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Article

Treatment with DHA improves epidermal keratinocyte differentiation and ameliorates inflammation in human keratinocytes and reconstructed human epidermis models

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Abstract: Atopic dermatitis (AD) is a chronic inflammatory skin disease, which can cause skin barrier function damaged. Although co-incubation with docosahexaenoic acid (DHA) exerts a positive effect in deficient skin model, there is no study to investigate the effects of topical treatment with DHA in inflammatory reconstructed human epidermis (RHE) model. The effects of DHA on monolayer normal human epidermal keratinocyte (NHEK) cells were evaluated via CCK-8, qPCR and ELISA. The skin related barrier function was assessed by hematoxylin-eosin (HE) staining, western blot (WB), Immunohistofluorescence (IF) and ELISA in normal and inflammatory RHE models. DHA upregulated filaggrin and loricrin expression at mRNA levels in addition to suppress overexpression of TNF-α, IL-1α and IL-6 stimulated by poly I:C plus LPS (stimulation cocktail) in cultured NHEK cells. After topical treatment with DHA, cocktail induced inflammatory characteristics of skin diseases including barrier morphological, differentiation proteins and TSLP secretion, which were alleviated in RHE models. Supplementation with DHA can improved related barrier function and have anti-inflammation effects in monolayer keratinocytes and RHE models, which indicated that DHA may have a potential value for the treatment of inflammation-associate skin diseases.

Keywords: DHA; reconstructed human models; filaggrin; skin barrier; inflammation

1. Introduction

The skin barrier consisted of dermis and upper epidermis is the first physical protective barrier of human body. The epidermis, a multi-layered compartment, protects human from terrible external environment through preventing the foreign pathogens, reducing the water loss and maintaining the homeostasis of skin through the cell death and differentiation of keratinocyte [1,2]. Filaggrin originates from its larger precursor profilaggrin existed in keratohyalin granules that is located in the stratum granulosum. During the differentiation of terminal, profilaggrin generates filaggrin monomers that can aggregate and link keratin to form cornified envelope (CE), which takes a critical role in maintaining the integrity of stratum corneum (SC) [3]. Meanwhile, filaggrin is a core epidermal protein that plays an important role for its intracellular metabolites that make contributions to epidermal barrier functions such as stratum corneum (SC) hydration and inhibition of UV-B irradiation [4].

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Some common skin barrier diseases such as atopic dermatitis (AD) and ichthyosis vulgaris (IV) are connected with filaggrin deficiency like filaggrin gene mutations [5]. It has been identified that loss-of-function FLG is the most important genetic risk factor for AD patients and is potential pathogenic factors for IV [6]. However, variation of FLG genes is not the only factor that causes filaggrin deficiency, the lack of filaggrin also can be observed in AD patients without FLG mutation status [7]. More and more study has found the modulatory effects of inflammatory environment in the epidermis. One of the major symptoms of AD patient is the overexpression of IL-4 and IL-13, which clearly decreases the filaggrin expression in normal human epidermal keratinocyte cells [8,9]. The dysregulation or the absence of filaggrin can cause skin barrier dysfunction, aggravate inflammation and increase the risk of microbial infections [10].

Traditionally, many studies have proposed effective programs including alleviating Th2-mediated inflammation and immunosuppressive drugs to treat skin diseases in AD patient. However, recent studies have shown that these traditional therapies can cause short or long-term risks [11,12]. There is increasing discovered that filaggrin deficiency is in the core of AD pathogenesis, hence upregulation of filaggrin expression is a more logical, effective and safe barrier repair therapy. Peroxisome proliferator-activated receptor (PPAR) agonists, especially PPARα and PPARγ, have drawn attention on the treatment of filaggrin-associated diseases including skin homeostasis and anti-inflammation, which make PPAR agonists further be explored [13,14]. However, most of the results of topical treatment are from mice model, which has interspecies-related differences [15]. Even if recent evidences show that PPARα + γ agonist docosahexaenoic acid (DHA) can upregulate the expression of FLG in organotypic, normal and deficient RHE skin models, all models are incubating with DHA [16,17]. More importantly, there almost no study exists whether the DHA works in inflammatory reconstructed human skin.

In this work, we firstly investigated the effects of DHA in human keratinocyte monolayer. Secondly, we investigated the impacts of topical treatment with a simple DHA formula in normal and inflammatory RHE skin models for the first time. We follow with interest the positive effects of DHA on the structural protein expression, skin barrier function and anti-inflammation, which is aiming for novel insights into the treatment with DHA in related skin disease.

2. Results

2.1. Effect of DHA, poly I:C and LPS on Cells Viability

The viability of NHEK cells was accessed by CCK-8 assay. The results of Figure 1 A showed that the NHEK cells viability was decreased with the increasing concentration of poly I:C and LPS. 30 ug/ml LPS and 80 ug/ml poly I:C significantly suppressed NHEK cells viability compared with the control group (p<0.05). As shown in Figure 1 B, 200 uM DHA indicated no apparent effect on cell proliferation. It is interesting that the cell proliferation is higher in 100 uM DHA supplement group than control group, which was not obviously statistically significant.

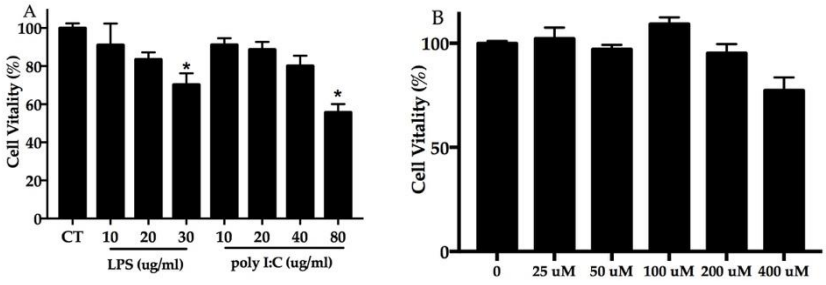

Figure 1. Effect of poly I:C, LPS and DHA on cells viability. NHEK cells exposed to poly I:C (0 ug/ml-80 ug/ml) and LPS (0 ug/ml-30 ug/ml) (A), DHA (B) for 24h. Data are expressed as mean ± standard deviation (SD), n = 5. *compared with blank control, p<0.05.

2.2. DHA decreased the cocktail-stimulated proinflammatory genes and cytokine secretion in NHEK cells

To evaluate the effect of DHA on stimulation cocktail (The concentration of cocktail is shown in Figure S1) and spontaneous proinflammatory cytokine expression in NHEK cells, this study measured IL-1α (Figure 2 A), TNF-α (Figure 2 B) and IL-6 (Figure 2 C) expression at levels of mRNA and protein. As can be seen from the Figure 2, the results of proinflammatory genes and secreted protein in the stimulation cocktail groups significantly (p<0.05) increased than control group. Interestingly, the addition of DHA significantly decreased the proinflammatory gene and cytokine expression induced by the cocktail (Figure 2) (p<0.05).

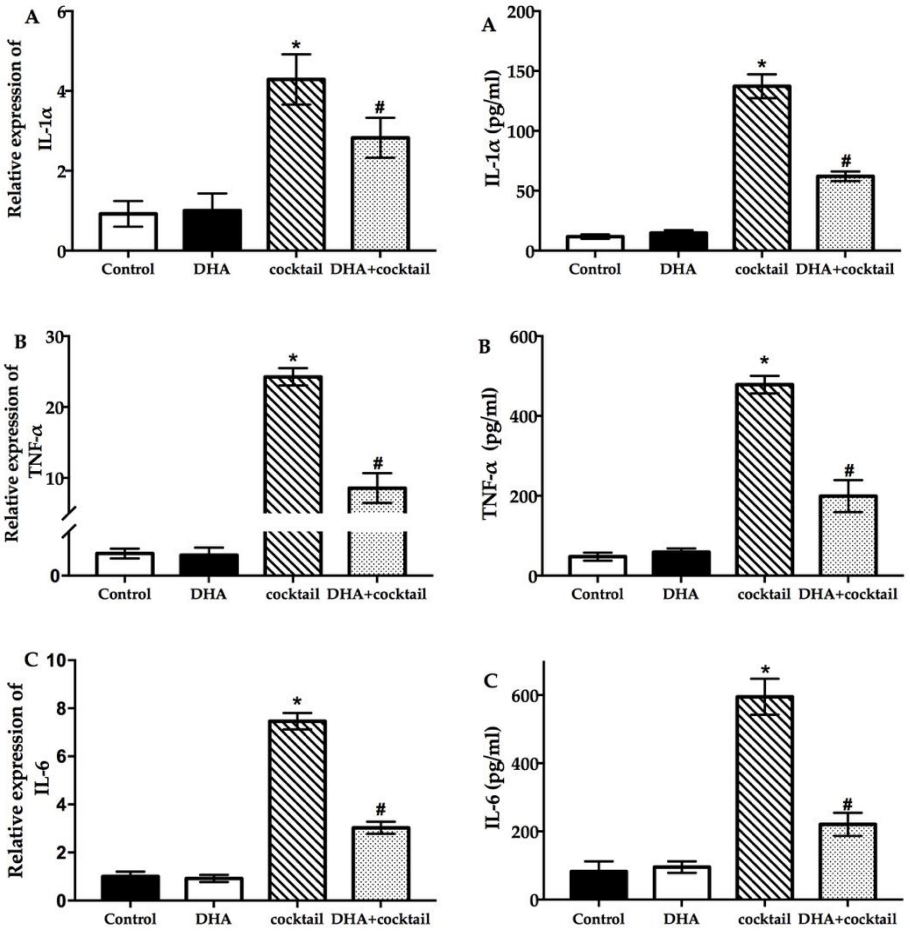

Figure 2. Effect of DHA on the inflammatory cytokine expression in normal human epidermal keratinocyte cells with or without cocktail (poly I:C plus LPS). The results of qPCR and ELISA show the changes of IL-1α(A), TNF-α(B), IL-6 (C) after incubation for 24h. Data were presented as mean ± SD, n = 5. * compared with the control group, p<0.05; # compared with the cocktail (LPS plus poly I:C) treated group, p<0.05. IL, interleukin; TNF, Cocktail, LPS + poly (I:C); tumor necrosis factor; LPS, lipopolysaccharide; poly I:C, polyinosinic-polycytidylic acid; qPCR, real-time quantitative polymerase chain reaction.

2.3. Effect of DHA on cultured NHEK cells with or without stimulation cocktail

The relative marker gene filaggrin (FLG), loricrin (LOR) and involucrin (IVL) expression was assessed for analysis of effect of the keratinocyte differentiation in response to DHA with or without stimulation cocktail. Incubation with DHA significantly increased the expression of FLG and LOR compared with blank group. (Figure 3) FLG and LOR were upregulated 2.7-fold and 7.2-fold after treatment with DHA. Interestingly, the expression of IVL was marginally influenced by DHA (Figure 3). In contrast, stimulation cocktail significantly inhibited the expression of FLG, IVL and LOR. After the DHA supplement, the amount of FLG and LOR in stimulation cocktail - treated group was 2.1-fold and 4.1-fold, which was higher than stimulation cocktail alone treated group. There was no difference observed in the IVL.

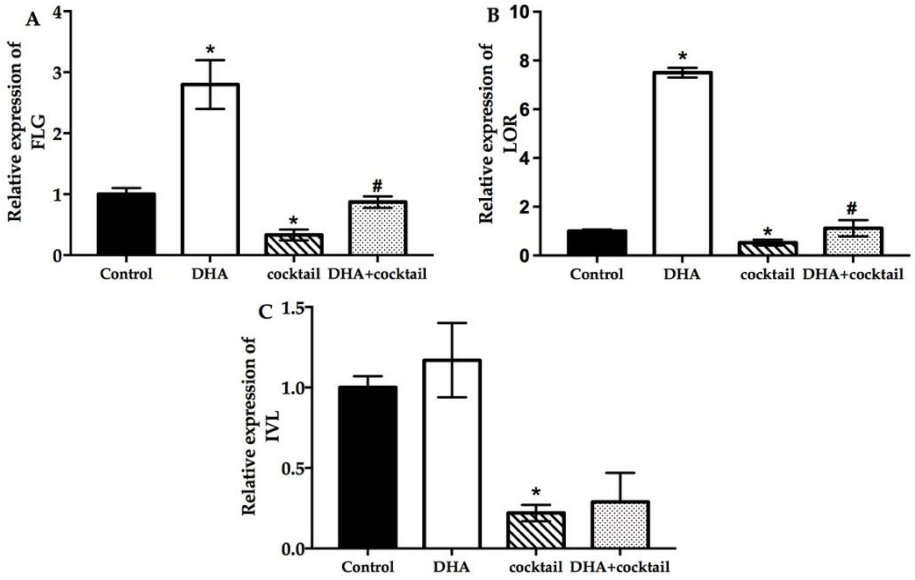

Figure 3. Effect of DHA on the differentiation of cultured NHEK cells with or without cocktail (LPS plus poly I: C). qPCR was used to evaluate the changes of FLG (A), LOR (B) and IVL (C) mRNA expression after incubation for 24h. Data were presented as mean ± SD, n = 5. * compared with respective blank group, $p<0.05$; # compared with their respective cocktail (LPS + poly I:C) alone treated group, $p<0.05$. Cocktail, LPS + poly (I:C); LPS, lipopolysaccharide; poly I:C, NHEK, normal human keratinocytes; qPCR, real-time quantitative polymerase chain reaction; FLG, filaggrin; LOR, loricrin; IVL, involucrin.

2.4. Topical treatment with DHA stimulates differentiation and improve barrier homeostasis

DHA was not stable to attach on the RHE, hence, we made a simple formula (Table S1) to investigate effect of the topical treatment with DHA. Firstly, we investigated the morphology of normal and inflammatory RHE models topically treated with DHA, which was used by haematoxylin and eosin staining. As show in Figure 4 A, the stratum corneum thickness of RHE significantly raised after topical treatment with DHA compared with normal group. Meanwhile, living layers of inflammatory RHE models induced by stimulation cocktail were looser and inflammatory RHE had spongiosis compared with normal models. Interestingly, the barrier disruption of inflammatory RHE models significantly improved after the topical treatment with DHA formula. In Figure 4 A, there is a clear trend of decreasing cavities in living layers and the status of spongiosis remarkably improved. Next, the effects of DHA on RHE differentiation protein were analyzed by immunohistofluoresence and western blot analysis. After topical treatment with DHA, the FLG and LOR amount significantly increased compared with normal group (Figure 4 B, C). The results of image quantification of immunohistofluoresence and western blot also revealed that FLG synthesis was significantly improved ($p<0.05$). But, no effect with IVL in all groups (Figure 4), which was according to with the result of qPCR. FLG, LOR and IVL expression in inflammatory RHE induced by stimulation cocktail were significantly decreased compared with the normal groups, which was shown in the results of WB and IF (Figure 4 B, C). In inflammatory RHE models, topical treatment with DHA significantly improved the FLG and LOR expression. Once more, DHA also showed no effect on expression of IVL in inflammatory RHE (Figure 4).

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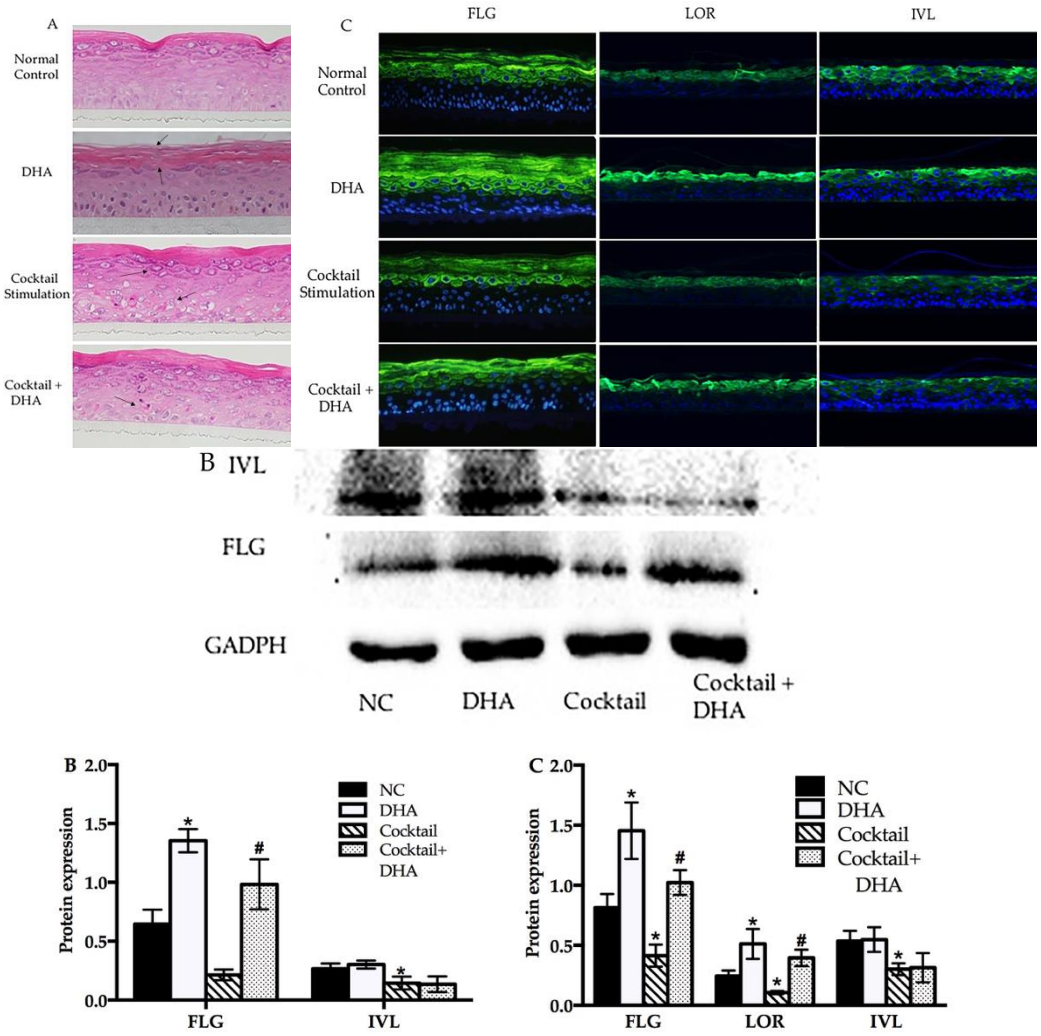

Figure 4. The topical treatment with DHA affect the morphology and differentiation protein expression in normal and inflammatory RHE models. (A) The morphology of normal, DHA, cocktail induced and DHA treated groups were evaluated by haematoxylin–eosin (HE) staining, (B) Western blot of FLG and IVL in normal control, DHA cocktail induced and DHA treated groups and relative optical densities of FLG and IVL. (C) Immunohistofluorescences analysis were executed for FLG, LOR and IVL in the above RHE models. Magnification 10×, mean ± SD, n = 4-5. * compared with the blank control groups, $p<0.05$; # compared with the inflammatory group, $p<0.05$. FLG, filaggrin; LOR, loricrin; IVL, involucrin; RHE, reconstructed human epidermis; Cocktail, LPS + poly (I:C); LPS, lipopolysaccharide; poly I:C, polyinosinic-polycytidylic acid.

2.5. Topical treatment with DHA downregulated the release of TSLP in inflammatory RHE model

It was necessary to prove that the inflammatory RHE model has reference value, we measured the level of TSLP by ELISA, which is related to inflammation characteristics observed in AD patient. As shown in Figure 5, supplementation with cocktail significantly increased the secretion of TSLP compared with normal RHE models (16 pg/ml vs. 274 pg/ml $p<0.05$). DHA alone showed a slight effect on TSLP expression in normal RHE models. On the contrary, the topical treatment with DHA significantly decreased the TSLP expression in inflammatory RHE models (90 pg/ml vs. 270 pg/ml, $p<0.05$).

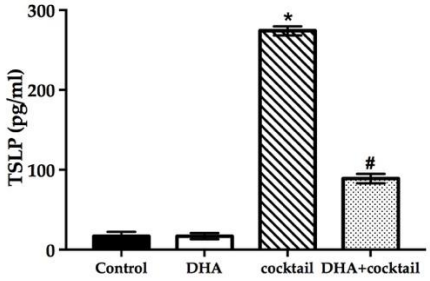

Figure 5. Effect of DHA on the TSLP secretion of RHE models with or without LPS plus poly I: C. The changes of TSLP were accessed by ELISA. Data are expressed as mean ± standard deviation (SD). * compared with the normal control, $p<0.05$; # compared with the inflammatory group, $p<0.05$. Cocktail, LPS + poly (I:C); LPS, lipopolysaccharide; poly I:C, polyinosinic-polycytidylic acid; TSLP, Thymic stromal lymphopoietin.

3. Discussion

The defect of filaggrin is a primary pathogenic factor for the atopic dermatitis (AD) [18]. Many studies have demonstrated that topical treatment with glucocorticoids and calcineurin inhibitor can decrease the integrity of stratum corneum and destroy the skin functions [12,19]. Therefore, the core of AD treatment is to repair skin barriers and restore relative function. The general approaches to cure AD contain hydration, regulation of SC pH [20] and application of epidermal barrier-improving agents such as LXR activators, AMP increasing agents and PPAR activators [21]. PPARs including PPARα, PPARβ/δ and PPARγ are expressed in human keratinocytes and skin, which play a critical role in keratinocyte differentiation and skin recovery [22]. All epidermal layers contain PPARβ/δ, but PPARα and PPARγ are present in suprabasal. Qiang [23] and Yan [24] have demonstrate that PPARγ agonists can stimulate cultured human keratinocytes differentiation and repair skin barrier in mice model. Meanwhile, PPAR-α agonist like WY14643 increases the expression of some epidermal differentiation structural protein, which may be critical in human keratinocyte differentiation. Besides, PPAR activators can alleviate and remedy the adverse effects of topical glucocorticoids (GC) like decreased keratinocyte proliferation and differentiation in skin [25]. Docosahexaenoic acid (DHA) can act as dual PPARα/γ agonists [26] and we investigate the impact of DHA in monolayer culture human keratinocytes. In the current study, we founded that DHA significantly increase the expression of FLG and LOR in the level of RNA in vitro, without effect of IVL (Figure 2). However, monolayer culture human cells cannot present complete epidermal maturation characteristic. Several reports have shown that PPAR agonists can reverse damaged barrier function in atopic dermatitis-like model, for example, Chiba and Yoshida have founded that topical application of PPAR- α agonist and DHA can treat atopic dermatitis in NC/Nga mice model [27,28]. Some study found that the rodent skin models may be able to explain some skin reactions to PPARs, yet there are restrictions on species specificity and interspecies difference [29,30].

Furthermore, it had been demonstrated that there is the presence of PPARs in reconstructed skin. The expression of filaggrin and other functional protein significantly increases with PPAR agonists supplement in normal models [31]. In reviewing the literatures, DHA is difficult to effectively adhere to the skin, hence, all data was found on the association between incubation with DHA and normal or FLG-deficient models, very little literature is found on the question of topical DHA application in reconstructed human epidermis model. Only a small clinical trial indicated a therapeutic effect in AD patients after topical application for two weeks [32]. In the present study, we firstly generate a normal reconstructed human epidermis (RHE) to investigate the topical effects of the DHA. Secondly, we make a simple formula (Table S1) to make sure that DHA can stably attach to the surface of the reconstructed human epidermis. We also observe that SC became thicker after the topical treatment with DHA (Figure 3), which is according to previous findings [31]. Another important function of filaggrin is its dephosphorylation degradation products called natural moisturizing factor (NMF), which affect multiple crucial functions tin the maintenance of epidermal homeostasis. NMF not only modulates the skin pH, acidification and increases retention

of water, but also shows the inhibitory influence of pathogenic microorganism colonization and has the positive effect of filaggrin-processing enzyme activity [33]. So, we demonstrated for the first time that upregulation of filaggrin works in reconstructed human epidermis models after topical treatment with a DHA formula and DHA also increases filaggrin and loricrin expression. Interestingly, IVL was not affected by DHA (Figure 3).

As mentioned in the literature review, inflammatory environment and microbial infection is another important risk factor to induce and aggravate AD. While there are some AD-like features RHE models induced by some regulatory factors in the previous studies [34], this study firstly developed an AD-associated features RHE model induced by poly I:C plus LPS. Lipopolysaccharide (LPS), a pathogen-associated molecular patterns (PAMPs), can trigger Toll-like receptor (TLR)4 to activate NF-κB to produce cytokines in human keratinocytes [35]. LPS can act as a microbial infection to generate an inflammatory environment in this model. Poly I:C is known as a TLR3 ligand stimulator that can imitate double-stranded RNA to induce an acute immune response in human keratinocytes. The concentrations of poly I:C and LPS were choose according to Figure S1and the previous literature [36,37]. After treatment of RHE with stimulation cocktail, the epidermal morphology was changed and bring out spongiosis, which agree with other AD-like models [38] and the characteristic of AD patients [39] (Figure 4). It is interesting to note that the results of HE is in well line with morphological characteristic of epidermal models induced by IL-3 and IL-14 [40]. Meanwhile, the stimulation cocktail decreased the distribution of barrier proteins like filaggrin (FLG), loricrin (LOR) and involucrin (IVL) (Figure 5), which is similar to other AD-like skin models in vitro [41,42]. Here, we first demonstrated that upregulation of FLG and LOR works in inflammatory RHE models after supplementation topical treatment with DHA (Figure 4).

Thymic stromal lymphopoietin (TSLP) that was abundantly expressed by keratinocytes plays an important part in atopic dermatitis and other allergic disorders [43]. Prior studies that have noted that TSLP is governed by the NF-κB pathway through TLR3. More interestingly, high level proinflammatory cytokines that are found in AD patient skin (IL-1α and TNF-α) can induce TSLP expression in keratinocytes [44]. Simultaneously, TSLP can brings out a Th2 inflammatory reaction via a vicious circle, which is potential underlying pathogenesis of AD and atopic march [36]. In this study, LPS can significantly stimulate the proinflammatory cytokines secretion in monolayer culture human keratinocytes (Figure 2), The cytokine levels of IL-1α were correlated inversely with NMF levels. TSLP is over-expressed in RHE models induced by stimulation cocktail further, which is associated inversely with FLG expression in AD patients. Conversely, the levels of cytokines and TSLP secretion were lower than DHA untreated group. It is possible, therefore, that DHA can increase FLG expression by decreasing the expression of TSLP and proinflammatory cytokines in inflammatory RHE models.

4. Materials and Methods

4.1. Monolayer cell culture

Normal human epidermal keratinocyte (NHEK) cells were purchased from Guangdong Biocell Co., Ltd (Guangdong, China) and cultured in Epilife (Gibico, Thermo Fisher Scientific, MA, USA) medium containing 60 uM Ca^{2+} and HKGS (Gibico, Thermo Fisher Scientific, MA, USA) in a 5% CO_2 incubator (Thermo Fisher Scientific, MA, USA) at 37°C. The medium was replaced every two days, the cells were used at 70% to 80% confluence.

4.2. Cell Viability Assay

Cell Counting Kit-8 (CCK-8) (Beyotime Biotechnology, Shanghai, China) was commonly used to test NHEK cells viability with high accuracy. NHEK cells (6×10^3 cells/well) were seeded in 96-well plates (Nunc, Thermofisher scientific, MA, USA). After stimulated with different concentrations of polyinosinic-polycytidylic acid (poly I:C), lipopolysaccharide (LPS) and docosahexnoic acid (DHA) for 24 hours, then the NHEK cells were incubated at 37°C for 4h adding 10 ul/100 ul CCK-8. Cell viability was used to count by the results of reading absorbance at 450 nm

with Microplate Reader (Molecular Devices, California, USA). The cell viability ratio was calculated, and the calculation formula is as follows: Viability (%) = (Optical Density OD $_{\text{treatment group}}$ - OD $_{\text{background group}}$)/OD $_{\text{control group}}$) x100%.

4.3. Study Design and DHA supplement

For LPS and poly I:C, we determined the optimum concentrations for subsequent studies. NHEK cells (6 x 10^3 cells/well) were seeded in 96-well plates (Nunc, Thermofisher scientific, MA, USA) and treated with different concentrations of poly I:C and LPS for 24 h according to the results of cells viability. We define the best concertation of LPS plus poly I:C by measuring the expression of TSLP (Figure S1).

NHEK cells were seeded into 6-well plates at a density 3×10^4 cells/ml. NHEK cells were cultured in Epilife medium with 1.3-1.5 uM Ca^{2+} and HKGS for 72h, and the medium was replaced with fresh Epilife medium containing different DHA (100 uM) for extra 24 h. For inflammatory stimulation, NHEK cells were incubated with HKGs and 1.5 uM Ca^{2+} in Epilife medium for 72h. After cocktail was added into medium to stimulate for 24h, then 100 uM DHA were added medium for another 24h.

4.4. Quantitative real-time PCR analysis

The total RNA was extracted from the NHEK cells following the TRIZOL reagent (Life Technologies, CA, USA) recommended protocol. The concentration and quality of RNA were measured using Qubit 3.0 (Thermofisher Scientific, MA, USA). This RNA was used for subsequent cDNA synthesis with the Prime script RT reagent Kit (Thermofisher Scientific, MA, USA). The changes of mRNA levels were measured by a Light Cycler 96 system (Roche) and SYBR Premix Ex Taq II (Takara Biotechnology, Dalian, China) following the manufacturer's recommended protocol. All primers of FLG, LOR, IVL, IL-1α, TNF-α, IL-6 and GADPH are listed in Table S1. The related data were analyzed using the delta cycle threshold method and the relative expression levels of each gene was normalized to Ct of the glyceraldehyde 3-phosphate dehydrogenase and calculated based on the $2^{-\Delta\Delta CT}$ method.

4.5. Construction of Reconstructed Human Epidermis (RHE) Models and DHA topical treatment

Reconstructed Human Skin (RHE) model was established by Complete Epilife growth medium and Cell culture Inserts (Thermofisher Scientific, MA, USA). Briefly, complete Epilife growth medium was prepared by adding 10 ng/ml keratinocytes growth factor, human keratinocytes growth supplement, 50 ug/ml ascorbic acid and 140 uM $CaCl_2$. According to the standard protocol, coating matrix was diluted by 1:100 and precoated. Cells were seeded in precoated inserts with 0.5 ml growth medium in both lower and upper compartment at a density of 7.5×10^4 cells/cm^2. The air-liquid inter face was established by adding complete growth medium with additional 1.5 mM $CaCl_2$ to the lower compartment and aspirating the upper compartment medium for 2 days incubation at 37℃ and 5% CO_2. The changes of subsequent medium were conducted by aspirating the medium from the lower compartment and replacing it with fresh medium supplemented with an additional 1.5 mM $CaCl_2$ (1.7 mM total $CaCl_2$). For inflammatory RHE, the cocktail was added 72h before DHA treatment.

According the Table S1, the simple formula was designed and used for next study. Dispense 32 μl/cm^2 of the 0.1% DHA cream formula on the top of each epidermis tissue. After 24 hours incubation, rinsing thoroughly 25 times with 1 mL PBS to remove all residual chemical from the epidermal surface before further analyzation.

4.6. Haematoxylin–eosin (HE) staining

Harvested RHE models were fixed in 10% formalin reagent (Sigma, St. Louis, MO, USA), dehydrated with ethanol and embedded in paraffin. RHEs sections were cut to 5um, deparaffinized and the slices were rehydrated using graded ethanol series. Finally, the slices were stained with

eosin and hematoxylin (Sigma, St. Louis, MO USA). The RHE slices were observed by EVOS FL auto (Thermofisher scientific, MA, USA) after mounting with neutral balsam.

4.7. Western blot

After treatment, the RHE models were washed with cold PBS and harvested with M-PER Mammalian Protein Extraction Reagent (Thermo fisher Scientific, MA, USA). A bicinchoninic Protein Assay kit (Pierce; Thermo Fisher Scientific) was used to measure the protein concentrations. 20 ug proteins were boiled and separated by 10% SDS-PAGE, then gels were transferred onto a PVDF membrane by using Power Blotter System (Thermofisher Scientific). Following 5% skimmed-milk blocking, the membranes were incubated with anti-FLG (1:2000; Thermofisher), anti-IVL (1:2000, Noves) and anti-GAPDH (1:5000; Invitrogen) antibodies overnight at 4°C. The membranes were washed and incubated with secondary antibody (1:20000) (Invitrogen) conjugated with horseradish peroxidase (HRP) in 0.5% PBST for 1 h. Proteins expression were detected by iBright FL1000 image system (Thermo fisher Scientific) and quantified by using iBright analysis software (Thermo fisher Scientific, USA).

4.8. Immunohistofluorescence assay

After different topical treatment, the RHE models were washed with cooled phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. After incubation in 0.05% Triton X-100 for 30 mins, the treated RHE slices were blocked with 5% BSA buffer for 60 min. The RHE models were incubated with primary antibody (FLG; 1:500, LOR 1:500, IVL 1:500, Abcam) overnight at 4 °C, followed incubation with an Alexflour 488-conjugated secondary Goat anti-rabbit IgG (1:200; Invitrogen) for 2 hours in the dark at 37 °C. The nuclei were stained by incubation for 10 min at room temperature with 4,6-diamidino-2-phenylindole (DAPI, 1:1000, Thermofisher Scientific, MA, USA). The images of immunohistofluorescence were visualized with Fluorescence Microscope (EVOS FL auto, Life Technology, Carlsbad, CA, USA) and analyzed using the EVOS browser imaging software (Thermofisher Scientific, MA, USA).

4.9. TNF-α, TSLP IL-α and IL-6 by Enzyme-Linked Immunosorbent Assay (ELISA).

The supernatant of NHEK cells or RHE models' samples were collected from the 6-wells plates. Proinflammatory mediators like tumor necrosis factor alpha (TNF-α), IL-1α, IL-6 and Thymic stromal lymphopoietin (TSLP) were determined by using ELISA kits (R&D, Minnesota, USA). The tests were performed strictly according to the manufacturer's instructions.

4.10. Statistical Analyses

All the values have been reported in terms of means± standard deviation (SD). The mean values were calculated based on data from at least three independent replicate experiments. The data were analyzed using the Student's t-test. P value less than 0.05 was statistically significant. All statistical analyses were performed using SPSS 25.0 (IBM Co., Armonk, NY, USA)

5. Conclusions

In conclusion, treatment with DHA can improved related barrier function and ameliorate inflammation in monolayer keratinocytes and inflammatory reconstructed human epidermis models, which indicated that DHA may have a potential value for the treatment of inflammatory-associate skin diseases.

Supplementary Materials: The following are available online, Figure S1: Effect of poly I:C, LPS and cocktail on the TSLP secretion in NHEK cells. Table S1: Primers for qPCR, Table S2: Formula Composition.

Author Contributions: methodology, T.J.; investigation, T.J., Q.Y. and W.Q.; validation, Q.W. and Q.Y.; writing—original draft preparation T.J. and W.Q.; writing—review and editing, W.W.; supervision and project administration, K.K.

Conflicts of Interest: The authors declare no conflict of interest.

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