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Fucoidan from seaweed *Fucus vesiculosus* inhibits 2,4-dinitrochlorobenzene-induced atopic dermatitis

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ABSTRACT

Fucoidan, one activator of scavenger receptor class A (SR-A), plays important roles in a variety of biological activities, including anti-inflammatory, antioxidant, and antitumor actions. However, the effects of fucoidan on atopic dermatitis (AD) have not been elucidated. To assess this, 2,4-dinitrofluorobenzene (DNFB)-treated BALB/c mice were painted with fucoidan. Results showed that fucoidan significantly ameliorated ear swelling, improved abdomen skin lesions, and decreased inflammatory cell infiltration. In addition, fucoidan significantly suppressed the serum levels of IgE and IL-4 in DNFB-induced AD mice. The infiltration of $CD4^+$ T cells in skin lesions and spleen was also reduced in fucoidan-treated AD mice. Furthermore, treatment with fucoidan promoted Treg cells but attenuated Th1/17 response in the spleens from DNFB-induced AD mice. Together, these results suggest that fucoidan, a natural seaweed-rich polysaccharide, has a potential therapeutic efficacy in the treatment of AD, correlates with the induction of higher anti-inflammatory response through inducing Treg cells.

1. Introduction

Atopic dermatitis (AD) is one kind of chronic skin disease characterized by inflammation, dryness, pruritus, and intense itching [1]. In recent years, the incidence of AD in China has gradually increased, especially up to 15% to 30% in children [2]. Current topical and systemic treatments for AD are reasonably effective but are associated with inconvenient side effects [3,4]. Therefore, there is an urgent need for the development of effective and safer drug therapy for clinical treatment of AD.

Studies suggested that immune cells, especially $CD4^+$ T cells, play an extremely important role in the progression of AD [5,6]. $CD4^+$ T cells express TCRαβ, and the recognition antigen is restricted by major histocompatibility complex class II (MHCII), and can differentiate into several subsets, including Th1 ($CD3^+CD4^+IFN\text{-}\gamma^+$), Th2 ($CD3^+CD4^+IL\text{-}4^+$), Th17 ($CD3^+CD4^+IL\text{-}17^+$), as well as Treg ($CD3^+CD4^+Foxp3^+$) cells under different microenvironments [7]. The acute phase of AD is a Th2-dominant immune response. The various allergens in the environment are primarily captured by Langerhans cells (LCs) after being exposed to the skin or mucous membranes. After a series of processing, $CD4^+$ T cells are activated and differentiation into Th2 cells and express type 2 cytokines (IL-4, IL-5, IL-10, and IL-13), which contribute to the pathogenesis of AD [8]. In the 24–48 h after the acute phase, the lesion area is transformed into a Th1-dominant immune response and induces high levels of cytokines such as IFN-γ and TNF-α [9]. In addition, the percentages of Th17 cells are increased both in the lesions and peripheral blood of AD patients, the expression of IL-17 is significantly positively correlated with the severity of such disease [10]; while SR1001, one antagonist of Th17 cell-specific nuclear transcription factor RORα/γ, can significantly inhibit dermatitis and IL-17

Abbreviations: AD, atopic dermatitis; SR-A, scavenger receptor class A; DNFB, 2,4-dinitrofluorobenzene; TNF-α, tumor necrosis factor α; IL-12, interleukin 12; IL-4, interleukin 4; Th cells, helper T cells; Treg, T regulatory; LC, Langerhans cell; ELISA, enzyme-linked immunosorbent assay; FACS, flow cytometry analysis

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production in MC903-induced AD mice [11]. Furthermore, the infiltrate of T regulatory cells, which inhibit skin Th1 cell responses, has been found to be reduced in the skin of AD patients [12]. All the above studies suggest that the imbalance of various subpopulations of $CD4^+$ T cells leads to a series of cytokine changes that are the main immunological mechanisms of AD.

Fucus vesiculosis fucoidan, which is a sulphated polysaccharide extracted from brown marine algae and is the most common source of experimental fucoidan used in the research. *Fucus vesiculosis* fucoidan has been reported to play critical roles in a variety of significant biological activities, such as anti-inflammatory, antioxidant, and anti-tumor activities [13,14]. *Fucus vesiculosis* fucoidan is known to be a natural ligand for class A scavenger receptors (SR-A), which play critical roles in regulating the body's innate immune response and adaptive immune responses [15,16]. However, whether *Fucus vesiculosis* fucoidan could suppress the development of AD through activating SR-A and affecting adaptive immune responses are still unknown. To address this, conventional Balb/c mice were subjected to a 2,4-dinitrofluorobenzene (DNFB) protocol to induce AD and further treated with fucoidan to assess the effects of fucoidan on a DNFB-induced AD-like mouse model.

2. Material and methods

2.1. Mice and atopic dermatitis model

Six-week-old female Balb/c mice (SPF) purchased from the Animal Core Facility of Nanjing Medical University were bred under SPF condition. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University and performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988.11.1).

Mice were weighed and distributed equally into 4 groups (n = 6) labeled with Ctrl (normal mice without any treatment), DNFB (atopic dermatitis without drug treatment), DNFB + fucoidan (atopic dermatitis plus fucoidan treatment), DNFB + Dexamethasone (topic dermatitis plus dexamethasone treatment).

AD was induced in BALB/c mice according to the previous study with minor modifications [17]. Briefly, 2,4-dinitrofluorobenzene (DNFB) (Sigma, Seelze, Germany) was dissolved in AOO (acetone:olive oil = 4:1) and used as an inducer of atopic dermatitis. The hair on abdominal skin was shaved with an area of about 4 cm^2. For sensitization, 100 μl 0.5% DNFB was topically applied to the shaved abdomen. Four days later, the mice were challenged by painting abdomen and the inner and outer surfaces of both sides of the ears with 20 μl 0.2% DNFB, and mice were rechallenged each day from day 5 to day 9 post sensitization to yield extensive disease. The treatment group was painting abdomen and ears with 50 μl 0.2% fucoidan (from *Fucus vesiculosus*, Sigma-Aldrich, St Louis, MO) during the experiment. On the other hand, mice in the control group were treated with an equal volume of AOO, and 0.1% dexamethasone was used as a positive control (Fig. 1a).

2.2. Ear swelling and ear erythema score

Ear thicknesses were measured with a thickness gauge at each time point. Based on the following scoring procedure, dermatitis that with dryness, erythema, edema, and excoriation was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The assessment was done by an investigator who was blind about the grouping of the animals.

2.3. Spleen indexes calculation

Ten days post first day's treatment, mice were weighed and sacrificed. The spleen was collected and weighed. The spleen indexes were defined as spleen weight *versus* body weight.

2.4. Histology by hematoxylin-eosin (H&E) and immunofluorescence staining

Mice ears were fixed in 4% formalin solution then embedded in paraffin blocks. Tissues were cut into 6 mm slices and stained with hematoxylin-eosin (H&E) and were analyzed using Axiovision software (Carl Zeiss, Hallbergmoss, Germany).

Paraffin sections (6 mm thickness) were deparaffinized and immunohistochemically stained for rat anti-mouse SR-A (R&D Systems, Minneapolis, MN USA) or Alexa Fluor FITC-rabbit anti-mouse CD4 (eBioscience, San Diego, CA). Then PE-conjugated goat anti-rat IgG (Cell Signaling Technology, Beverly, MA, USA) was added to detect SR-A. Quantification of CD4 and SR-A in each group was achieved by Image J software.

2.5. Detection of IgE and IL-4 by ELISA

Total serum samples were prepared from the blood of each group and were determined *via* ELISA using a commercially available IgE or IL-4 kit according to the manufacturer's instructions (Abcam, Cambridge, MA).

2.6. Real-time PCR

RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis and Real-time PCR was performed using Power Syber Green PCR Master Mix (Applied Biosystems, Foster City, CA). The sequences of primers were as follows: SR-A, F: AGAAGAACA AGCGCACGTGG, R: CCCAACAGCACCCAGGGTTA; TNF-α, F: CATCTT CTCAAAATTCGAGTGACAA, R: TGGGAGTAGACAAGGTACAACCC; IL-12p35, F: GACAGTGGAGGCACCAGGCC, R: CAGACATCGCTGTCCCG GCG; IL-4, F: ATGGGTCTCACCTCCCAACTG, R: TCAGCTCGAACACTT TGAATAT; TGF-β, F: ATGCTAAAGAGGTCAAAAGC, R: CCAAGGTAAC GCCAGGAATT; IL-10, F: ACTTTAAGGGTTACTTGGGTTGC, R: ATTTT ACAAGGGGAGAAATCG; GAPDH, F: GGTGAAGGTCGGTGTGAACG, R: ACCATGTAGTTGAGGTCAATGAAGG. Data were processed using SDS software (Applied Biosystems). Results were normalized to the expression of GAPDH.

2.7. Cell proliferation assay

Cell proliferation assay was performed with a Cell Counting Kit-8 (CCK-8, Sigma-Aldrich). Briefly, splenocytes (5×10^4/100 μl in each well) from each group were seeded into 96-well plates, and then added ConA (100 μl, with a final concentration of 5 mg/l) in each well and cultured at 37 °C for 24 h. Then, the plates were removed from the incubator, CCK8 solution (10 μl/well) was added, and reincubated at 37 °C for another 2 h. After that, the optical absorbance was measured at 450 nm.

2.8. Isolation of splenocytes and flow cytometry

Spleens were rinsed with an erythrocyte lysis buffer, meshed through a 100-mm cell strainer, and washed and counted. Flow cytometry was performed as described previously with some modifications [16]. For Th1/Th2/Th17 cells, 2×10^6 of single-cell suspension were stimulated with 25 ng/ml PMA (Sigma) and 1 mg/ml ionomycin (Sigma) in the presence of 0.66 μl/ml Golgistop (BD, San Jose, CA) for 6 h. Cells were stained with surface makers CD3-APC and CD4-FITC (both from eBioscience), fixed and permeabilized with Cytofix/Cytoperm buffer and then intracellularly stained with PE-conjugated IFN-γ, IL-4, IL-17 or isotype antibody, respectively. For Treg cells detection, 2×10^6 of single-cell suspensions were surface stained with CD3-APC and CD4-FITC. Then, cells were fixed and made permeable with fixation-permeabilization buffers (eBioscience) and blocked with Fc-receptor (eBioscience). Finally, cells were stained with PE-conjugated

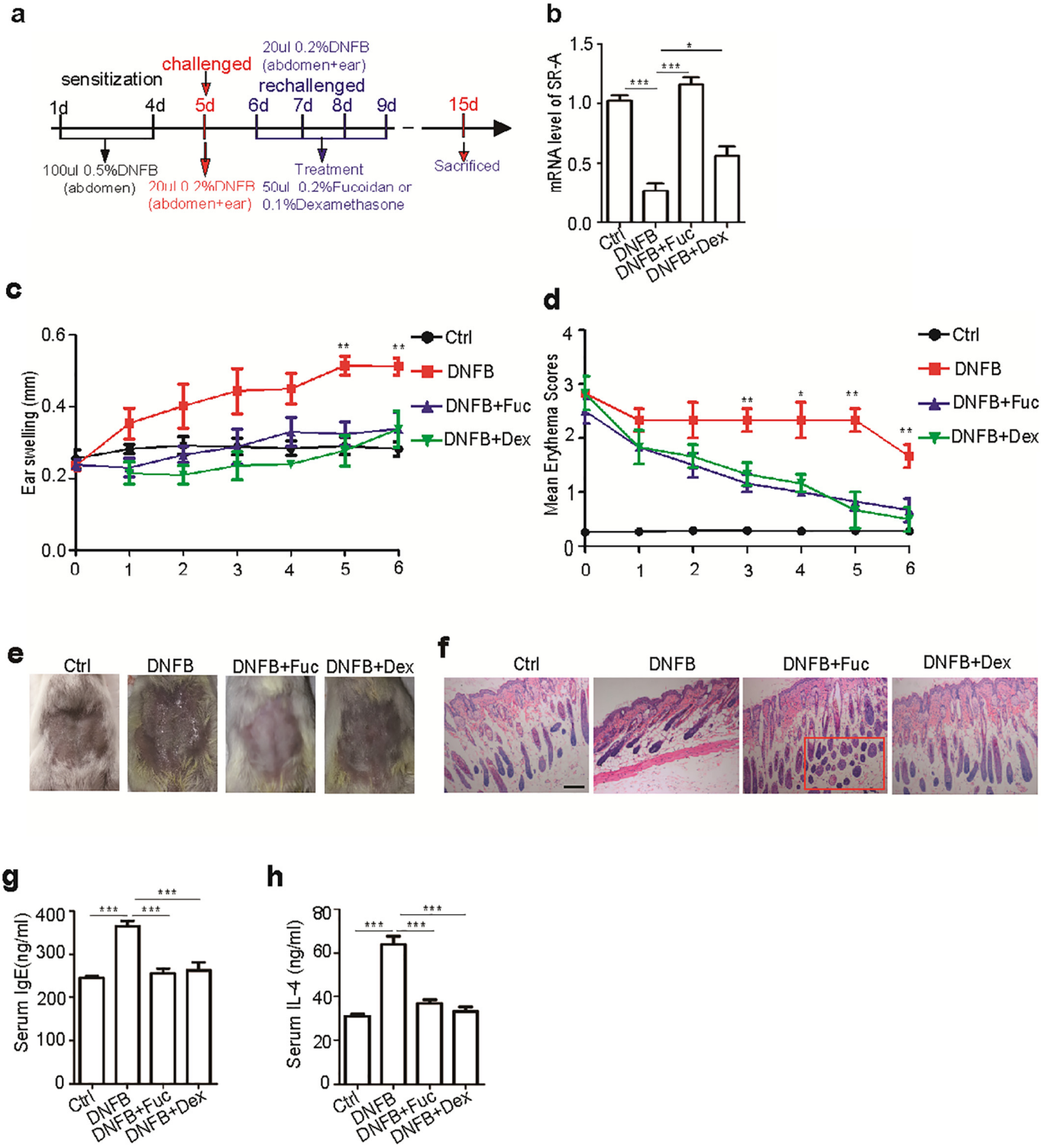

Fig. 1. Fucoidan improved atopic dermatitis (AD)-like symptoms in DNFB-induced mice.
(a) Fucoidan or dexamethasone was used to treat DNFB-induced AD in the mouse model according to the diagram. The AD response was analyzed at time points indicated following elicitation. (b) RT-PCR analysis of the mRNA level of SR-A in the ears of each group. (c) Ear thickness was detected after treatment in AD mice by using a caliper micrometer. (d) Erythema scores as a function of treatment in mice with AD. (e) Representative macroscopic photographs of DNFB-induced skin lesions of atopic dermatitis. (f) Cross-sections of peritoneum were stained with hematoxylin & eosin in each group. Levels of serum IgE (g) and IL-4 (h) were detected by ELISA. Data are expressed as the mean ± SD of each group (n = 6), and are representative of one typical experiment out of three, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

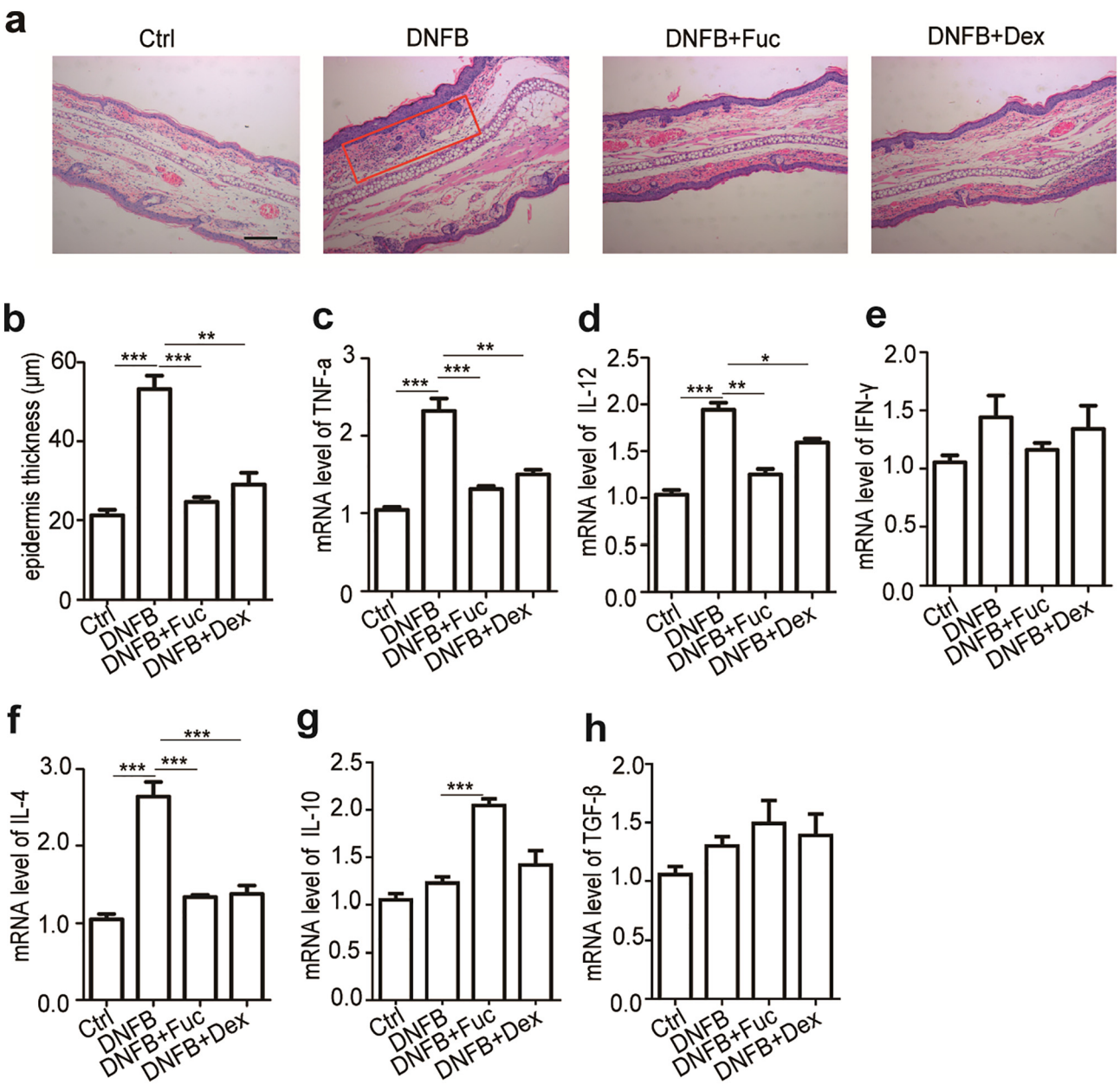

Fig. 2. Fucoidan reduced the inflammatory response in DNFB-induced atopic dermatitis mice.
(a) Representative cross-sections of ears were stained with hematoxylin & eosin in each group. (b) Epidermal thickness in each group. (c-h) mRNA levels of inflammatory cytokines the ears from each group were detected by RT-PCR. Data are expressed as the mean ± SD of each group (n = 6), and are representative of one typical experiment out of three, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

anti-Foxp3 antibodies (eBioscience). Cells were detected using a BD FACSVerse flow cytometer (BD Biosciences, Heidelberg, Germany) and analyzed by FlowJo software (Treestar, Inc., San Carlos, CA).

2.9. Statistics analysis

All analyses were carried out with the SPSS 21.0 software. Results were expressed as mean ± SD. Multiple comparisons were performed by one-way ANOVA and followed by LSD post-test for comparison between two groups. Correlation analysis was made by using the Pearson correlation coefficient. p values < 0.05 were considered significant: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Result

3.1. Effects of fucoidan on DNFB-induced AD-like symptoms in Balb/c mice

To determine whether SR-A involved in the development of AD, the expression of SR-A in DNFB-induced AD mice was assessed. Results showed that the mRNA level of SR-A in the ears of AD mice was significantly decreased (Fig. 1a and b). To further investigated the impact of SR-A on mice's dermatitis, we used *Fucus vesiculosis* fucoidan, one agonist of SR-A, to treat DNFB-induced AD mice. Dermatitis severity was estimated based on swelling and the dermatitis score, and results showed that DNFB-treated mice showed progressive symptoms of erythema, edema, and dryness of the ear skin than those normal mice. In contrast, treatment with fucoidan improved the symptoms of erythema, edema, and dryness (Fig. 1c and d), which was accompanied by increased mRNA levels of SR-A (Fig. 1b). In addition, histological results

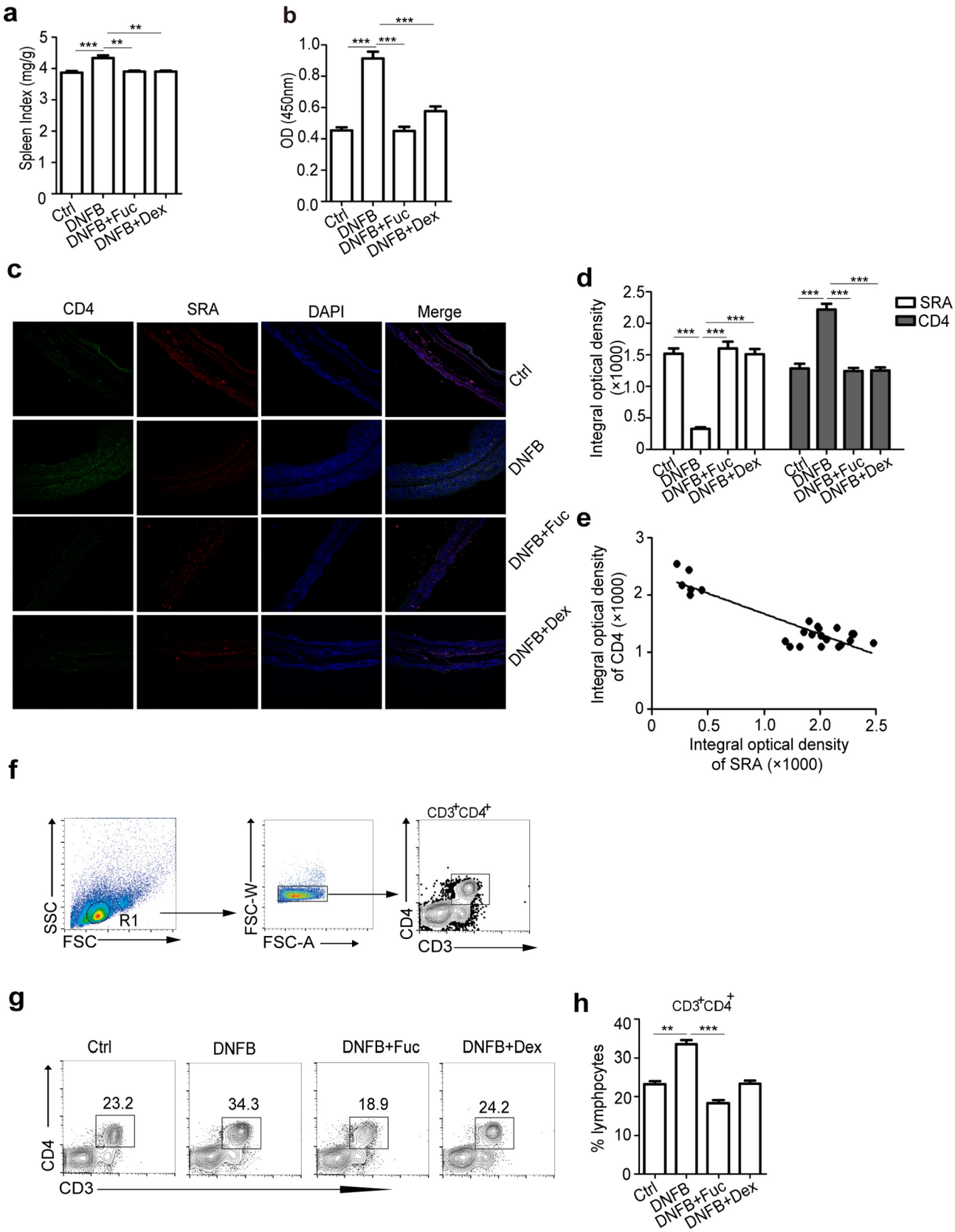

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Fig. 3. Fucoidan attenuated spleen index, splenocyte proliferation and $CD4^+$ T cell in DNFB-induced AD mice.
(a) The spleen index in each group was defined as spleen weight *versus* body weight. (b) Splenocytes from each group were prepared and examined their proliferation by CCK-8. Data are expressed as the mean ± SD of each group (n = 6), and are representative of one typical experiment out of three, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$. (c) Representative data of immunofluorescence of SRA (red) and CD4 (green) in the ears of each group are shown, original magnification, ×400. (d) Quantification of SRA and CD4 in the image above (from ten random fields of view per sample). (e) Pearson correlation analyzed the relationship between the expression of SRA and CD4 in the ears from each group. (f–h) Single-cell suspensions of mouse spleens from each group were prepared. Then cells were stained with CD3-APC and CD4-FITC and then were analyzed of $CD3^+CD4^+$ cells. (f) Gating strategy for $CD3^+CD4^+$ cells. Representative dot plots (g) and percentage (h) of $CD3^+CD4^+$ cells in each group. Data are expressed as the mean ± SD of each group (n = 6), and are representative of one typical experiment out of three, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of abdominal skin displayed an absence of skin structures such as sweat glands and hair follicles in DNFB-induced AD mice, whereas treatments with *Fucus vesiculosis* fucoidan resulted in an increase of sub-epithelial stromal cells (Fig. 1e and f). Furthermore, the levels of serum IgE and IL-4, which are closely associated with the clinical severity of AD, were significantly enhanced in DNFB-induced AD mice; however, the expression of IgE and IL-4 were remarkably decreased after *Fucus vesiculosis* fucoidan treatment (Fig. 1g). No adverse effects were found in the *Fucus vesiculosis* fucoidan treated mice group.

3.2. Fucoidan inhibits the infiltration of inflammatory cells

Pervious study showed that there existed cutaneous inflammatory response at the DNFB-challenged sites, therefore, we examined whether *Fucus vesiculosis* fucoidan could ameliorate the inflammatory reactions during the development of dermatitis. As shown in Fig. 2a–b, the skin histopathology in DNFB-induced AD mice displayed an increase in epidermal thickening and inflammatory cell infiltration (as showed in the red frame) in the ears when compared with control mice; however, the inflammatory cell infiltration was markedly decreased after treatment with *Fucus vesiculosis* fucoidan. In addition, the mRNA levels of pro-inflammatory TNF-α and IL-12 but not IFN-γ were significantly attenuated after treated with *Fucus vesiculosis* fucoidan in DNFB-induced AD mice (Fig. 2c–e). Furthermore, the mRNA expression of anti-inflammatory IL-4 was also inhibited in AD mice treated with *Fucus vesiculosis* fucoidan (Fig. 2f). However, the mRNA levels of IL-10 and TGF-β were induced in AD mice treated with *Fucus vesiculosis* fucoidan (Fig. 2g and h).

3.3. Effects of fucoidan on splenocyte proliferation and $CD4^+$ T cells infiltration

The spleen index was significantly increased in AD mice than that of control mice, while treatment with *Fucus vesiculosis* fucoidan significantly attenuated the spleen index in DNFB-induced AD mice (Fig. 3a). To investigate the role of *Fucus vesiculosis* fucoidan for potential anti-proliferative effects *in vitro*, concanavalin A (ConA)-stimulated mouse splenocytes was assessed. Results showed that the splenocytes proliferation were remarkably increased in DNFB-induced AD mice, but were significantly suppressed after treated with *Fucus vesiculosis* fucoidan (Fig. 3b).

It is reported that adaptive $CD4^+$ cells are highly involving in the development of AD [5,6]. We next want to address the role of *Fucus vesiculosis* fucoidan on $CD4^+$ cells infiltration. As shown in Fig. 3c and d, $CD4^+$ T cells were found to infiltrate into the ears of DNFB-induced AD mice (as showed in green), however, these cells were dramatically decreased after *Fucus vesiculosis* fucoidan treatment. Interesting, the expression of SR-A was negative associated with $CD4^+$ T cells in the ears (Fig. 3e), suggesting that *Fucus vesiculosis* fucoidan may play a key role in suppression of $CD4^+$ T cells growth. In addition, the expression of $CD3^+CD4^+$ T cells in the spleen of AD mice was significantly increased, but was downregulated after *Fucus vesiculosis* fucoidan treatment (Fig. 3f–h). These data indicate that treatment with fucoidan could inhibit $CD4^+$ cells response during the development of AD.

3.4. Fucoidan triggers $CD4^+CD25^+Foxp3^+$ regulatory T cells in atopic dermatitis mice

To further investigate the cellular mechanism underlying the anti-dermatitis roles of fucoidan, we next examined the $CD4^+$ T cell subsets to understand whether $CD4^+$ T cell differentiation involve in this process (Fig. 4a–e). Results showed that the percentages of Th1 and Th17 cells were significantly increased in DNFB-induced AD mice when compared to normal control; however, these cells were decreased after *Fucus vesiculosis* fucoidan treatment (Fig. 4a, b, d). In addition, Th2 cells were significantly increased in DNFB-induced AD mice, while they were slightly enhanced after *Fucus vesiculosis* fucoidan treatment (Fig. 4c). Interesting, a significant increase of Treg cells were observed in *Fucus vesiculosis* fucoidan treated AD mice (Fig. 4e).

Taken together, these data suggest that *Fucus vesiculosis* fucoidan could regulate $CD4^+$ T cell subsets differentiation, involving a mechanism of inducing regulatory T cells.

4. Discussion

Topical steroid therapy is effective for the treatment of AD, which is characterized by erythema, edema, excoriation, and scaling [1], however, it is suggested that not be used in the long-term due to its side effects [18]. Thus, new small molecules are frequently used for studying AD pathology and searching for new drugs for AD.

As one typical agonist of SR-A, fucoidan is a kind of non-toxic nature from the production of brown seaweed, which is widely consumed as part of the normal diet in East Asia, particularly China, Japan, and Korea [19]. Fucoidan has been known to play many effects, including antiviral, antioxidant, antitumor, and anti-inflammatory properties in humans and animals [19–23]. Our data showed that both mRNA and protein levels of SR-A were decreased in the ears of AD mice, which made us believe that something that fucoidan may improve the symptoms of AD. To our knowledge, this is the first study to examine the roles of fucoidan in the process of AD using a mouse AD model, and we found that fucoidan can relief ear swelling and improved the skin lesions.

A growing body of evidence has demonstrated that AD is related to an increased in the production of pro- and anti-inflammatory cytokines, such as IL-4, TNF-α, and IFN-γ [24,25]. In addition, allergen-specific IgE plays a central role in skin reactions (pruritus, erythema) through interacting with high-affinity receptors to activate basophils and mast cells [26]. Previous study showed that *Fucus vesiculosis* fucoidan inhibited IgE production by B cells *in vitro* stimulation or *in vivo* OVA immunized mice [27]. Consistence with this, treatment with fucoidan markedly suppressed serum IgE and mRNA level of inflammatory cytokines in the ear tissue of AD mice. Furthermore, our results also indicate that *Fucus vesiculosis* fucoidan suppressed the spleen index and splenocytes proliferation in DNFB-induced AD mice, suggesting that hypersensitivity reactions in AD mice were weakened after fucoidan treatment.

Adaptive immune cells have been confirmed for the major contributors and regulators of AD [28,29]. Infiltrating immune cells in AD lesions can switch from Th1 in the acute phase to Th2 cells in the chronic phase [30]. The percentage of Th17 cells was increased in peripheral blood of AD patients and associated with the severity of AD

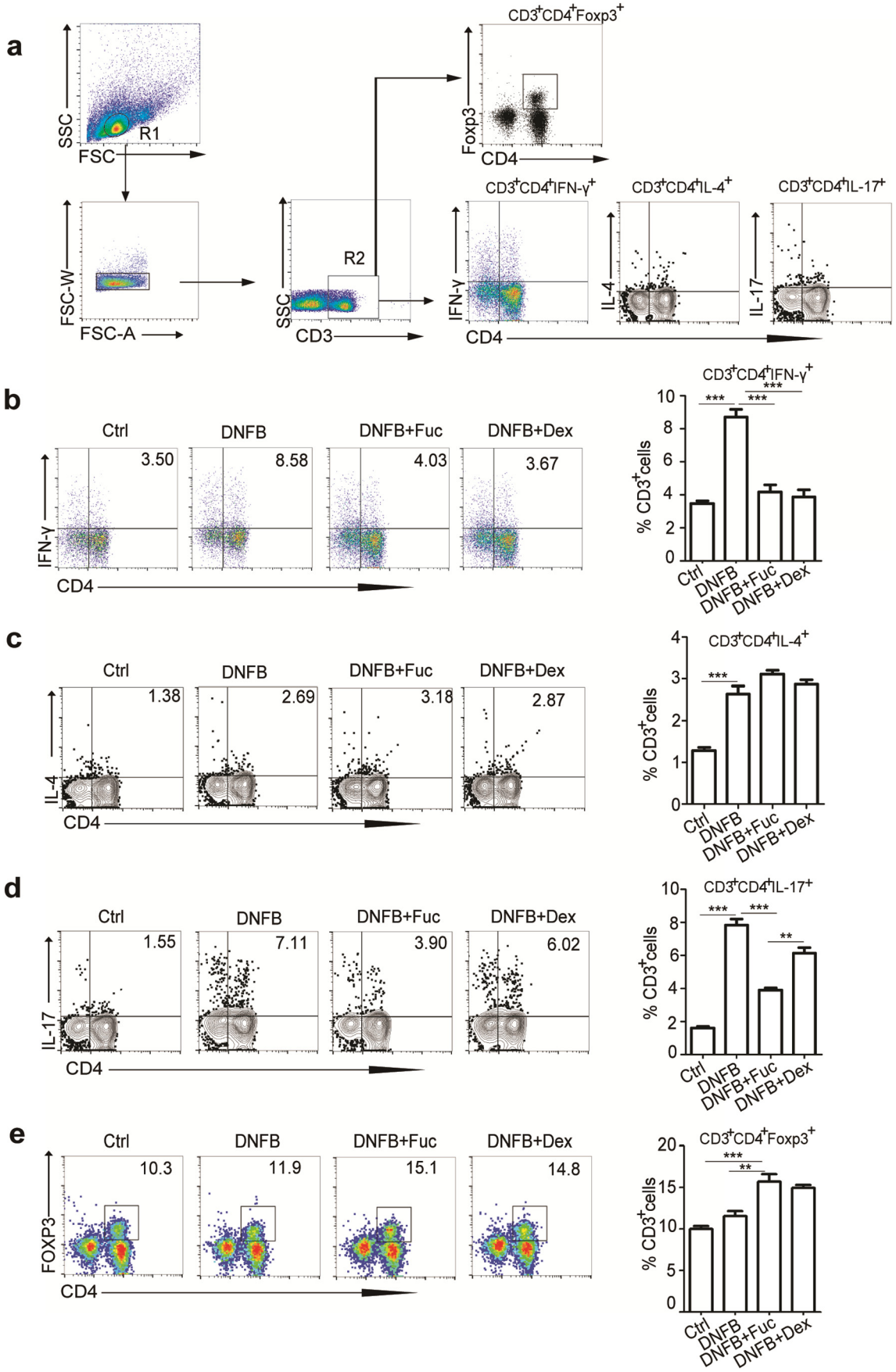

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Fig. 4. Effect of Fucoidan on $CD4^+$ T cell subsets.
(a–e) Single-cell suspensions of mouse spleens from each group were prepared, cells were stained with CD3-APC and CD4-FITC, and then intracellularly stained with PE-conjugated antibodies against IFN-γ, IL-4, IL-17, and Foxp3 for FACS analysis of $CD3^+CD4^+IFN\text{-}\gamma^+$ (Th1), $CD3^+CD4^+IL\text{-}4^+$ (Th2), $CD3^+CD4^+IL\text{-}17^+$ (Th17), and $CD3^+CD4^+Foxp3^+$ (Treg). (a) Gating strategy for above cells. (b–e) Representative dot plots and percentage of $CD3^+CD4^+IFN\text{-}\gamma^+$ (b), $CD3^+CD4^+IL\text{-}4^+$ (c), $CD3^+CD4^+IL\text{-}17^+$ (d), and $CD3^+CD4^+Foxp3^+$ (e) in each group. Data are expressed as the mean ± SD of each group (n = 6), and are representative of one typical experiment out of three, $^{***}p < 0.001$, $^{**}p < 0.01$.

[31]. Regulatory T cells, which were reduced suppressive capacity in AD subjects, play an important role in the regulation of Th cells to common allergens [32]. Our previous study has shown that SR-A plays a critical role in regulating adaptive $CD4^+$ T cell differentiation during helminth infection [16]. Consistently, the expression of SR-A was found negatively related to $CD4^+$ T cell in AD lesions, suggesting that activation of SR-A maybe one potential target for AD treatment through affecting $CD4^+$ T cell response. Indeed, *Fucus vesiculosis* fucoidan significantly inhibited the infiltrated $CD4^+$T cells into AD lesions, probably due to the decreased production of chemokines [33]. In addition, our data indicated that *Fucus vesiculosis* fucoidan also suppressed T helper cell subsets, which may relate to the enhanced Treg cells in the spleen of AD mice. We found that Th2 cells were slightly increased but the expression of IL-4 was significantly decreased after fucoidan treatment, probably due to the expression of IL-4 was also produced by other immune cells, such as mast cells and macrophages [34,35]. Chao-Huei Yang et al. used oligo-fucoidan to treat blood mononuclear cells from allergic asthmatic subjects, and found that the Treg/Th17 cell ratio was increased [36]. The pulmonary inflammation was relieved by Mekabu fucoidan injection intraperitoneally, which accompanied by the downregulated Th2-dominated responses in OVA challenged mice [37]. These above studies supported our research in some way. To gain insights into the mechanism of regulation by Treg cells, we also detected the expression of IL-10 and TGF-β, both of which were closely related to the suppression function of Treg cells [38]. We found that both of the mRNA levels of IL-10 and TGF-β were increased in the ears from fucoidan-treated AD mice, suggesting that fucoidan-mediated protection of AD probably due to the production of IL-10 and TGF-β. Since fucoidan is one specific activator of SR-A, which is typically expressed in antigen-presenting cells (such as macrophages and dendritic cells [39]) but not T lymphocytes [40], we speculate that fucoidan might activate antigen-presenting cells through SR-A, and indirectly regulate T cell response. However, the detailed mechanism of inflammation inhibition in fucoidan induced-Treg cells in atopic dermatitis needs further investigation.

In summary, we reported that fucoidan can ameliorate atopic dermatitis, accompanied by the decreased inflammatory cell infiltration, splenocytes proliferation, and $CD4^+$ T cell response. Our data suggest a new therapeutic potential of fucoidan for the treatment of AD.

Declaration of competing interest

The authors have no conflict of interest to declare.

Acknowledgements

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Data availability

All data of this study are available from the first author upon reasonable request.

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