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Topical application of fucoidan derived from *Cladosiphon okamuranus* alleviates atopic dermatitis symptoms through immunomodulation

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ABSTRACT

Atopic dermatitis (AD) is a T helper (Th) 2 cell-mediated allergic disease, which features increased number of immunocytes and level of Th2-associated cytokines. Fucoidan is well known a naturally occurring agent effectively ameliorating many AD symptoms. Though these alleviative effects are exhilarating, the mechanisms behind, however, are still rather limited. In this study, we report that fucoidan derived from *Cladosiphon okamuranus* (FT) inhibits nitric oxide (NO) production by exerting its anti-inflammatory ability. Topical application on animals show that FT promotes skin repair, reduces immunocyte proliferation, and decreases serum IgE level. In histological analysis, FT favorably reduces epidermal hyperplasia and eosinophilic infiltration. The pharmacodynamics mechanism of FT is determined by means of down-regulating AD-associated cytokines (IL-4, IL-5, IL-22, IL-33, and TSLP) and up-regulating TGF- β 1 level. Moreover, FT can regulate systemic immunity by enhancing tolerogenic dendritic cells (Tol-DCs) to activate regulatory T cells (Treg) differentiation and to decrease the population of Th22 and memory B cells. Overall, topical application of FT is able to enhance Treg secreting TGF- β 1 and to down-regulate Th2 cell-mediated immunity so that AD symptoms are significantly alleviated. Thereby, FT is an ideal drug candidate potentially replacing or complementing corticosteroids to be developed and used as a therapeutic agent to treat AD.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammation disease as a consequence of skin hypersensitivity, which is characterized by intense itching, dry, scaly skin lesions, and highlighted by high serum levels of Th2-associated cytokines and IgE [1]. The current mainstream treatment for AD is topical administration of corticosteroids by suppressing adverse immune-inflammatory responses. Unfortunately, these drugs often cause unfavorable side effects for long-term use and are not able to improve epidermal hyperplasia [2,3]. These facts directly reflect the insufficiency of the current treatments, thus underscoring that there is a desperate need of new drugs and/or therapeutic strategies to compensate or supplement the corticosteroids therapy.

AD generally upregulates the Th2-mediated immune responses.

Allergens that are recognized by antigen-presenting cells (APC) are brought to the lymphatic system. Having associated with naive T cells, the cells start to differentiate into Th2 cells, thus secreting a series of Th2-associated cytokines (IL-4, IL-5, IL-13). These cytokines then signal B cells to produce IgE [4,5]. In skin, these immune responses in no time increase expressions of epidermal innate cytokines IL-25, IL-33, and thymic stromal lymph-protein (TSLP), as a whole exacerbating both severe skin inflammation and skin physical damage [6]. In chronic stage, AD additionally develops serious epidermal hyperplasia owing to dysregulation of IL-22, which has a synergistic effect with Th1 and Th17 simultaneously [7].

Fucoidan is a marine polysaccharide, rich in fucose and sulfate groups, derived from several edible brown seaweeds, such as *Saccharina japonica*, *Undaria pinnatifida*, *Fucus vesiculosus*, *Cladosiphon okamuranus*,

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etc. [8–11]. It exhibits a wealth and variety of biological activities, including anti-tumor, anti-viral, antimicrobial, and antioxidant activities [12–15]. Previous studies have reported that topical application of fucoidan could reduce erythema, inflammation, and UV-induced dehydration [16,17]. In addition, fucoidan has been shown to be conducive to conventional AD treatment in the NC/Nga mice model [18]. It was parallelly demonstrated that fucoidan can lower down IgE level in peripheral blood mononuclear cells in AD patients [19].

Despite copious beneficial evidence of fucoidan, the regulation of Th2 cell-mediated immunity in AD is not well elucidated. In this study, we report that topical application of fucoidan derived from *Cladosiphon okamuranus* (FT) can ameliorate AD symptoms by activating Tol-DCs to elevate Treg cells and the TGF- β 1 level.

2. Materials and methods

2.1. Material

Fucoidan derived from *Cladosiphon okamuranus* (FT) was provided by Kanwa Healthcare Ltd. (New Taipei, Taiwan); it was manufacture by South Product Co., Ltd. (Uruma, Japan). The characteristics of fucoidan, with average molecular weights of 49.8 kDa, contained 52.7 % fucose, 18% uronic acid, and 17.6% sulfate residues [20].

2.2. Measurement of cytotoxicity

RAW264.7 cells at 2×10^5 cells / well were re-suspend and plated in each well of a 96-well plate for 24 h. Then, the cells were treated 100 μ L a series of concentrations (31.25, 62.5, 125, 250, 500, and 1000 μ g/mL) of fucoidan in a medium containing 2 % FBS at 37 °C for 24 h. After the treatment, they were added with 20 μ L of the MTS re-agent, which incubated for 1 h at 37 °C. The cell proliferation was assayed with a spectrophotometer, measured at 490 nm.

2.3. NO assay

During the examination, RAW264.7 cells at 2×10^5 cells / well were implanted into a 96-well plate. After the cells adhesion, added fucoidan with a series of concentrations into the well plate, and separately added 1 μ g/mL LPS to induced cells producing nitrate for 24 h. And then, transferred part of the supernatant to the other 96 well plate. After mixed with Griess reagent A and Griess reagent B reacting in the dark, measured the absorption under 550 nm.

2.4. Animal subjects

Male BALB/c mice of 6 weeks' old were supplied from the National Laboratory Animal Center (Taipei, Taiwan). All the mice were maintained at the Institutional Animal Care and Use Committee, College of Life Sciences, National Taiwan Ocean University (Keelung, Taiwan). They were individually housed in an environment-controlled room under conditions of controlled temperature 22 ± 2 °C, relative humidity 50 ± 10 %, and a 12:12 light–dark cycle. They were acclimated to the new environment and diet for 5 days, during which free intake of water and food was allowed.

2.5. Induction of AD in animal model

Before the mice developed AD-like skin lesions, the dorsal skin area was shaved with depilatory cream 2 days prior to the experiment. For AD induction process, the mice were applied with 100 μ L of 1 % DNCB, dissolved in acetone: olive oil (4:1) mixture on days 0 and 4. Then, they were applied with 100 μ L of 0.2 % DNCB twice a week until sacrificed.

During the experimental period, the blood of the mice was sampled on days 0, 9, and 28. The serum was used for measuring the IgE levels. The mice were sacrificed on day 28. The dorsal skin tissues were isolated

for histological analysis, and the weights of subiliac lymph nodes were used to evaluate the proliferative responses of the lymph nodes. Mice were divided into five groups of 5 mice each: the control group, only vehicle was used; the DNCB group, the mice were applied with DNCB during the experiment; the dermovate (Der.) group, the same operation was applied to as to the DNCB group. 100 mg of dermovate ointment (0.05 % clobetasone propionate) was applied topically to each mouse; the FTL and FTH group, the same operation was applied to as to the DNCB group. Besides, they were topical applications of 100 mg the cream (7:3 w/w mixture of deionized water and olive oil with 2 % w/w emulsifier containing 3.5 % fucoidan and 7 % fucoidan) since day 14, respectively.

2.6. Evaluation of dermatitis scores

The severity of skin lesions was evaluated by Matsuda *et al.* (1997) [21]. The dermatitis scores of skin severity were defined as the sum of the individual scores, for each of the following scoring procedure for AD syndromes as previously described for dryness/scaling, erythema/hemorrhage, and excoriation/erosion: 0, no symptoms; 1, mild; 2, moderate; and 3, severe (12, the maximum score). The skin lesion of a mouse was observed and evaluated on days 0, 4, 7, 11, 14, 18, 21, 25, and 28.

2.7. Histopathological analysis

After the mice were sacrificed, the skin was sampled from the dorsal area of each mouse. The samples were fixed in 10 % formalin for 24 h at room temperature. The fixed skin tissues were trimmed into an appropriate size; and paraffin sections were cut at 4 μ m. The sections were stained with hematoxylin-eosin (H & E) and Giemsa for observing epidermal hyperplasia and counting the number of eosinophils in five ran-dom fields. Microscopic observations were carried out at 400 $\times$ magnifications.

2.8. Level measurements of cytokines

Cytokine analysis was performed by using ProcartaPlex® Multiplex Immunoassay (Affymetrix, eBioscience, San Diego, USA) as specified by the manufacturer: IL-4, IL-5, IL-13, IL-22, IL-33, TSLP, and TGF- β 1. Readings were performed on the MAG-PIX®-Luminex platform (Merck Millipore, Darmstadt, Germany).

2.9. Flow cytometry analysis

After Spleen were homogenized, the spleen cells filtered by 40 μ m cell strainer (BD Biosciences, San Diego, USA). Used red blood cells lysis buffer to lyse erythrocytes for 3 min at room temperature and washed in 10 % fetal bovine serum (Gibco, Glasgow, UK). Then, they centrifuged at 1000g for 10 min at 4 °C, supernatants were discarded carefully, and pellets re-suspended in 10 % fetal bovine serum. Splenocytes were harvested, and diluted to 1×10^6 cells per tube. They were labeled with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD80 (BD Biosciences, San Diego, USA), a FITC-conjugated anti-mouse CD86 (BD Biosciences, San Diego, USA), an allophycocyanin (APC)-conjugated anti-mouse CD273 (BD Biosciences, San Diego, USA), or a phycoerythrin (PE)-conjugated anti-mouse CD274 (BD Biosciences, San Diego, USA). Th22 cells (CD4⁺/CCR10⁺) from splenocytes were detected by staining with a FITC-conjugated anti-mouse CD4 (BD Biosciences, San Diego, USA) and a PE-conjugated anti-mouse CCR10 (R&D Systems, Minneapolis, USA). Memory B cells (CD19⁺/CD27⁺) were detected by staining with a FITC-conjugated anti-mouse CD19 (BD Biosciences, San Diego, USA) and a PE-conjugated anti-mouse CD27 (BD Biosciences, San Diego, USA). Plasma cells (CD27⁺/CD138⁺) were detected by staining with a PE-conjugated anti-mouse CD27 (BD Biosciences, San Diego, USA) and an APC-conjugated anti-mouse CD138 (BD Biosciences, San Diego,

USA). Mast cells (CD117⁺/FcεRI⁺) were detected by staining with a PE-conjugated anti-mouse CD117 (BD Biosciences, San Diego, USA) and a FITC-conjugated anti-mouse FcεRI (eBioscience, San Diego, USA). The labeled splenocytes were assessed by recording at least 10,000 events, using an Attune NxT Flow cytometer (Thermo Fisher Scientific, IL, USA).

2.10. Statistical analysis

The quantitative data were expressed as mean ± the standard deviation (S.D.). The results were undergone an analysis of variance (ANOVA) followed by a Tukey's test. In each case, a p-value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of fucoidan on cytotoxicity and nitric oxide (NO) production in RAW264.7 cells

First of all, there is no apparent cytotoxicity to the RAW 264.7 cells when fucoidan was provided even as high as 1000 µg/mL in comparison to the control group by the MTS assay (Fig. 1A). To determine the inhibition effect of fucoidan on NO production, the LPS-induced NO production in RAW264.7 cells was measured after addition with a series of different concentrations of fucoidan, in which a significant difference starts at 31.25 µg/mL. The NO production appears inversely proportional to the increased concentrations of fucoidan in a dose-dependent manner (Fig. 1B).

3.2. Improvement efficacy of topical application of FT on DNCB-induced mice

In the animal examination, mice were divided into 5 groups, namely, control, dinitrochlorobenzene (DNCB), dermivate (Der.), FTL, and FTH. SCORing AD (SCORAD) was used to score wound severity for DNCB-induced mice. Symptoms of erythema/hemorrhage, edema, excoriation/erosion and dryness/scaling were recorded; the severity of each symptom was scored as 0 (no symptom), 1 (mild), 2 (moderate), and 3 (severe). We started to apply fucoidan on day14, and the skin damage began to improve on day18. We observed that the SCORAD values of the FT groups were significantly lower than that of the DNCB group on day25 and 28. It is noticeable that the Der. group showed epidermal thinning, causing mice to scratch the skin. Its score was slightly higher than those of the FT groups (Fig. 2).

After mice sacrifice, we measured the weights of subiliac lymph nodes to explore the proliferative responses of lymph nodes. The results show that DNCB causes proliferation of lymphocytes in the lymph nodes in the DNCB group. For both FT groups, the weights of subiliac lymph nodes are relatively lighter, suggesting that FT can reduce lymphocyte

proliferation. By contrast, the weight of subiliac lymph nodes in the Der. group is the lowest amid all testing group, suggesting Der. strongly inhibited inflammatory responses (Fig. 3).

Next, the serum IgE level was monitored on days 0, 9, and 28. We first sensitized with DNCB and followed by challenging with FT/Der. on the dorsal skin of mice. DNCB is known to induce Th2 polarization of skin and to cause elevated IgE level. In general, the serum IgE level is consistent as high as 2000 ng/mL amid all testing groups on the 9th day, indicating DNCB successfully induces an allergic reaction in mice. The DNCB-induced mice were then treated with topical application of FT or Der. for 14 days. In both the FT and Der. groups, the production of IgE is significantly suppressed, while FT outperforms Der. (Fig. 4), highlighting that topical application of FT greatly ameliorates the symptoms of AD.

3.3. Effects of topical application of FT on epidermis and cell infiltration in DNCB-induced mice

Epidermal thickness was measured by using H&E staining as an index for DNCB-induced skin lesions. As shown in Fig. 5B, the DNCB group shows significant epidermal hyperplasia. By contrast, the epidermal thickness of all topical treatment groups tapers in varied extent; the Der. Group is particularly thinner than the control (Fig. 5A, C, F). The fact is that Der. has a strong side effect causing skin atrophy and increasing the risk of skin infections because of skin barrier disruption (Fig. 5G). The Giemsa staining further contrasts that the DNCB treatment results in apparent eosinophils infiltration, which, nonetheless, is significantly ameliorated in the groups receiving Der. or FT (Fig. 6).

3.4. Reduction of immune co-stimulatory molecules and splenocytes by topical application of FT

To evaluate the improvement of topical application of FT in the systemic immunity, we examined the level of immunocytes and co-stimulatory molecules. Splenocytes were isolated from all testing groups for flow cytometry analysis. First, CD80 and CD86 are co-stimulatory molecules for T-cell activation in tandem. When compared with that of the DNCB group, the CD80 and CD86 expression levels are significantly lower those of the FTH group (Fig. 7 A, B). Second, the expression levels of CD273 (PD-L2) and CD274 (PD-L1) are significantly up-regulated in the FTH group (Fig. 7 C, D). The fact is that CD273 and CD274 are inhibitory receptors, which inhibit T cell proliferation and inflammatory cytokine production. Third, we examined Th22 cells, memory B cells, plasma cells, and mast cells, which all are associated with the Th2 cell-mediated immune responses. The populations of plasma cells (CD27⁺/CD138⁺) and mast cells (CD117⁺/FcεRI⁺) are not significantly decreased, while the populations of Th22 cells (CD4⁺/

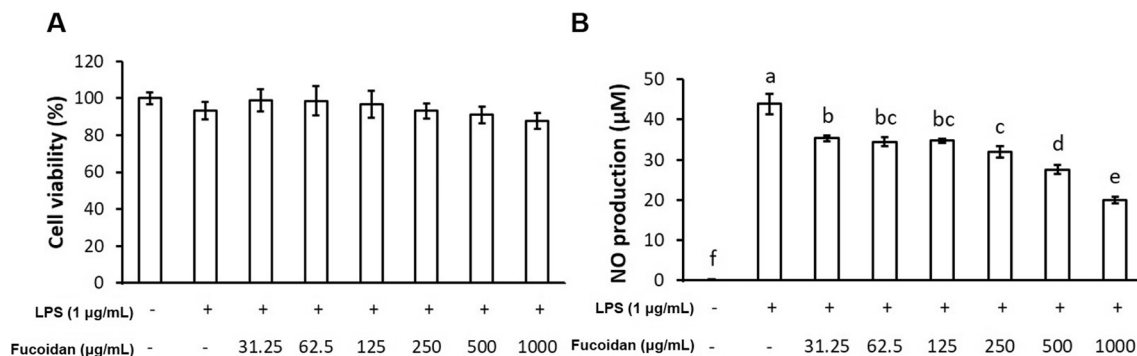


Fig. 1. The inhibitory effect of fucoidan on LPS-induced nitric oxide (NO) production. (A) Cytotoxicity of FT on RAW264.7 cells. (B) nitric oxide production in RAW264.7 cells treated with fucoidan. Four independent experiments were performed, and values were calculated as mean ± SD in each group. Means with different letters above the bars stands for significant differences at p < 0.05.

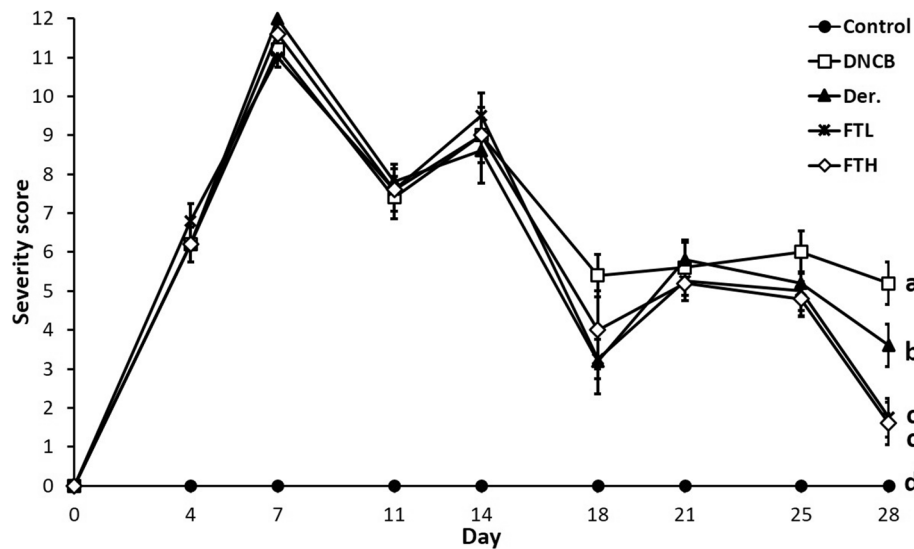


Fig. 2. The dermatitis score was measured twice a week for erythema/hemorrhage, scaling/dryness, edema, and excoriation/erosion. Each column shows the mean $\pm$ SD of 5 mice. Means with different letters above the bars stands for significant differences at $p < 0.05$.

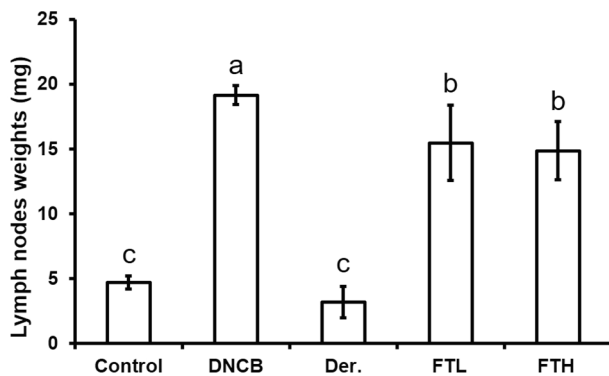


Fig. 3. Effects of FT on the subiliac lymph nodes in the DNCB-induced mice. Each column shows the mean $\pm$ SD of 5 mice. Means with different letters above the bars stands for significant differences at $p < 0.05$.

CCR10⁺) and memory B cells (CD19⁺/CD27⁺) are significantly decreased (Fig. 8). Added together, topical application of FT improves the Th2 cell-mediated immune responses in AD through positive

immunomodulation.

3.5. Reduction of AD-associated cytokines by FT in DNCB-induced mice

To explore the immunomodulation of FT for AD, we performed cytokine analysis for the FTH and the DNCB groups. Given that IL-4 and IL-13 are the main cytokines that cause allergic reactions, these cytokines, as expected, are highly expressed in the DNCB group. Although the level of IL-13 in the DNCB group is no difference to that in the FTH group, the level of IL-4 is significantly lower than that in the DNCB group (Fig. 9 A, C). We found here that the topical application of FT can effectively inhibit production of IL-5 (Fig. 9 B) in agreement with the Giemsa histological analysis. One should also be aware that cytokine disordered could result in skin barrier disruption, inflammation, and skin thickness. Here, we noticed that cytokines IL-22, IL-33, TSLP, and IFN- γ all are elevated to significant extent in the DNCB group. In contrast, these cytokines are significantly downregulated in the FT groups (Fig. 9 D, E, F, H). The expression levels of TGF- β 1 in the FTH group is significantly increased, which is higher than that in the DNCB group (Fig. 9 G). Added together, the topical application of FT is concluded favorably to improve AD symptoms by suppressing AD-

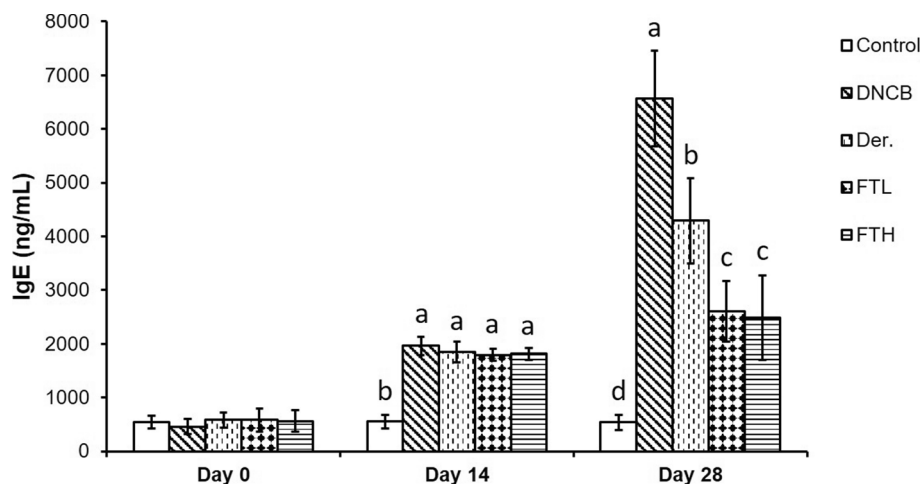


Fig. 4. The serum IgE levels in the DNCB-induced mice were determined by using ELISA assay. Results are expressed as the mean $\pm$ SD ($n = 5$). Means with different letters above the bars stands for significant differences at $p < 0.05$.

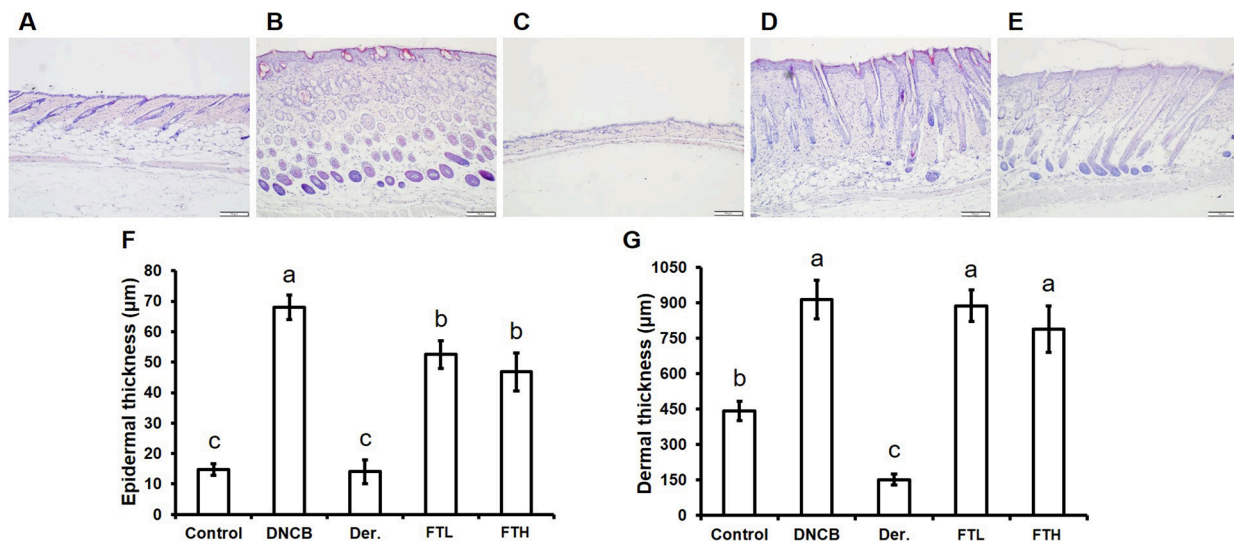


Fig. 5. Effects of FT on the dermis in the DNCB-induced skin lesion. (A-E) H & E stain to contrast dermis changes, (F) epidermal thickness, and (G) dermal thickness. Original magnification $\times 100$. Means with different letters above the bars stands for significant differences at $p < 0.05$.

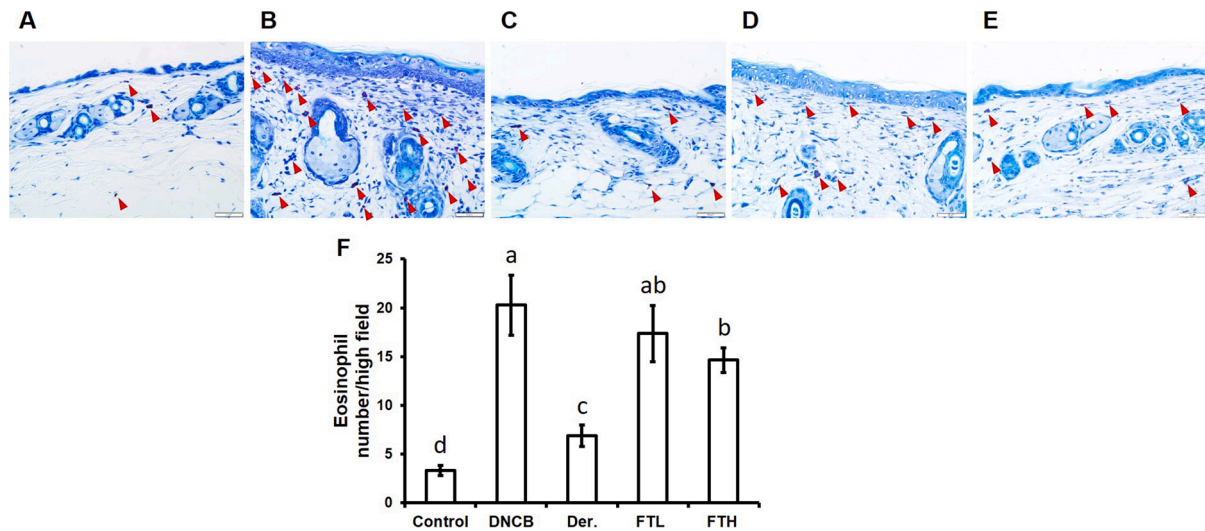


Fig. 6. Effects of FT on the DNCB-induced skin lesion by Giemsa staining (A-E). The red arrows indicate the presence of eosinophil granulocyte in the dermis of skin. (F) The number of eosinophil granulocytes in skin lesion was measured and graphed. Original magnification $\times 400$. Means with different letters above the bars stands for significant differences at $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

associated cytokines.

4. Discussion

It has been well documented that the biological activity of fucoidan is affected by its size and the content of subcomponents, e.g., the number, ratio and position of fucose, glucuronic acid, and sulfate [22,23], because these factors inference its absorption and physiological activity. Topical application of fucoidan has also been investigated, while the investigation was limited to physical symptoms, such as skin wound repair, burn repair, and reduction of UV damage [16,17,24], covering relatively less immunology. Nonetheless, Yang (2012) [18] ever reported that fucoidan down-regulates AD-associated chemokines, such as thymus- and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC); Tian *et al.* (2019) [25] reported that topical application of fucoidan promotes regulatory T cells (Treg) and suppresses Th2 immune responses in AD-induced mice. Some recent studies also reported that the fucoidan ointment can penetrate skin barrier and accumulate in muscles, and then go into systemic

circulation, thus highlighting that the topical application of fucoidan meets desirable pharmacokinetics [26]. The previous reports showed that the cream containing 3–4% fucoidan regulated immune response, alleviating AD symptoms in the animal model [18] and improving patients with oral herpes [27]. Therefore, we established two groups: the cream containing 3.5% fucoidan as basis dose the cream containing 7% fucoidan as double in this study. NO is involved in several biological functions, including vasodilation, apoptosis, and immune response; moreover, NO production assay is one of the methods commonly used to determine the anti-inflammatory activity of compounds. Clinical studies have indicated that serum NO levels in patients with atopic dermatitis are higher than those in the healthy control group [28,29]. Taniuchi *et al.*, (2001) [30] demonstrated that NO is related to skin vasodilation and erythema in AD patients. On top of that, we demonstrate here that FT could reduce NO production and improve anti-inflammation [31]. Most importantly, fucoidan can reduce pro-inflammatory cytokines (IL-1 β , IL-6, and NO) by down-regulating NF- κ B in nucleus.

Glucocorticoids are medicines widely applied to treat AD, while they inevitably bring on some adverse effects if long-term use, such as weight

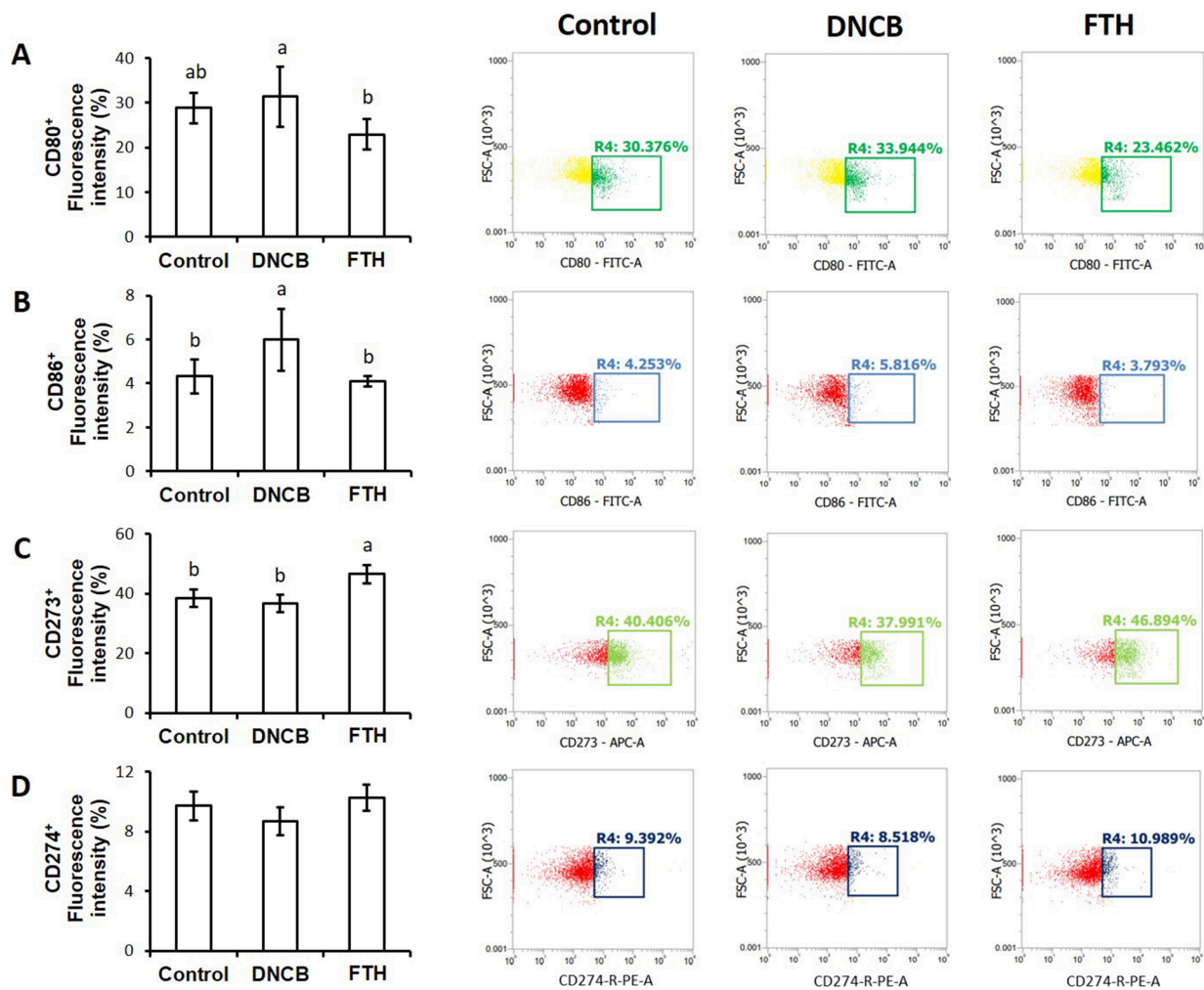


Fig. 7. CD80 (A), CD86 (B), CD273 (C), CD274 (D) expressions in DNCB-induced mouse splenocytes. Results are expressed as the mean $\pm$ SD ($n = 5$). Means with different letters above the bars stands for significant differences at $p < 0.05$.

loss and skin thinning [32,33] as shown in Der. group. In this context, it underlines an unmet need and thus prompts one to develop or search for new drugs, adjuvant and/or alternative treatments.

Topical application of FT is subsequently demonstrated favorably to reduce skin damage, suppress proliferation of lymph nodes, and inhibit serum IgE production at macroscopic level. When APC, such as macrophages, B cells, or dendritic cells, presents antigens to T cells, the CD28 receptor of T cells could associate with CD80 and CD86 co-stimulatory molecules of APC, thereby promoting T cells proliferation and leading naïve T cells polarization toward Th2 cells [34]. In the present study, we demonstrate that topical application of FT significantly reduces co-stimulatory molecules (CD80 and CD86). In parallel, we show that the CD273 and CD274 expression were up-regulated in the FTH group. CD273 (PD-L2) and CD274 (PD-L1) are typical cell surface receptors of Tol-DCs, which negatively regulate adaptive immune responses [35,36]. In addition to downregulate expression of co-stimulatory molecules (CD80 and CD86) on the surface and upregulate expression of inhibitory surface molecules (CD273 and CD274), Tol-DCs promote proliferation and activation of Treg cells [37,38]. These results together account for that FT could suppress a wide range of Th2-mediated immune responses through decreasing APC presenting allergens to naïve T cells and activating Treg cells for negative regulation.

On the other hand, Th2 cells stimulate B cells to differentiate into memory B cells and plasma cells upon the first-time exposure to allergen. When the second-time exposure to allergen, the memory B cells rapidly produce specific IgE antibodies. They also differentiate into

plasma cells, which could secrete large quantities of specific IgE [39]. Mast cells remain inactive until specific IgE binds to FcεRI on the surface of mast cells. Then, mast cells release large amounts of degranulated mediators, e.g., IL-4, histamine, chemokines, etc., which result in severe allergic inflammation [40]. We thus analyzed populations of memory B cells, plasma cells, mast cells in splenocytes. In comparison with the DNCB group, we found that the reduction of memory B cells and serum IgE level is only taking place in the FT group.

Epidermal hyperplasia, a histological sign of AD, is caused by the expression of Th22, Th1 and Th17 cytokine upregulation. Several reports have confirmed that IL-22 plays a pivotal role in keratinocyte-related diseases. In fact, IL-22 increases keratin-17 expression in keratinocytes via the STAT3 and ERK1/2 signaling pathways, leading to epithelial proliferation. Beyond that, both IL-22 and IFN- γ greatly enhance epithelial proliferation by a synergistic effect [41]; moreover, the clinical report demonstrated that the chronic AD skin lesions increase the levels of keratin 16 mRNA and Ki67 protein, which cause proliferation and hyperplasia; simultaneously, the expressions of Th22, Th1, and Th17 cytokines also increasing were observed [7]. The increased expression of IL-22 from Th22 cells in AD patients is another factor to exacerbate epithelial proliferation [42]. Topical application of FT, however, is determined here that it can reduce the overall population of Th22 and downregulate serum IL-22 and IFN- γ levels, both as a whole to suppress epidermal hyperplasia.

In summary, topical application of FT efficiently suppresses Th2-associated immunocytes so as to suppress the releases of downstream

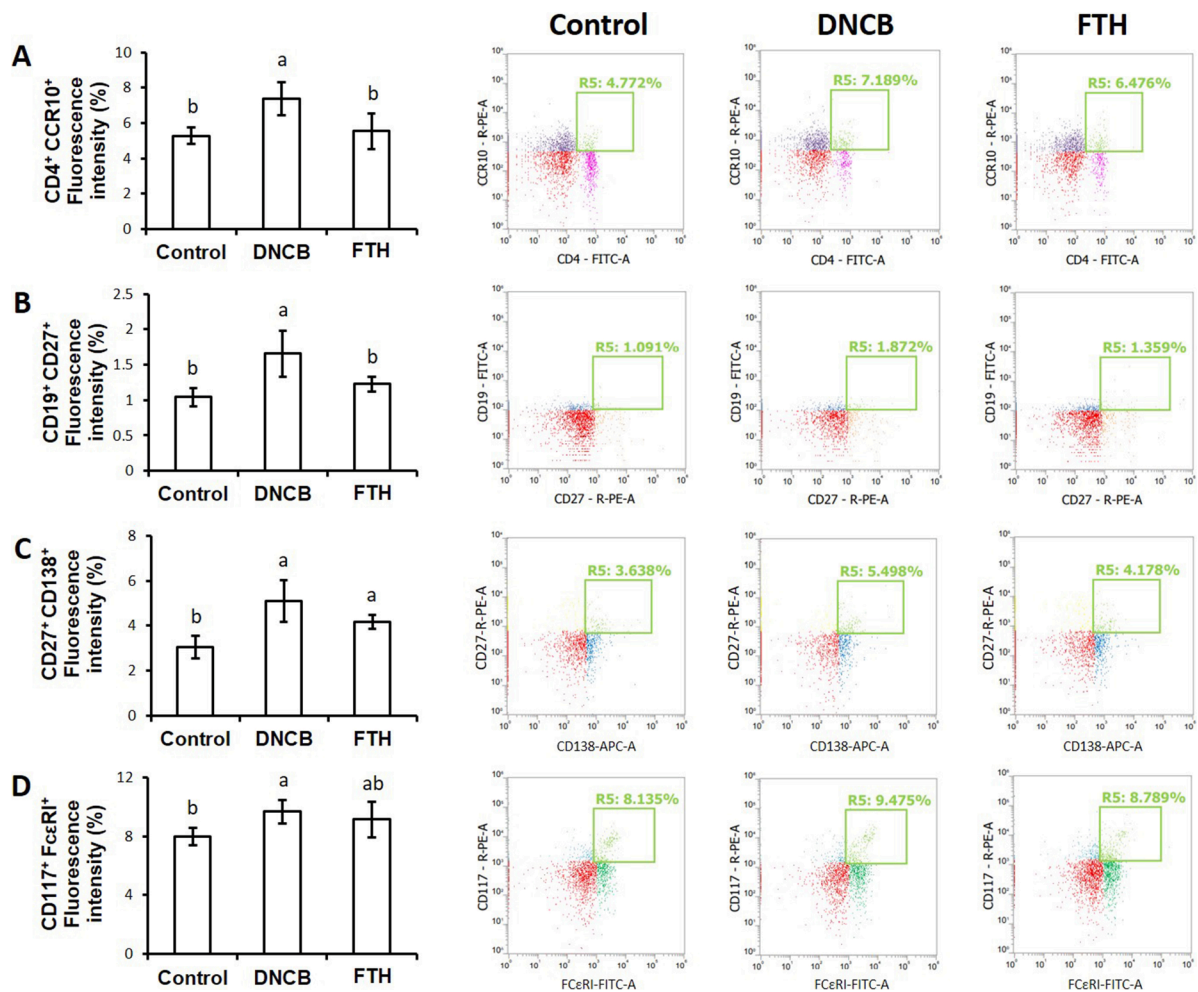


Fig. 8. Representative flow cytometry plots of CD4⁺CCR10⁺ Th22 cells (A), CD19⁺CD27⁺ memory B cells (B), CD27⁺CD138⁺ Plasma cells (C), CD117⁺FcεRI⁺ mast cells (D) in the spleen, and graphs representing the percentage of these cells in DNCB-induced mice. Means with different letters above the bars stands for significant differences at $p < 0.05$.

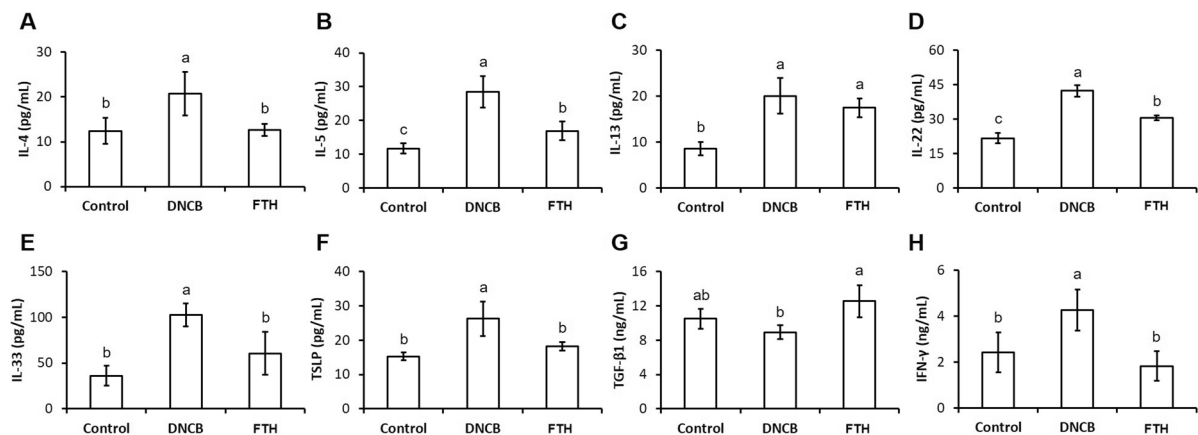


Fig. 9. The serum cytokine levels in DNCB-induced mice were determined using the ProcartaPlex Mouse IL-4 (A), IL-5 (B), IL-13 (C), IL-22 (D), IL-33 (E), TSLP (F), TGF-β1 (G), and IFN-γ (H) Simplex kit. Results are expressed as the mean $\pm$ SD ($n = 5$). Means with different letters above the bars stands for significant differences at $p < 0.05$.

cytokines. IL-4 and IL-13 play important roles in allergic reaction by activating T and B cells proliferation, and promoting B cells differentiation into plasma cells, which, nonetheless, are the key regulators in humoral and adaptive immunity [43,44]. Both Th2 cells and mast cells produce IL-5, which intrinsically engages in multiple allergic reactions

and induces a vast number of eosinophils infiltration [45]. Owing to Th2-associated cytokines that damage skin barrier, the epithelial and epidermal innate cytokines (IL-33, and TSLP) are up-regulated resulting in skin inflammation [46,47]. Although most allergic reactions are mainly from Th1/Th2 imbalance, the previous discussion shows that the

synergistic effect of Th1 cytokines and IL-22 causes epidermal hyperplasia in the chronic stage of AD. It means that by up-regulating Th1 cytokines, suppressing Th2 immune response is less effective in improving atopic dermatitis. In this study, we observed that the expression of Th1 and Th2 cytokine (except IL-13) downregulated to a remarkable extent in the FTH group. We speculated that regulatory T cells produce TGF- β 1 for negative regulation of Th1 and Th2; therefore, we measured the expression of TGF- β 1 in serum. In contrast, the serum level of TGF- β 1 produced by Treg cells was up-regulated in the FTH group, which has a critical role in the inhibition of proliferation and differentiation of allergy-related T and B cells [25,48].

5. Conclusions

With the aforementioned results, FT is substantiated to be able to reduce NO productions in RAW264.7 cells. Topical application of FT activated Tol-DCs to promote Treg cells differentiation, secreting TGF- β 1, which suppressed Th2-mediated immune response in AD. In addition, FT reduced the population of memory B cells to decreased IgE production. Notably, we also observed that topical application of FT ameliorated epidermal hyperplasia via Th22 down-regulation. This pilot study observed that topical application of FT modulated Th2 cell-mediated immunity, which alleviates AD symptoms in the DNCB-induced mice model.

Author contributions

Chang-Jer Wu and Yi-Lin Chan designed the whole experiments and manuscript. Bo-Rui Chen and Kung-Ting Hsu performed experiments and analyzed data. Tsung-Lin Li reviewed the manuscript. Bo-Rui Chen, Tsung-Lin Li, Yi-Lin Chan and Chang-Jer Wu engaged in discussion and writing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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