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The protective effects of human milk components, 2′-fucosyllactose and osteopontin, against 2,4-dinitrochlorobenzene-induced atopic dermatitis in mice

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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease with high morbidity in infants. Several bioactive components of human milk, such as 2′-fucosyllactose (2′-FL) and osteopontin (OPN), are reported to exhibit anti-inflammatory properties. This study determined that oral administration of 2′-FL and OPN significantly attenuated the 2,4-dinitrochlorobenzene (DNCB)-induced AD symptoms in BALB/c mice. 2′-FL and OPN treatment markedly downregulated the serum IgE level and decreased the numbers of mast cells and eosinophils in the dermal tissues from DNCB-sensitized mice. Interestingly, we found that the combination of OPN with 2′-FL produced a great therapeutic effect on AD in mice. Additionally, the result showed that the components suppressed Th2 response, resulting in an attenuated cutaneous inflammatory response. Furthermore, *in vitro* study validated that 2′-FL and OPN inhibited Th2 cells differentiation. Together, we conclude that 2′-FL and OPN ameliorates DNCB-induced AD-like skin inflammation, and its supplement might help to prevent AD in infants.

1. Introduction

Atopic dermatitis (AD) is a very common skin disease in children caused by environmental exposures and immune system disorder (Hay et al., 2014; Kabashima, 2013), which is associated with elevated serum immunoglobulin E (IgE) as well as infiltration of immune cells. Generally, the infiltrate of activated $CD4^+$ Th cells is critical for the development of AD by initiating or sustaining skin immune responses (Turner et al., 2012). The early expression of Th2 cytokines has been considered as a primary cause for the pathogenesis of AD (Simpson et al., 2016). Of note, the disorders of Th2 cells and their cytokines (e.g., IL-4, IL-5, and IL-13) increased inflammation in the AD skin lesions (Chan et al., 2001). Transgenic mice overexpressing IL-4 or IL-13 in the skin spontaneously developed symptoms associated with AD (Chan et al., 2001; Zheng et al., 2009). IL-4 and IL-13 drive the pathogenesis of AD by modulating several key immunological features, including the decrease in the skin barrier integrity, causing the enhanced exposure to allergens and pathogens (Hanel et al., 2013). Notably, dupilumab, an antibody that can block both IL-4 and IL-13, is highly effective for the treatment of adult patients with moderate-to-severe AD (Gooderham et al., 2018). Targeting the activated $CD4^+$ Th2 cell subtypes is a promising strategy to control the initiation of AD pathology.

Human breast milk provides nutrition for infants and also contains a variety of distinct bioactive molecules that protect against inflammation and infection and contribute to immune development (Ballard and Morrow, 2013). Osteopontin (OPN) is a multifunctional glycoprotein, present at high concentration in human milk, which contributes to the protection of the infant from pathogens and supports the maturation of the immune system (Jiang and Lonnerdal, 2016). It is worth noting that exogenous OPNs could be detected in mice serum at 3 h after oral

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administration with OPN (Jiang et al., 2021). Clinical trial on infants fed with formula supplemented with OPN exhibited significantly reduced levels of serum inflammatory cytokines (i.e., TNF-α and IL-6), as compared with the infants fed with the regular formula (Lonnerdal et al., 2016). Of note, OPN expression is normally limited to the bone and epithelial surfaces and secreted in body fluids such as milk, blood, and urine (Chen et al., 1993). Ashkar et al. observed that draining lymph node cells from HSV-infected OPN-deficient mice produced exaggerated amounts of Th2 cytokines in comparison with the wild-type (WT) controls (Ashkar et al., 2000). 2′-fucosyllactose (2′-FL) is the most prevalent human milk oligosaccharide (HMO), which makes up about 30% of all of HMOs and is considered safe for infant nutrition. Several studies showed that HMOs have the capability of developing immune system and modulating immune responses (Goehring et al., 2016; Hegar et al., 2019; Sprenger et al., 2017). Infants fed with formula supplemented with 2′-FL exhibited low plasma concentration of inflammatory cytokines compared to the infants fed with control formula supplemented with galactooligosaccharides (Goehring et al., 2016). Interestingly, the children who fed on a formula with 2′-FL had a lower risk of developing IgE-associated allergic disorders than that fed unsupplemented formula (Sprenger et al., 2017). Dietary intervention with 2′-FL improves both humoral and cellular immune responses in a murine influenza vaccination model (Xiao et al., 2018). The percentages of Th1 and Tregs in spleen were substantially increased in mice receiving 1% dietary 2′-FL compared to vaccinated mice receiving control diet (Xiao et al., 2018). Also, administration with 2′-FL enhanced the vaccine-specific $CD8^+$ T cell responses. Rudloff et al. observed that ^{13}C-labelled HMOs can reach the infant's systemic circulation, and are excreted intact in the infant's urine (Rudloff et al., 2012). It should be noted that 2′-FL is detected in the systemic circulation after oral administration, indicating the potential biological function of 2′-FL serving as a direct modulator for the activation of immune cells (Vazquez et al., 2017).

In this study, we orally administrated 2′-FL and/or OPN to 2,4-dinitrochlorobenzene (DNCB)-induced AD skin lesions in BALB/c mice and investigated whether 2′-FL and OPN has the preventive efficacy on AD-like symptoms. We evaluated the disease by quantifying skin pathology, serum IgE production, and infiltration of mast cells and eosinophils. Furthermore, we assessed the effect of 2′-FL and OPN on $CD4^+$ Th2 responses during the AD pathogenesis.

2. Materials and method

2.1. Animals

Six-week-old BALB/c mice were purchased from the Animal Center of Southern Medical University (Guangzhou, China). The animals were housed under a 12-hour light/dark cycle in a specific pathogen-free animal condition at a constant temperature (20–25 °C) and humidity (50 ± 5%). All animal experiments in our study were approved by the Welfare and Ethical Committee for Experimental Animal Care of Southern Medical University.

2.2. Grouping and treatment

Dinitrochlorobenzene (DNCB) solution (dissolved in a 1:3 mixture of olive oil and acetone) was topically applied on dorsal skin and ears of mice to induce AD-like skin lesions. At the first day, 150 μl of 2% DNCB solution was applied symmetrically on shaved dorsal skin, and 10 μl were applied on the back of both ears. On the fifth day after the sensitization, 0.5% DNCB solution was applied to stimulate the dorsal skin (150 μl) and the back of both ears (10 μl each). 2′-FL and/or OPN (Jennewein Biotechnologie, Germany) were completely dissolved in sterilizd saline before use. DNCB sensitized mice were randomized to four groups (n = 5 mice per group for each experiment): (1) DNCB + saline: intragastric administration with saline from day 1 to day 21; (2) DNCB + 2′-FL: intragastric administration with 2′-FL (600 mg/kg/day) from day 1 to day 21; (3) DNCB + OPN: intragastric administration with OPN (37.5 mg/kg/day) from day 1 to day 21; (4) DNCB + 2′-FL + OPN: intragastric administration with OPN (37.5 mg/kg/day) and 2′-FL (600 mg/kg/day) from day 1 to day 21. The control mice without DNCB sensitization were gavaged with saline from day 1 to day 21.

2.3. Evaluation of skin lesions and ear thickness

The each ear thickness was measured by vernier caliper (Vogel, German). The severity of dorsal skin lesions were evaluated according to the four manifestation: edema, excoriation/erosion, erythema/hemorrhage, and scarring/dryness, and added up each score (0, no symptoms; 1, mild; 2, moderate; 3, severe). These visual assessments were carried out every seven days.

2.4. Scratching behavior test

Mice were placed into cages for 1 h for habituation. After habituation, the number of scratching episodes for 15 min was counted macroscopically. The total scratching behaviours were calculated within 15 min. Scratching behaviors were tested on day 7, day 14, and day 21 of the experiment.

2.5. Histopathological analysis

Ears and dorsal skin lesions were fixed in formalin (4%) and then embedded in paraffin blocks. Deparaffinized skin sections were stained with hematoxylin and eosin (H&E) to evaluate the epidermal thickeness and inflammatory infiltration. To assess the infiltration of mast cells, the histologic sections were stained with toluidine blue (TB) (Beyotime Biotechnology, Shanghai, China). Immunohistochemical staining with eosinophil peroxidase (EPX) (bs-3881R, Bioss, Beijing, China) was used to identify eosinophils. The slides were observed under automatic fluorescence microscope (Olympus BX63, Japan). The staining intensity of TB and EPX of each specimen was counted by two independent observers in ten high power fields. Each filed was randomly selected in the dermal inflammatory infiltrate of the histologic lesions. Cells exhibiting specific blue or brown were defined as positive, respectively. The number of positive cells in each field was counted and the data were statistically analyzed.

2.6. Measurement of serum immunoglobulin E

Mice serum samples were collected on the last day of the experiment. Serum levels of IgE were detected and quantified by using the commercial uncoated enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, San Diego, CA, USA) according to the manufacturer's instruction.

2.7. Flow cytometry

On the last day of the experiment, single-cell suspensions isolated from skin draining lymph nodes (dLNs) (axilla and groin) which mainly reflect the inflammatory response status of the back skin were prepared. For intracellular staining, 1×10^7 lymphocytes were cultured in flat-bottomed 48-well plates with cell stimulation cocktail and protein transport inhibitor (eBioscience, San Diego, CA, USA) for 6 h. To exclude dead cells, cells were first stained with fixable viability stain (eBioscience) for 30 min at 4 °C. After surface staining with FITC-labeled rat anti-mouse CD4 (eBioscience), permeabilized cells were stained with APC-labeled rat anti-mouse IL-4 mAb (eBioscience). Flow cytometry was performed on LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA), 5×10^4 events per sample were evaluated, and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.8. RNA isolation and quantitative real-time PCR

Total RNA from dorsal skin was isolated by using TRIzol (TransGen Biotech, Beijing, China). 500 ng RNA was quantified for the reverse transcription reaction with TranScript All-in-One First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Gene expression analyzed by quantitative real-time PCR (RT-PCR) was amplified by TransStart Green qPCR SuperMix (TransGen Biotech). The levels of the target gene were normalized with GAPDH gene expression.

2.9. In vitro induction of Th2 cell differentiation

Naïve $CD4^+$ T cells were sorted out from BALB/c mice splenocytes by the $CD4^+CD62L^+$ T cell isolation kit (Miltenyi Biotech, Germany) following the manufacturer's instructions. 6×10^5 cells in 250 μl of culture medium were plated in 48-well polystyrene round-bottom plate precoated with anti-CD3 antibody (1 μg/ml, 145-2C11, eBioscience, San Diego, CA, USA) in the presence of anti-CD28 antibody (1 μg/ml, 37.51, eBioscience). For Th2 cell differentiation, rmIL-4 (20 ng/ml, 214–14, Peprotech, Rocky Hill, NJ, USA), rmIL-2 (10 ng/ml, 575402, Biolegend, San Diego, CA, USA) and anti-IFN-γ (10 μg/ml, XMG1.2, Biolegend) were added. To assess whether 2′-FL and OPN affect the Th2 differentiation, the cells were differentiated in the medium containing 2′-FL (1 mg/ml) or OPN (0.2 mg/ml), either alone or in combination, for 3 days. The intracellular expression of IL-4 and GATA3 was measured by flow cytometry.

2.10. Statistical analysis

Data was expressed as mean ± standard error of mean (SEM). One-way ANOVA was used for comparisons among multiple groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. 2′-FL and OPN alleviated the DNCB-induced AD-like symptoms in BALB/c mice

To investigate the potential improving effect of OPN and 2′-FL on AD, a BALB/c AD model mice was established by topical application of DNCB on both ears and the dorsal skin (Fig. 1A). Edema, excoriation, erythema, and scarring were apparent on the skin of DNCB-challenged mice after multiple sensitizations of DNCB. The ear thickness in DNCB-treated mice was substantially decreased upon dietary 2′-FL and/or OPN administration (Fig. 1B). The ears of 2′-FL and OPN fed AD mice showed less swollen than those of the control mice (Fig. 1C). The severity of dorsal skin lesions was attenuated in mice orally administered with 2′-FL and OPN compared with those in mice treated with saline (Fig. 1D and 1E). Besides, the infiltration of inflammatory cells in dermal and the epidermis thickness of dorsal skin was markedly reduced in the 2′-FL and OPN treated AD mice compared to the control AD mice (Fig. 1F and 1G). Interestingly, we found that the combination of OPN with 2′-FL exhibited more beneficial effects in the treatment of mice with AD. Together, the data indicate that oral administration of OPN and 2′-FL ameliorates the AD-like skin lesions and ear thickening. Filament

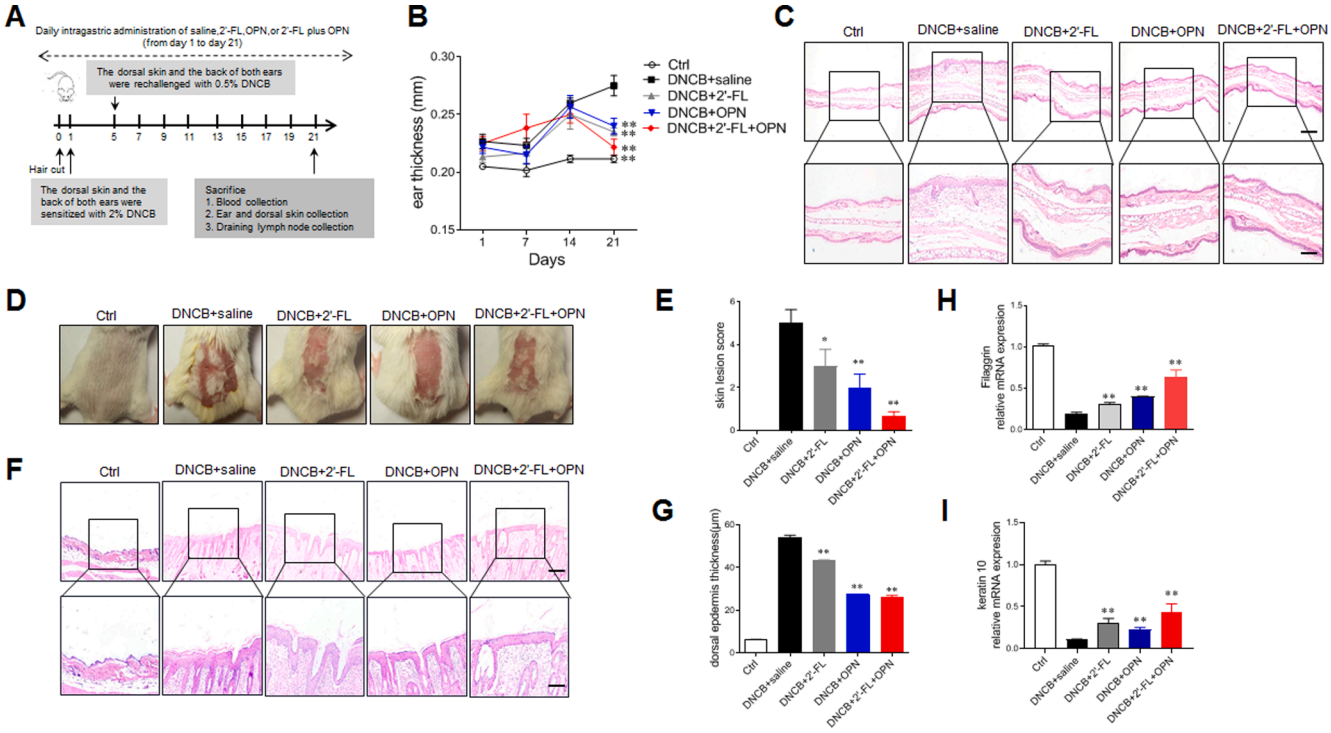

Fig. 1. 2′-FL and OPN treatment attenuated the DNCB-induced AD-like symptoms in BALB/c mice. (A) Schematic of atopic dermatitis induction with 2′-FL and/or OPN treatment. To induce AD-like symptoms, the hair-shaved dorsal skin and ear of BALB/c mice (n = 5) were sensitized with 2.0 % DNCB. 0.5% DNCB was re-challenged at the dorsal skin and ears at the indicated time points. 2′-FL and OPN dissolved in normal saline was orally administrated to BALB/c mice daily from day 1 to the last day of the experiment. (B) Ear thickness was measured and compared at the indicated day post DNCB sensitization. (C) The ear was removed on the last day of the experiment, and the pathological changes were determined by hematoxylin and eosin (H&E) staining, scale bar (top) = 200 μm (bottom) = 100 μm. (D, E) Images of skin lesions from AD mice with or without 2′-FL and/or OPN treatment were taken on the last day of experiment (D), and the dermatitis scores were presented (E). (F, G) Histological analysis of mouse dorsal skin lesion taken on the last day of the experiment was performed using H&E staining, scale bar (top) = 200 μm, (bottom) = 100 μm (F), and the epidermis thicknesses were measured (G). (H, I) The skin tissues were collected from DNCB-treated mice on the last day of the experiment. Total relative mRNA expression levels of filaggrin (H) and keratin 10 (I) were measured by quantitative RT-PCR analysis and expressed as a ratio to GAPDH. *$p < 0.05$, **$p < 0.01$, compared to the DNCB-sensitized mice treated with saline. Data shown represent three independent experiments with similar results.

aggregation protein (Filaggrin) is a critical epidermal barrier protein which is reduced in AD mice (Otsuka et al., 2014). Additonally, keratin proteins are major constituents of the cytoskeleton in epidermis. The expression of these proteins was downregulated in AD lesional skin (Totsuka et al., 2017). Herein, we found that 2′-FL and OPN treatment restored the expression of filaggrin and keratin-10 in AD mice (Fig. 1H and 1I).

Monitor of spontaneous scratching behavior in AD mice is a possible approach to assess the efficacy of anti-pruritics. We recorded and quantified the time the mice spent rubbing their nose, ears, and dorsal skin with their hind paws. The result showed that treatment with 2′-FL and OPN significantly reduced the DNCB-increased scratching time (Fig. 2).

3.2. 2′-FL and OPN limited serum IgE elevation and reduced mast cells and eosinophils infiltration in AD mice

Next, we investigated whether 2′-FL and OPN influence the DNCB-induced increase in serum IgE level in BALB/c mice. The blood samples were collected on the last day of the experiment. Repeated application of DNCB strongly enhanced the serum IgE levels in AD mice compared to the control mice. Treatment with 2′-FL and/or OPN significantly decreased the serum IgE level in DNCB-induced AD mice (Fig. 3A). Moreover, we evaluated the infiltration of mast cells and eosinophils, which closely links with the AD pathogenesis (Liu et al., 2011). Mast cells in skin lesions were stained with toluidine blue, and the result showed that upon stimulation with DNCB, infiltrated mast cells increased in the dorsal skin. By contrast, less infiltrated mast cells were shown in the dermis from the 2′-FL and OPN treated mice (Fig. 3B). Similarly, immunohistochemical staining showed that the amount of eosinophils in the dermis from DNCB-sensitized mice decreased significantly after OPN and 2′-FL administration (Fig. 3C). These results suggest that 2′-FL and OPN treatment decreased the risk of allergic disorders in the DNCB-induced AD mouse model.

3.3. 2′-FL and OPN decreased DNCB-induced inflammatory response and Th2 polarization in BALB/C mice

On the last day of the experiment, the total RNA was isolated from dorsal skin lesion. Subsequently, the mRNA expressions of pro-inflammatory cytokines (eg., TNF-α, IL-6, and IL-1β) were measured by RT-PCR. The result showed that the expressions of the pro-inflammatory cytokines were substantially reduced in the 2′-FL and/or OPN-treated AD mice than the control mice (Fig. 4A). Additionally, we measured the skin mRNA expression of thymic stromal lymphopoietin (TSLP), a cytokine produced by keratinocytes, which is believed to propagate AD lesions through Th2 polarization (Jariwala et al., 2011). The elevated TSLP expression in AD mice was significantly was markedly suppressed upon 2′-FL and OPN orally administration (Fig. 4A). Together, these results indicate a potential role of OPN and 2′-FL in limiting the inflammatory response in the mouse model of AD.

The persistence and function of Th2 cell subtype in AD have a central role in modulating skin inflammation (Turner et al., 2012). We further analyzed the differentiation of $CD4^+$ Th2 cells in the dLNs of the DNCB-treated BALB/c mice. The result showed that 2′-FL and OPN treatment significantly suppressed the upregulation of IL-4 producing $CD4^+$ Th2 lymphocytes in AD-like mice (Fig. 4B). Furthermore, we evaluated the mRNA level of Th2 cell-specific factors in the AD skin lesions. The mRNA levels of IL-4 were significantly inhibited in the skin lesion from 2′-FL and OPN-treated AD mice compared to those from control mice (Fig. 4C). Consistently, the 2′-FL and OPN-treated AD mice had significantly lower gene expressions of the Th2 transcription factor GATA3 in the dorsal skin than that in the saline-treated group (Fig. 4C). The data suggest that 2′-FL and OPN inhibited the DNCB-induced increases in Th2 response in BALB/c mice.

3.4. 2′-FL and OPN directly inhibited the Th2 differentiation in vitro

HMOs are resistant to digestion in the gastrointestinal tract and may be taken up into the body. OPN is absorbed in the colon mucosa and represented in the circulation (Christensen et al., 2020). Besides, the concentration of 2′-FL was around 1.5 mg/l in plasma and 100 mg/l in urine from breastfed infants (Goehring et al., 2014). Therefore, we raised the question of whether 2′-FL and OPN directly modulate the differentiation and activation of Th2 cells, respectively. We assessed the effect of OPN and 2′-FL on Th2 activation by an *in vitro* differentiation model, in which naive $CD4^+$ T cells cultured under a standard Th2 polarization condition. We found that the 2′-FL and OPN prohibited differentiation of naive $CD4^+$ T cells into IL-4-producing Th2 cells (Fig. 5A). Also, the analysis of Th2 cell-specific GATA3 validated the direct inhibitory effect of 2′-FL and OPN on Th2 cell differentiation (Fig. 5B). These results indicate that 2′-FL and OPN directly regulate the differentiation of Th2 lymphocytes.

4. Discussion

AD is a common chronic inflammatory skin disease, which is characterized by intense itch and inflammatory (Leung and Bieber, 2003). Infants fed regular formula had significantly higher serum concentrations of the pro-inflammatory cytokine TNF-α than infants fed formula supplemented with OPN (Lonnerdal et al., 2016). Also, a previous randomized controlled trial showed that infants fed with the 2′-FL enriched formula have lower inflammatory responses (Goehring et al., 2016). In the present study, we have demonstrated that both 2′-FL and OPN significantly alleviate DNCB-induced AD-like symptoms in BALB/c mice. OPN and 2′-FL treatment profoundly suppressed IgE-mediated mast cell activation. Surprisingly, 2′-FL and OPN exhibit a great therapeutic effect on AD in mice. Moreover, our studies revealed that 2′-FL and OPN mainly limit Th2 cell polarization in the AD mice model, indicating that these compounds shape the T helper cell responses during the pathogenesis of AD.

The skins of the ear and the back are commonly selected sites in skin research using mouse models. Dorsal skin of mice has a complete layered structure, which is closer to human skin physiologically and suitable for

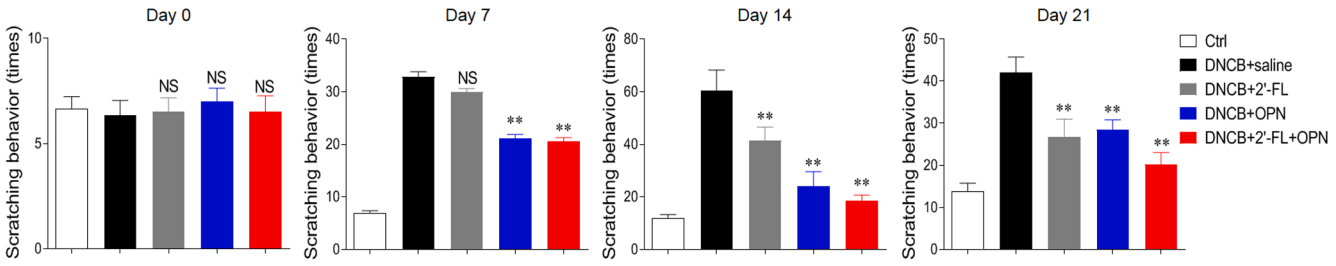

Fig. 2. 2′-FL and OPN alleviated es incidence in BALB/c mice. The scratching time of BALB/c mice (n = 5) was recorded and analyzed at the indicated day post DNCB challenge. NS, not significant, **p < 0.01, compared to the DNCB-sensitized mice treated with saline. Data shown represent three independent experiments with similar results.

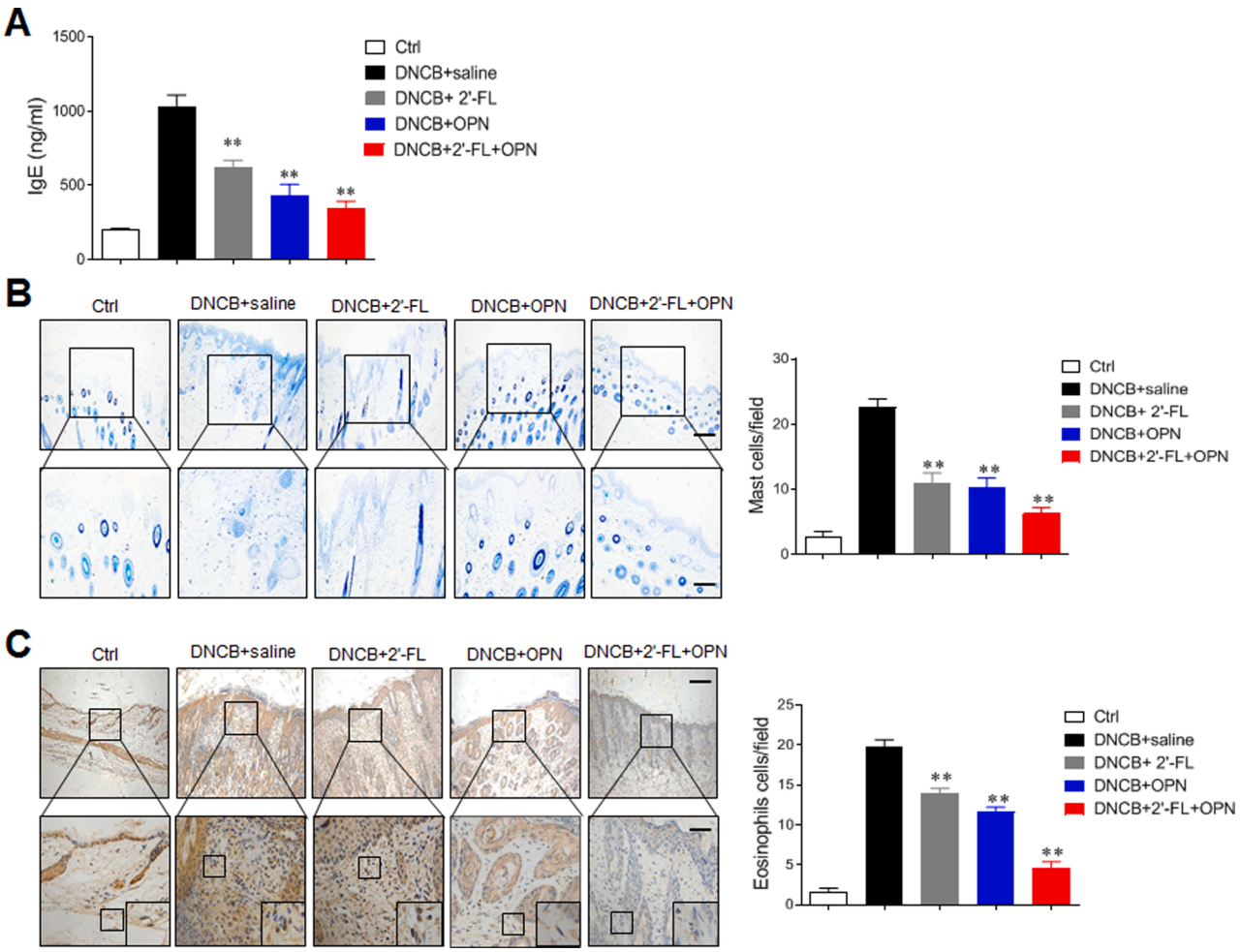

Fig. 3. Orally administration of 2′-FL and OPN decreased IgE elevation and skin infiltration of mast cells and eosinophils in DNCB-induced AD mice. (A) Blood was collected from DNCB-induced AD mice (n = 5) on the last day of the experiment. Serum levels of IgE were assessed by ELISA. **p < 0.01, compared to the DNCB-sensitized mice treated with saline. (B) Toluidine blue (TB) staining of skin lesions from DNCB-treated BALB/c mice was used to identify mast cells, scale bar (top) = 200 μm, (bottom) = 100 μm. The cells exhibiting specific blue represented positive cells. (C) Immunohistochemical staining against eosinophil peroxidase (EPX) was used to identify eosinophils. Scale bar (top) = 200 μm, (bottom) = 50 μm. The cells exhibiting specific brown in cytoplasm represented positive cells. **p < 0.01 compared to the DNCB-sensitized mice treated with saline. Data shown represent three independent experiments with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

discussing the infiltration of inflammatory cells. Both the epidermis and dermis of the ear are very thin, the dermis with fewer cells especially. Therefore, it is suitable to discuss hyperplasia after inflammation. Our study indicated that OPN and 2′-FL administration significantly improve the pathogical changes in dorsal skin and ear of AD mice. Actually, the two significant mechanisms of AD pathogenesis are abnormalities of the skin barrier and cutaneous inflammation due to immune responses to antigens in the skin. Spontaneous scratching is a typical symptom of AD, and the vicious itch–scratch cycle aggravates skin barrier dysfunction in AD (Hu et al., 2021). Here, we found that OPN and 2′-FL treatment not only reduced the inflammatory response to AD, but also improved the integrity of the skin barrier. After oral administration of OPN and 2′-FL, the expression of filaggrin and keratin 10 increased, which might be related to the reduction of scratching behaviours and skin inflammation.

It has been widely accepted that altered immune responses highly associated with the increased onset of atopic disease seen in AD. During the development of AD, a dense activated $CD4^+$ Th cell infiltration was observed in the dermis tissue (Leung and Bieber, 2003). Indeed, the immunological hallmark of atopic dermatitis (AD) is a Th1/Th2 dysbalance. Th2 dominance in the tissue samples from AD patients was well documented, with the increased production of Th2 cytokines (i.e., IL-4, IL-5, and IL-13) in T cells and skin samples (Brandt and Sivaprasad, 2011; Moreno et al., 2016). As an example of Th2-associated pathology, Th2-type cytokines inducing activation of IgE-producing B cells and promoting the recruitment of eosinophils play a critical role in AD development. Our results showed that oral administration of 2′-FL and OPN extensively suppressed the Th2 differentiation in DNCB-treated BALB/c mice, which consequently led to the reduction of serum IgE level and decreased the infiltration of eosinophils and mast cells. Xanthou et al. reported that intranasal administration of recombinant OPN decreased the levels of Th2 cytokines upon pulmonary antigenic challenge and protected mice from allergic airway disease (Xanthou et al., 2007). In a mouse model of food allergy, daily administration with 2′-FL alleviated the allergy symptoms associated with the reduction in the activation and infiltration of mast cells (Castillo-Courtade et al., 2015). An increased PCNA-activity of the mast cells in the epidermis of AD lesions resulted in the activation of keratinocytes and stimulation of endothelial growth (Groneberg et al., 2005). Therefore, 2′-FL and OPN-mediated biological changes in Th2 and mast cell activation led to a highly inhibited epidermal and dermal thickness in the AD mice. Keratinocytes activation and subsequent soluble pro-inflammatory mediators release attract immune cells into the dermis and contribute to the skin inflammation (Albanesi et al., 2018). TSLP is primarily expressed by activated keratinocytes in the skin. Notably, studies in mice and humans

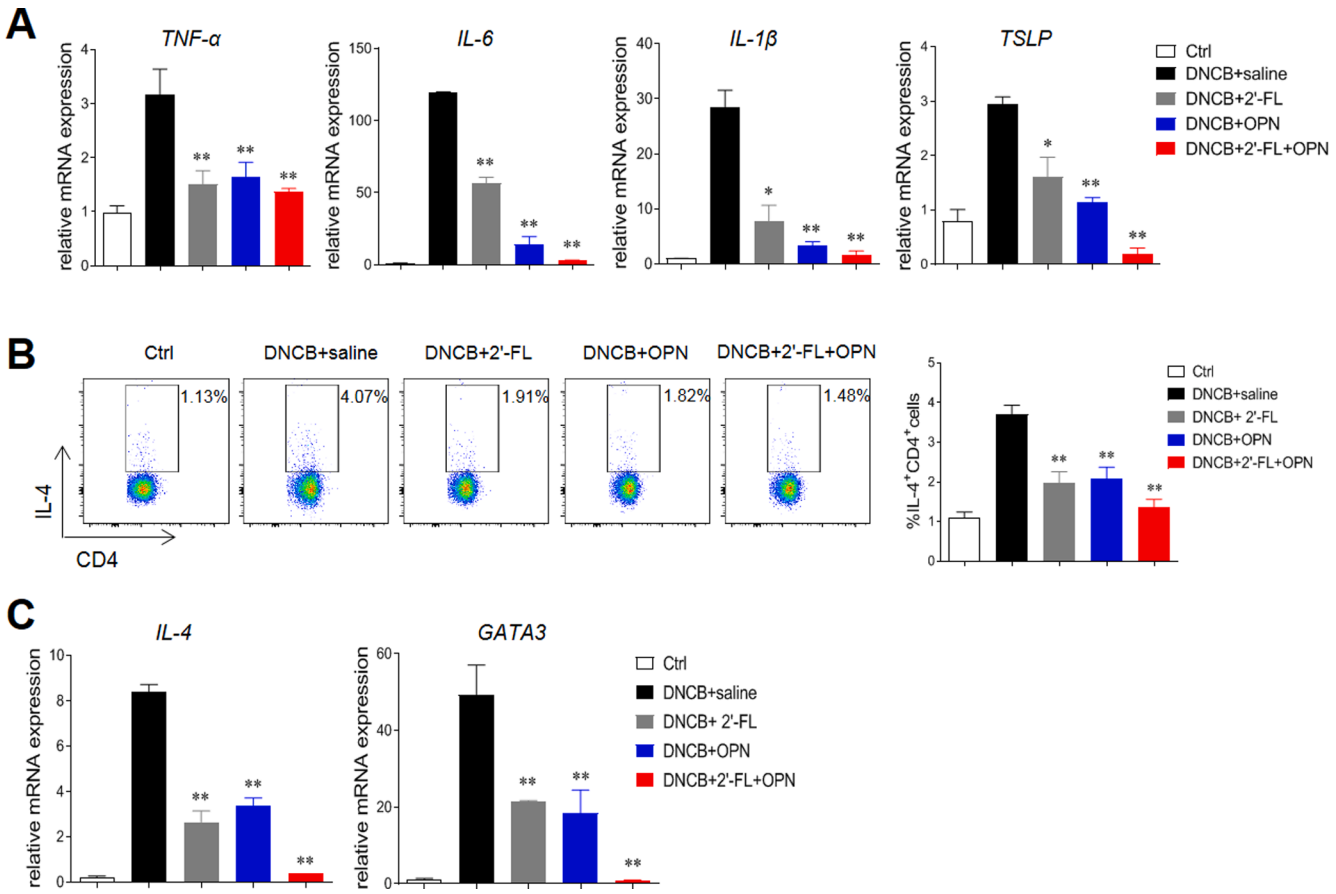

Fig. 4. 2′-FL and OPN treatment affected the DNCB-induced inflammatory responses and Th2 responses in BALB/c mice. The skin tissues were collected from DNCB-treated mice (n = 5) on the last day of the experiment. (A) Total relative mRNA expression levels of TNF-α, IL-6, IL-1β, and TSLP were measured by quantitative RT-PCR analysis and expressed as a ratio to GAPDH. *$p < 0.05$, **$p < 0.01$, compared to the DNCB-sensitized mice treated with saline. (B) The dLNs were extracted from AD-like BALB/c mice (n = 5) on the last day of the experiment, and a single-cell suspension was prepared. (B) The Th2 ($CD4^+IL\text{-}4^+$) cell subset was measured and compared by FACS analysis. **$p < 0.01$, compared to the DNCB-sensitized mice treated with saline. Data are representative of three independent experiments. (C) Relative mRNA expression levels of IL-4 and GATA3 were measured by quantitative RT-PCR analysis and expressed as a ratio to GAPDH. **$p < 0.01$, compared to the DNCB-sensitized mice treated with saline. Data are representative of three independent experiments.

have demonstrated TSLP as a major contributor that initiates Th2-dominant allergic inflammatory response in AD (Liu, 2006). Besides, the pro-inflammatory and Th2 cytokines (e.g., TNF-α, IL-1β, IL-4, and IL-13) in the lesional skin in AD patients could synergize to induce TSLP expression by keratinocytes, implicating a feed-forward loop of the skin inflammatory response (Indra, 2013). Our finding of 2′-FL and OPN-limited hypersensitivity to DNCB-induced AD skin lesion correlated with the decreased epidermal TSLP expression. Additionally, the induced expression of inflammatory cytokines in DNCB-treated mice was significantly suppressed upon the 2′-FL and OPN administration. It is plausible to assume that both 2′-FL and OPN treatment modulates and shapes the Th2 immune responses during the development of AD.

OPN exists both as a component of the extracellular matrix and as a soluble cytokine that mediates diverse biological functions. After oral administration, OPN can reach various tissues and organs such as epidermal cells of the small intestine and brain tissue through blood circulation (da Silva et al., 2009). It is noteworthy to mention that OPN is also expressed in naive T cells and strongly upregulated in response to T cell receptor ligation. Shinohara et al. observed that OPN gene expression in T cells is controlled by T-bet, a master regulator of Th1 cells. Importantly, the T-bet-dependent OPN expression is crucial for the efficient skewing of $CD4^+$ T and $CD8^+$ T cells toward Th1 and type 1 $CD8^+$ T (Tc1) cells, respectively (Shinohara et al., 2005). Mice deficient in OPN exhibited decreased levels of Th1 cytokines but the enhanced expression of Th2 cytokines in response to the intracellular bacterium and virus challenge (Ashkar et al., 2000; Cantor and Shinohara, 2009). It is reasonable to assume that the dynamics of OPN production and autocrine uptake by activated T cells is essential for the Th cell differentiation. CD44 has been best characterized as an adhesion receptor engaged by migrating T cells, and also function as a physiological receptor for OPN. Klement et al. found that the OPN-CD44 axis suppressed $CD8^+$ T cell activation and controlled the immunosuppression in the tumor microenvironment (Klement et al., 2018). 2′-FL can be absorbed intact into the circulation, suggesting that its systemic effect may depend on direct interaction with the immune system outside of the gastrointestinal tract (Goehring et al., 2014). Zehra et al. found that 2′-FL regulates human epithelial cell responses to the antigen-antibody complex, thereby influencing the progression of allergic diseases (Zehra et al., 2018). In vitro studies have shown that 2′-FL directly suppresses LPS-mediated inflammation in intestinal epithelial cells through the limitation of CD14 induction (He et al., 2014). PBMCs stimulated with 2′-FL tended to proliferate less than the unstimulated PBMCs. By contrast, compared with PBMC stimulated with LPS alone, cells co-stimulated by a combination of HMO and LPS proliferated more and tended to have fewer detectable $CD4^+$ T cells (Comstock et al., 2014). In a murine influenza vaccination model, 2′-FL treatment significantly improved the vaccine-specific $CD4^+$ and $CD8^+$ T-cell responses (Xiao et al., 2018). It would be of great interest to define whether 2′-FL and OPN are directly interacting with immune cell receptors and directly triggering the changes in the immune cells, and

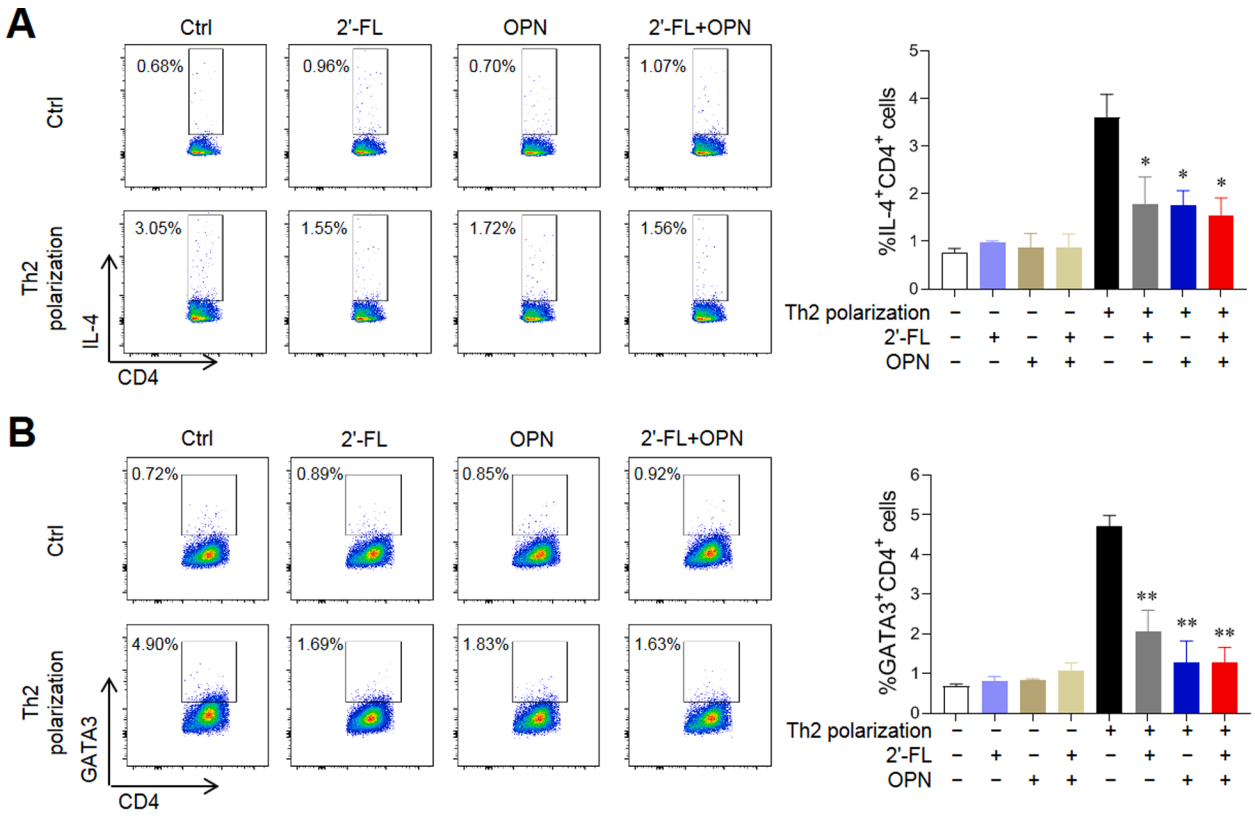

Fig. 5. 2′-FL and OPN suppressed the Th2 cell polarization *in vitro*. Freshly harvested naive T cells from BALB/c mice were polarized to Th2 cells in the presence or absence of 2′-FL and/or OPN for 3 days. The intracellular expression of IL-4 (A) and GATA3 (B) was evaluated by flow cytometry. *$p < 0.05$, **$p < 0.01$, compared to the group of cells under Th2 differentiation condition without 2′-FL or OPN treatment. Data are representative of three independent experiments.

further investigation is warranted.

DNCB-induced skin inflammation is currently recognized as an effective, rapid and reproducible AD mouse model (Kim et al., 2014; Lin et al., 2017). It was characterized by higher serum IgE, infiltrating inflammatory cells in skin lesions, and increased production of IL-4 in circulation. However, this model is not physiologically relevant to AD lesions in the human. Additionally, the number of animals involved in the study was limited. Further studies using mice model that spontaneously develop AD-like skin lesions or human clinical disease to determine the function of 2′-FL and OPN on AD pathogenesis are warranted. On the other hand, *in vitro* models based on human tissues (Martel et al., 2017) to study the effect of 2′-FL and OPN on AD-like skin inflammation remain to be investigated.

Overall, the human milk components do have a promoting benefit against infectious disease and may influence immune system development in infants. In the current study, we demonstrate that 2′-FL and OPN treatment could ameliorate AD pathology through limiting serum IgE levels, suppressing eosinophils, and mast cell infiltration. These components can inhibit Th2 differentiation/activation and decrease pro-inflammatory cytokine expression induced by DNCB in the AD mice model. These findings substantiate the concept that human breast milk components have beneficial effects for attenuating AD-like skin lesions.

Ethical statement

The procedures of animal care and use was approved by the Welfare and Ethical Committee for Experimental Animal Care of Southern Medical University (Guangzhou, Guangdong, China) and complied with all applicable institutions and government regulations regarding the ethical use of the animals.

CRediT authorship contribution statement

Xi Chen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Chunyan Yang:** Conceptualization, Methodology, Validation, Formal analysis, Investigation. **Jiaqi Zeng:** Methodology, Formal analysis, Investigation. **Zhengyumeng Zhu:** Methodology, Formal analysis, Investigation. **Liyun Zhang:** Methodology, Investigation. **Jonathan A. Lane:** Conceptualization, Resources. **Xueling Wu:** Conceptualization, Methodology, Supervision, Funding acquisition. **Daming Zuo:** Conceptualization, Methodology, Supervision, Resources, Funding acquisition, Writing – review & editing.

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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