 14, and 17. (A) Topical treatment with SDE (30 mg/mL) or Dex (20 μ g/mL) was provided daily from Day 6. (B) Oral administration of SDE (200 mg/kg) or PS (10 mg/kg) was provided daily from Day 5.

Culture of OVA-sensitized splenocytes

BALB/c mice (female, 6 weeks old) were sensitized by intraperitoneal (i.p.) injection with 10 μ g ovalbumin (OVA) adsorbed in 1 mg of Inject Alum on Days 0 and 7. After 1 week, splenocytes were prepared by aseptically removing the spleens from the mice. Splenocytes were diluted to 5×10^6 cells/mL in RPMI 1640 medium. The splenocytes were then cultured in the presence or absence of OVA (100 μ g/mL) with SDE. The plates were incubated at 37°C for 72 h. The supernatant are used for ELISA analysis.

Statistical analysis

Results are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis of the differences between experimental groups were assessed by one-way analysis of variance (ANOVA) followed by F-protected Fisher's least significant difference tests.

Results and discussion

Topical treatment of SDE attenuates TMA-induced ear swelling and IgE production in mouse serum

Contact dermatitis is commonly divided into two subtypes: irritant contact dermatitis and ACD. The

former is attributed to reversible local immune responses, whereas ACD involves the induction of hapten-specific T cell-mediated immune response and enhancement of IgE levels [23–26]. Different types of chemical allergens called haptens are able to induce different classes of T cell-mediated immune responses. In particular, TMA has been known to elicit strong Th2-mediated immune response [27–29]. Furthermore, in a previous study, we investigated TMA-induced ACD-related immune responses. TMA treatment of mouse skin induced an increase in serum IgE levels as well as the production of cytokines such as IL-1 β , TNF- α , and IL-4 in the ear tissue and IL-4 production in the dLNs [22]. Because these immune responses are similar to T cell-related allergic immune responses, we investigated the effects of SDE on the TMA-induced ACD mouse model to confirm potential of SDE as an alternative agent to treat ACD or atopic dermatitis (AD).

The first, to investigate whether topical treatment with SDE affects the ACD mouse model, TMA was applied to mouse ears. In general, because exposure to haptens (including TMA) increases skin thickness and serum IgE levels, we evaluated these parameters as symptoms of TMA-induced ACD. Figure 2 shows that TMA treatment of mouse ears caused an increase in both ear thickness and serum IgE levels, whereas topical

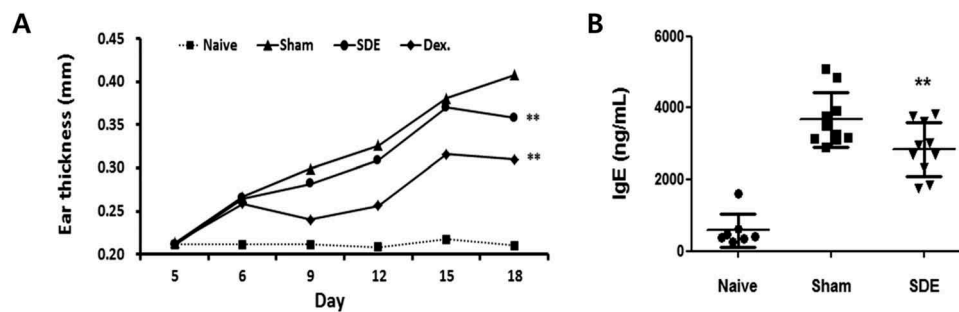


Figure 2. Effect of topical treatment with SDE on the ear thickness and serum IgE levels in TMA-treated mice.

Experimental ACD was induced on the ears of BALB/c mice by sensitization with 5% TMA on Day 0 and treatment with 2% TMA on Days 5, 8, 11, 14, and 17. Topical treatment with SDE (30 mg/mL) or Dex (20 μ g/mL) was provided daily from Day 6. (A) Ear swelling was measured 24 h after TMA treatment. Each symbol of square, triangle, circle, and rhombus indicates the naïve group, sham group, SDE group, and Dex group, respectively. (B) TMA treatment-induced increase in serum IgE levels was measured by ELISA. Results are shown as mean $\pm$ SD. Asterisks (**) indicate significant differences, at $p < 0.01$, between the SDE-treated and non-treated groups of TMA-treated mice.

treatment of SDE significantly reduced the symptoms, compared with sham group.

SDE suppresses the production of pro-inflammatory and Th2 cytokines in the ear tissue and dLNs

When the skin is exposed to haptens, keratinocytes produce TNF- α [30], and then, blood monocytes, tissue macrophages, and dendritic cells secrete IL-1 β [31]. These pro-inflammatory cytokines have been known to play a crucial role in the development of ACD by stimulating the migration and maturation of LCs as well as the production of chemokine (C-C motif) ligand (CCL)27/CTACK (cutaneous T cell-attracting chemokine) in keratinocytes. CCL27, which is a ligand for CC-chemokine receptor 10 (CCR10), selectively attracts CCR10+ memory T cells to the site of inflammation [32–34]. IL-4 is also closely linked to ACD because it induces IgE

secretion by B cells through the regulation of B cell class switching [35,36] as well as stimulates the production of CCL26, which further induces the chemotaxis of CCR3+ cells (especially eosinophils), in keratinocytes [37]. In our previous study, we confirmed that the infiltration of CCR3+ cells was increased at the sites of TMA-induced inflammation in mice, and that IL-4 treatment increased CCL26 production in HaCaT cells, a human keratinocyte cell line, by the phosphorylation of STAT6 [22]. Therefore, to reveal the effects of SDE in TMA-induced ACD model, it was essential to evaluate the levels of IL-1 β , TNF- α , IL-4, and IL-12. Therefore, we investigated whether the production of IL-1 β , TNF- α , and IL-4 is reduced by topical treatment of SDE. Topical treatment of SDE significantly suppressed IL-1 β , TNF- α , and IL-4 protein levels in mouse ear tissue (Figure 3(a)). In a previous study, we found that TMA treatment induced ACD through a Th2 immune response, rather than a Th1 immune

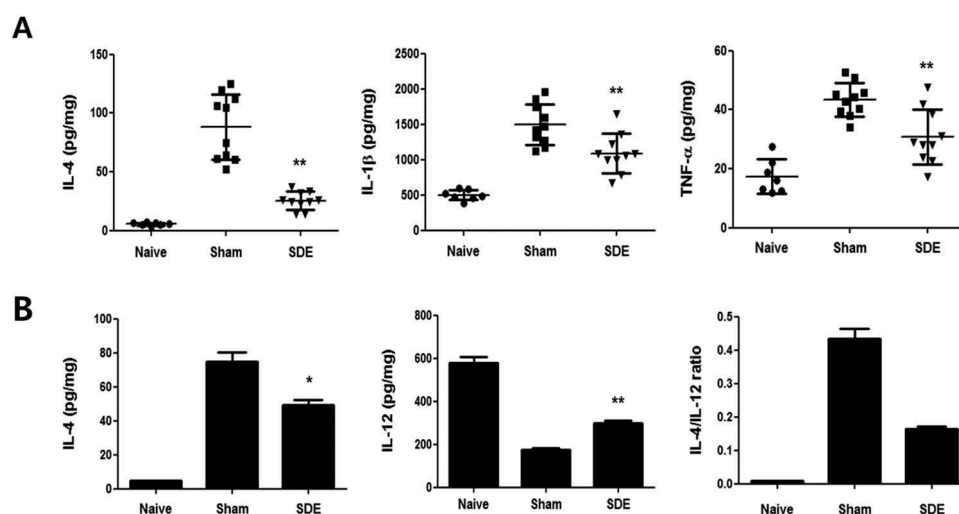


Figure 3. Effect of topical treatment with SDE on TMA-induced cytokine production in ear tissue and dLNs.

Ear tissue was isolated, and the proteins were extracted by lysis buffer for cytokine analysis. (A) The production of IL-4, IL-1 β , and TNF- α cytokines in the ear tissue was analyzed by ELISA. (B) The production of IL-4, IL-12 cytokines and IL-4/IL-12 ratio in the dLNs was analyzed. Results are shown as mean $\pm$ SD. Asterisks (*) and (**) indicate significant differences, at $p < 0.05$ and $p < 0.01$, respectively, between the SDE-treated and non-treated groups of TMA-treated mice.

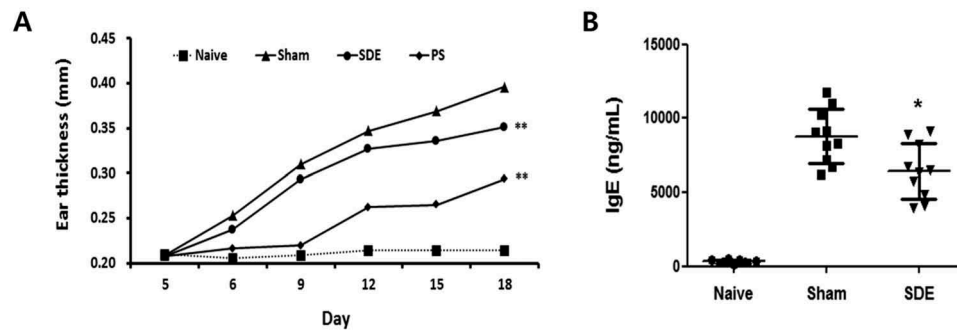


Figure 4. Effect of oral administration of SDE on TMA-treated ear thickness and serum IgE levels.

Experimental ACD was induced on the ears of BALB/c mice by sensitization with 5% TMA on Day 0 and treatment with 2% TMA on Days 5, 8, 11, 14, and 17. Topical treatment with SDE (200 mg/kg) or PS (10 mg/kg) was provided daily from Day 5. (A) Ear swelling was measured 24 h after TMA treatment. Each symbol of square, triangle, circle, and rhombus indicates the naïve group, sham group, SDE group, and PS group, respectively. (B) TMA treatment-induced increase in serum IgE levels was measured by ELISA. Results are shown as mean $\pm$ SD. Asterisks (*) and (**) indicate significant differences, at $p < 0.05$ and $p < 0.01$, respectively, between the SDE-treated and non-treated groups of TMA-treated mice.

response (data not shown). Therefore, we additionally investigated the production of IL-4 and IL-12 as the main cytokines of Th2 and Th1 immune responses in dLNs isolated from mice, respectively. As a result, the SDE attenuated IL-4 production and enhanced IL-12 production in the dLNs (Figure 3(b)). Thus, topical treatment of SDE could attenuate Th2-related allergic responses by ameliorating abnormal immune responses in the ACD mouse model.

Effects of oral administration of SDE on the TMA-induced ACD mouse model

We confirmed that topical treatment of SDE attenuated ACD symptoms by regulating the immune responses activated by TMA exposure. The next, we examined whether SDE ameliorates TMA-induced inflammation by oral administration. As

a result, oral administration of SDE significantly attenuated the TMA-induced increase in ear thickness (Figure 4(a)) and serum IgE levels (Figure 4 (b)). Furthermore, we evaluated the levels of pro-inflammatory cytokines and Th1-/Th2-associated cytokines in the ear tissue and dLNs. Figure 5 shows that oral administration of SDE significantly inhibited the secretion of IL-1 β , TNF- α , and IL-4 in the ear tissue and suppressed IL-4 production in the dLNs. However, in the dLNs, IL-12 production was enhanced by the oral administration of SDE. These results indicated that the treatment of SDE can ameliorate the symptoms of TMA-induced ACD by suppressing inflammation and regulating Th1/Th2 immune balance.

In the present study, we showed the anti-allergic effects of SDE administered topically or by oral

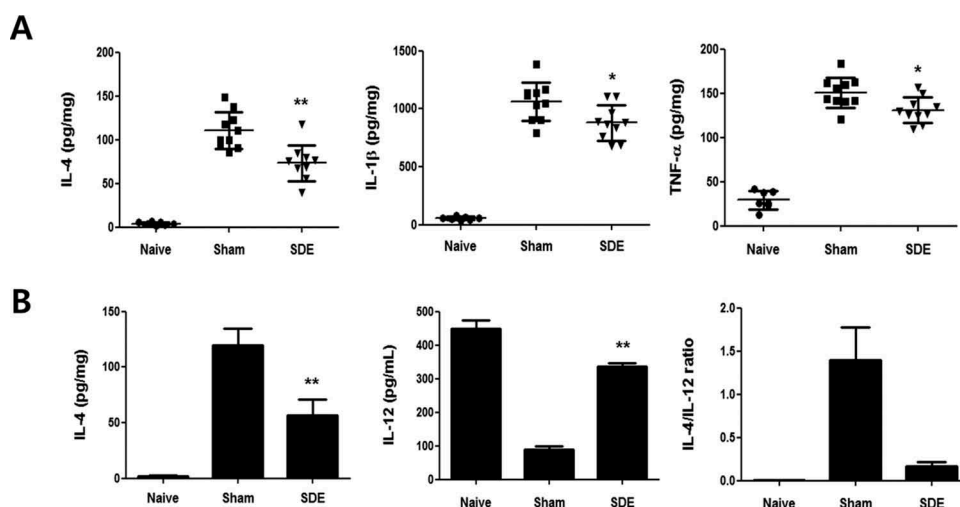


Figure 5. Effect of oral administration of SDE on TMA-induced cytokine production in ear tissue and dLNs.

Ear tissue was isolated, and the proteins were extracted by lysis buffer for cytokine analysis. (A) The production of IL-4, IL-1 β , and TNF- α cytokines in the ear tissue was analyzed by ELISA. (B) The production of IL-4, IL-12 cytokines and IL-4/IL-12 ratio in the dLNs was analyzed. Results are shown as mean $\pm$ SD. Asterisks (*) and (**) indicate significant differences, at $p < 0.05$ and $p < 0.01$, respectively, between the SDE-treated and non-treated groups of TMA-treated mice.

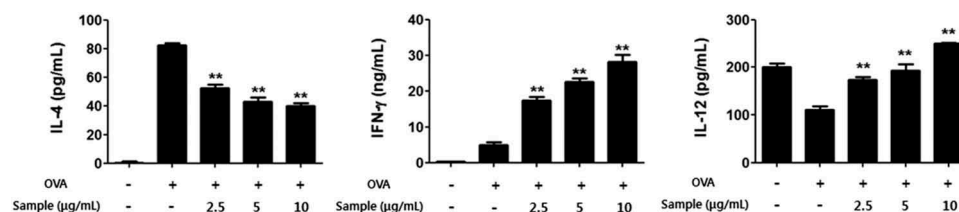


Figure 6. Effect of SDE on IL-4, IFN- γ , and IL-12 production in splenocytes isolated from OVA-sensitized BALB/c mouse.

Splenocytes were seeded to 5×10^6 cells/mL and cultured in the presence or absence of OVA (100 μ g/mL) with SDE (2.5, 5 and 10 μ g/mL). After 72 h, IL-4, IL-12, and IFN- γ production were measured by ELISA analysis. Results are shown as mean $\pm$ SD. Asterisks (**) indicate significant differences at $p < 0.01$ between the SDE- treated and non-treated groups of OVA-treated splenocytes.

administration in a mouse model of ACD. SDE application to attenuate ACD symptoms is possible by both routes. When the inhibitory effects of SDE exerted via the two routes were compared, topical treatment of SDE was found more potent than oral gavage in local lesions (ear tissue): (topical treatment vs. oral administration, IL-1 β ; 27% vs. 17%, TNF- α ; 29% vs. 13%, IL-4; 71% vs. 34%). However, oral administration of SDE was more potent than topical treatment in peripheral lymph organs such as dLNs: (topical treatment vs. oral administration, IL-4; 34% vs. 53%, IL-12; 173% vs. 384%). Therefore, we suggest that topical treatment of SDE might directly affect ACD-related factors in inflammatory lesions, whereas oral administration of SDE might indirectly affect ACD-related factors through gut-associated lymphoid tissues and mucosa-associated lymphoid tissues.

Immunomodulatory effect of SDE on Th1/Th2 immune balance in splenocytes

In this study, we found that SDE ameliorated the symptoms of TMA-induced ACD by regulating Th1/Th2 (IL-12/IL-4) immune responses. However, the IL-12 is not able to directly represent Th1 immune response because IL-12 is produced from antigen presenting cells (APCs). To verify direct evidence of Th1 immune response, we investigated the production of interferon (IFN)- γ because the IFN- γ is produced from Th1 cells. Therefore, we evaluated IFN- γ and IL-4 as a direct evidence of Th1/Th2 immune balance using splenocytes of ovalbumin (OVA)-sensitized BALB/c mice. As a result, SDE increased IL-12 and IFN- γ production and decreased IL-4 production induced by OVA (Figure 6). This result showed the regulatory effects of SDE on Th1 (IFN- γ) and Th2 (IL-4) cytokine production induced by antigen-specific T cell immune responses. We suggest that the SDE is able to regulate Th1/Th2 immune

balance by modulating antigen-specific immune responses between APCs and T cells.

Silkworm droppings contain various components such as lipids, lignans, chlorophyll, and flavonoids, each having a physiological functionality [17–20]. For example, it was reported that β -sitosterol, a phytosterol, was identified in silkworm droppings [38], and that it suppressed IL-2 production in mouse primary splenocytes [39]. Moreover, β -sitosterol showed anti-AD effects by reducing the levels of histamines, IgE, and IL-4 in AD-like NC/Nga mouse model [40]. IL-4 has been known to drive Th2 cell development by directly acting on the precursor T cells and downregulating IL-12 production in antigen-presenting cells including dendritic cells [41]. IL-12 inhibits IL-4 production in allergen-specific human CD4+ T cells [42]. Th2 antagonists such as anti-IL-4 antibody and IL-12 showed suppressive effects on allergic immune responses in TMA-induced ACD mouse model, whereas IFN- γ did not affect these responses [43]. Taken together, our results suggest that the anti-ACD effects of SDE may be associated with the regulation of Th1/Th2 immune balance by upregulating IL-12 production via suppression of Th2 cell activation.

In conclusion, SDE shows great potential to function as an alternative agent to treat Th2 immune response-mediated skin diseases such as ACD and AD. However, how SDE regulates Th1/Th2 balance remains a big question because there are various factors linked to T cell activation. This aspect warrants further studies to demonstrate the effects of SDE on T cells activated by antigen presentation.

Author contribution

D.W.C. and D.A.K. designed the study. D.W.C., D.A.K., H. J.S., and S.Y.J. performed the experiments and analyzed data. D.W.C., S.K.J., and D.H.S. discussed the data. D.W.C. and H.S.S. wrote the paper. All authors checked and approved the paper.

Disclosure statement

No potential conflict of interest was reported by the authors.

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