

Atopic dermatitis and filaggrin

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Filaggrin has a key structural and functional role in the epidermis with important impact on the homeostasis of the skin. Inherited or acquired filaggrin deficiency has been described to essentially contribute to the pathogenesis of atopic dermatitis (AD), one of the most frequent chronic eczematous skin diseases in child-hood and adult-hood. Increasing knowledge has been gained during the last years about direct and indirect consequences of filaggrin deficiency. Furthermore, with the help of novel murine and human experimental models important steps forward have been made towards the characterization of filaggrin structure and function. Future approaches aimed at restoration of filaggrin expression may open novel mechanism-based therapies for AD.

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Introduction

Filaggrin (filament aggregation protein) is a critical epidermal protein. It derives from its larger precursor pro-filaggrin, which is present in keratinocytes in the stratum granulosum. Profilaggrin is constituted by a central region of filaggrin-repeat units and its post-translational processing during the differentiation of keratinocytes from granular to cornified cells, yields individual filaggrin monomers. Filaggrin is capable to aggregate keratin filaments which contributes to the flattening of corneocytes. Moreover, filaggrin and its breakdown products are essential parts with a key role in the epidermal homeostasis [1] (Figure 1).

Filaggrin deficiency plays an important role in the pathogenesis of AD. Variations in filaggrin genotype, the skin microenvironment or environmental factors have been described as key contributors to decreased levels of

filaggrin. The deficiency of filaggrin in AD patients impacts on the function of the epidermis and increases the risk for microbial infection or development of other atopic diseases. In recent years, important advances in the understanding of the causes and consequences of filaggrin deficiency in AD have been made. Animal models of filaggrin deficiency and novel therapeutic approaches for AD through the modulation of filaggrin expression have been described.

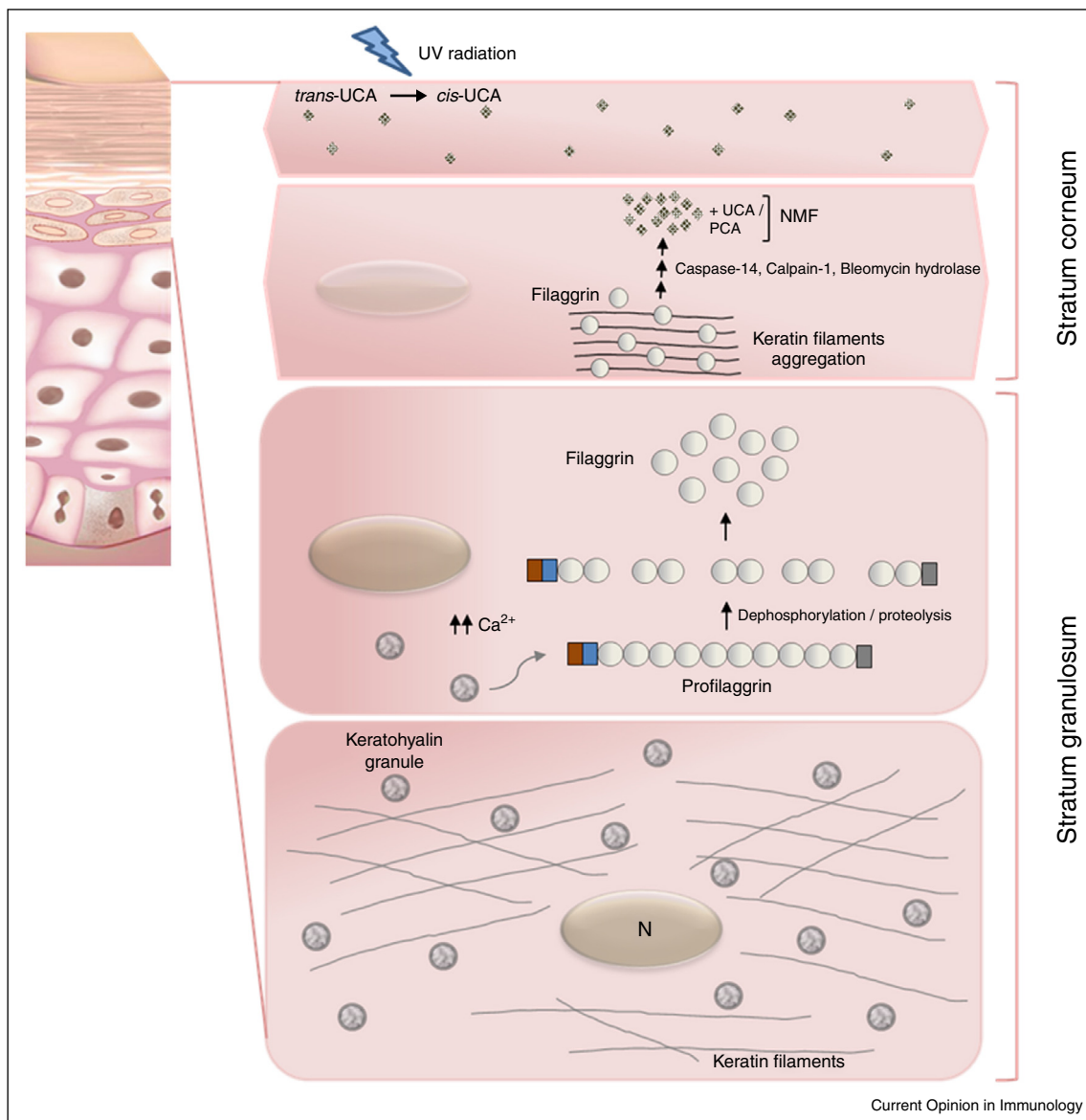
Causes of filaggrin deficiency

Multiple factors can modulate the levels of filaggrin and its breakdown products. Genetic modifications, such as mutations in human filaggrin gene (*FLG*) have been demonstrated to be associated with skin barrier disease such as ichthyosis vulgaris, AD or irritative contact dermatitis. However epidermal filaggrin deficiency can also be observed in AD patients independently from *FLG* mutation status. Therefore, other factors such as the cutaneous cytokine milieu or environmental influences including low humidity or mechanical damage are capable to secondarily modulate filaggrin expression [2].

Genetic factors

FLG is located in the epidermal-differentiation complex (EDC) on chromosome 1q21. The EDC contains genes that encode S100-fused type proteins (SFTP), which include profilaggrin, hornerin, filaggrin-2, repetin, cornulin, trichohyalin, and trichohyalin-like 1. Loss-of-function (LOF) mutations in *FLG* have been identified as the strongest genetic risk factor for AD in European and Asian populations described so far [3]. However, in populations from Africa or with an African ancestry, *FLG* mutations common in European patients seem to be rare. In line with this observation, a recent study showed absence of LOF mutations in *FLG* in a population of 69 AD-children from South Africa. However, levels of filaggrin breakdown products, urocanic acid (UCA) and pyrrolidone-5-carboxylic acid (PCA) were significantly decreased in those AD patients [4]. In a cohort of African-American children with AD, *FLG* LOF mutations common in European AD populations were rare as well [5]. Due to low frequencies of common LOF mutations in *FLG* in African populations, other candidate genes, such as *FLG2* have been investigated for their association with AD. This finding is interesting because structure and function of filaggrin-2, a SFTP, is similar to filaggrin. Two mutations in *FLG2*, rs12568784 and rs16833974 were recently demonstrated to be associated with AD persistence in African American children. rs12568784 represents a stop-gain mutation in exon 3, which might impair the production of filaggrin-2 [6].

Figure 1



Filaggrin in healthy skin. Filaggrin derives from the precursor profilaggrin, a ~500 kDa protein constituted by a central area of filaggrin-repeat units, a N-terminal S100 calcium-binding domain and a B-domain. Profilaggrin is highly phosphorylated and rich in histidines. It is expressed as keratohyalin granules in keratinocytes in the stratum granulosum. During differentiation, keratinocytes accumulate keratinocyte-specific proteins including profilaggrins. In the stratum corneum (SC), cells denude and form differentiated corneocytes. Profilaggrin is dephosphorylated and cleaved to filaggrin monomers by proteases such as kallikrein 5, caspase-14, elastase-2, matrilysin, and prostasin during the differentiation progress from granular to cornified cells [39]. The number of filaggrin units (each unit with a MW of ~35 kDa), which derive from profilaggrin is not fixed and is genetically determined. Filaggrins but not profilaggrins aggregate keratins, which are a family of proteins that form intracellular filaments that contribute to compaction and mechanical strength of the cells. The filaggrin-aggregated keratin filaments form tight bundles that promote the flattening of corneocytes. Transglutaminases cross link filaggrin and other differentiation-linked proteins into the cornified envelope. In the outer layers of the SC, filaggrin monomers are degraded by proteases such as caspase-14, calpain-1, and bleomycin hydrolase into free amino acids, including histidine or glutamine among others which are further metabolized to urocanic acid (UCA) and pyrrolidone-5-carboxylic acid (PCA) respectively. These filaggrin breakdown products constitute the natural moisturizing factor (NMF) with the function of hydration or UV protection [1]. The nonoxidative deamination of histidine produces *trans*-UCA, which has an important role in the acidification of the SC through the 'Histidine-to-UCA pathway'. Furthermore, upon UV radiation *trans*-UCA is converted to *cis*-UCA, with potential immunomodulatory effects. The acidic environment in the SC is key for the formation of ceramides, an important lipid class in the intercellular lipid matrix in the SC essential for the regulation of the skin permeability. The corneocytes enriched in proteins surrounded by a continuous intercellular lipid matrix are key for epidermal barrier function [15].

Beyond the role of *FLG* inheritance in AD, 'parent-of-origin' effects have been described. Maternal *FLG* mutations increased the risk of AD in the offspring. The results were consistent for four European *FLG* mutations studied in 2 independent populations. Interestingly, this effect was only significant when mothers were allergic but not in non-allergic mothers, which suggests that changes in the maternal immune system induced by *FLG* mutations might increase the risk for AD in the child [7*].

Besides mutations in *FLG* or *FLG*-related genes, variations in *FLG* copy number or epigenetic regulation of *FLG* also modulate filaggrin expression [2].

Proinflammatory cytokines

The cytokine environment in the skin exerts important modulatory effects on filaggrin expression. It is well-established that Th2 cytokines such as IL-4, IL-13, IL-17, IL-22, and IL-31 negatively modulate filaggrin expression in keratinocytes. Regulatory functions of cytokines on filaggrin have been extended to IL-33, IL-25 or thymic stromal lymphopoietin (TSLP). IL-33 is a cytokine increased in the skin of AD patients. IL-33 decreased filaggrin mRNA and protein levels of keratinocytes and skin samples, in particular from AD patients. However, the skin barrier-modulating effects of IL-33 were weaker than the effects mediated by IL-4 [8]. In line with these findings, the pro-Th2 cytokine IL-25 acted synergistically with Th2 cytokines to inhibit expression of filaggrin and its breakdown products. The acidic environment induced by filaggrin breakdown products reduced viral entry into keratinocytes. Consequently, an IL-25-dependent decrease in filaggrin breakdown products could modify local pH and increase thereby viral entry into keratinocytes and risk for viral infections and viral spreading [9].

TSLP, a cytokine highly expressed by keratinocytes in the acute and chronic phase of AD, was investigated in relation to its effects on filaggrin regulation. TSLP downregulated filaggrin expression in normal human epidermal keratinocytes. The comparison of the effect of TSLP in combination with other inflammatory cytokines such as IL-4, IL-17A, or IL-22 on filaggrin expression showed that TSLP and IL-4 had a higher effect on filaggrin expression than IL-17A and IL-22. The downregulatory effect of filaggrin by TSLP was mediated by STAT3-dependent pathways, ERK-dependent pathways, or both [10]. Genetic variations that result in diminished TSLP protein have been recently associated with less persistent AD in *FLG* LOF carriers [11].

Besides the regulatory effects on filaggrin expression, inflammatory cytokines such as IL-4, IL-13, and IL-25 downregulate the expression of other filaggrin-like proteins, such as hornerin, and filaggrin-2 [12*].

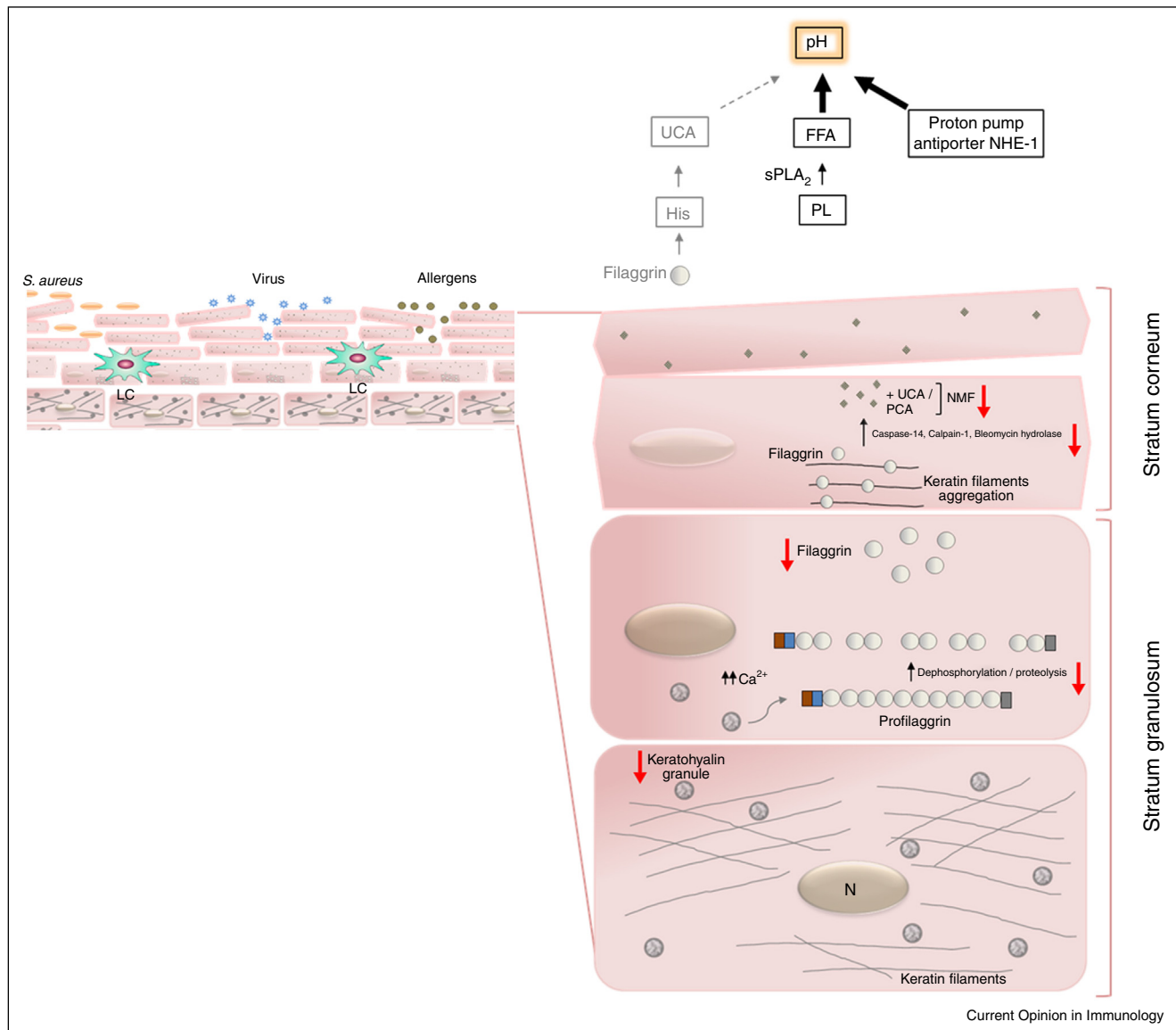
Filaggrin deficiency: consequences

Filaggrin deficiency has multiple consequences on epidermal organization and function with important implications such as higher risk for other atopic diseases or microbial infection (Figure 2).

A recent study showed that the deficiency of filaggrin in a fibroblast-free and immunological cell-free reconstructed human epidermis, reproduced some of the alterations observed in AD patients. For instance, lower number of keratohyalin granules and a reduction in the epidermal thickness could be observed. Also, the intracellular matrix of corneocytes was disturbed which led to increased SC permeability. Enzymes involved in filaggrin degradation, such as caspase-14 and bleomycin hydrolase were down-regulated. The concentration of UCA and PCA was reduced. However, proteins of cellular junctions were upregulated as a possible compensatory mechanism to restore permeability barrier defects [13]. Compensatory up-regulation of tight junctions proteins in a filaggrin-deficient skin equivalent has been demonstrated recently [14]. In line with these results, it has been shown with the help of a *FLG* knockdown (*FLG*-) skin construct that although histidine-to-UCA pathway was decreased, the pH of the skin surface remained unaffected. That effect was due to a compensatory upregulation of NHE-1 and the PL-to-FFA pathway via secreted sPLA₂, which maintained correct pH to ensure ceramide processing (Figure 2). This dysregulation of the skin acidification pathway due to filaggrin deficiency, however, had an impact on skin lipids, with increased amounts of FFA which led to less ordered intercellular lipid lamellae and increasing skin permeability to lipophilic drugs in the *FLG*- constructs [15].

The dysregulation of skin lipids in AD lesions together with enrichment of LCs has been suggested to increase CD1a-mediated lipid presentation to T cells. Data of a recent study implicate that PLA₂ derived from house dust mite (HDM) might interfere skin lipid processing for CD1a-mediated presentation to T cells and increase activity of CD1a-reactive T-cells. Interestingly, this PLA₂ activity was inhibited by recombinant filaggrin, which suggests that the loss of inhibition of HDM derived PLA₂ activity due to decreased levels of filaggrin could provide neolipid signals to CD1a-reactive T cells with potential inflammatory consequences [16]. The higher exposure of immune cells to danger signals due to deficient skin barrier increased maturation of antigen-presenting cells. Consequently, higher number of mature LCs was observed in the skin of *FLG* LOF carriers with and without AD. *cis*-UCA downregulated costimulatory molecules on the surface of dendritic cells. Less mature dendritic cells conditioned by *cis*-UCA induced higher proportion of CD4 T cells with regulatory T-cell phenotype. Therefore, deficiency of *cis*-UCA in *FLG* LOF carriers could lead to higher LC maturation and reduced induction of regulatory T-cell populations [17*].

Figure 2



Filaggrin deficiency in AD. Multiple consequences derive from the deficiency of filaggrin. Lower number of keratohyalin granules in the stratum granulosum together with a disturbed intracellular matrix of corneocytes. Enzymes, such as caspase-14 and bleomycin hydrolase, involved in filaggrin degradation, are downregulated. As a consequence reduced concentrations of filaggrin breakdown products such as UCA and PCA are observed [13]. Although the 'histidine-to UCA pathway' of acidification is altered in filaggrin-deficient skin, other pathways restore the acidic pH environment in the SC, such as the sodium/hydrogen antiporter-1 (NHE-1) and the phospholipid (PL)-to-free fatty acids (FFA) pathways via secreted phospholipases (sPLA₂) [15]. Dysregulation of the acidification pathways impacts on skin lipids, with less ordered intercellular lipid lamellae, affecting skin permeability. Moreover, in filaggrin-deficient skin, morphology of corneocyte surface is altered [18]. The skin barrier deficiency due to decreased levels of filaggrin and its breakdown products can lead to higher exposure to danger signals such as *S. aureus*, HSV-1, VV or to food-allergens and aero-allergens. Increased activation and maturation of LC with reduced capacity of induction of regulatory T-cell populations might result [17]. All these factors can contribute to the higher risk for other atopic diseases or microbial infections in subjects with filaggrin deficiency.

Furthermore, filaggrin deficiency goes along with an altered morphology of corneocyte surface. Higher number of villus-like projections, as an abnormality of corneocyte morphology was observed in *FLG* LOF carriers with AD. These structural differences negatively correlated with NMF levels and persisted even after clinical

improvement of AD [18]. Filaggrin deficiency has also been related to other alterations such as paracellular defects or a stress response mediated by type 1 interferon, which may represent a functional mechanism responsible for the augmented viral infections in filaggrin deficient patients [19]. Other alterations include increased transepidermal

water loss (TEWL) [20]. TEWL has been recently analyzed in order to predate the clinical development of AD in children. Detection of increased TEWL reading at day 2 or at 2 months of age in asymptomatic infants was associated with increased prevalence of AD at 12 months. This effect was not dependent on *FLG* LOF mutations but was enhanced by [21[•]].

Microbial infections

AD patients are susceptible to viral dissemination such as in eczema vaccinatum (EV) caused by vaccinia virus (VV) or eczema herpeticum (EH) caused by herpes simplex virus (HSV). Skin barrier dysfunction as a result of altered filaggrin expression may impact on the propensity of viral penetration and dissemination. Support for this hypothesis comes from experiments carried out in flaky tail (*ft/ft*) mice showing that filaggrin deficiency is associated with a greater dissemination of VV, which results in EV. The effects were amplified after inoculation of the skin of *ft/ft* mice topically sensitized with OVA with VV. The data showed that synergy of filaggrin deficiency and allergic inflammation increased VV dissemination. IL-17A was found to be at least in part responsible for increased susceptibility to viral dissemination in *ft/ft* mice [22]. In line with this observation, a study demonstrated that the inhibition of filaggrin expression and its breakdown products by pro-Th2 cytokines enhanced HSV-1 replication *in vitro* due to a modification of the local pH [9].

Filaggrin has been also found to play an important role in skin protection against α -toxin, the most important virulent factor produced by *Staphylococcus aureus* (*S. aureus*). Bacterial infections caused by *S. aureus* frequently exacerbate AD skin lesions, and reduced filaggrin levels have been associated with increased epidermal colonization with *S. aureus* [23]. Recently it has been shown that the expression of filaggrin in epidermal cells is important to prevent α -toxin-induced cell death. The mechanism involved sphingomyelinase, an enzyme that reduces the number of α -toxin binding sites, whose secretion is mediated by filaggrin. These results showed that filaggrin exerts a crucial function in the protection against *S. aureus* infection [24].

Risk of other atopic diseases

LOF mutations in *FLG* have been associated with a higher risk for allergic rhinitis or asthma. In the case of asthma, previous studies have shown association of R501X mutation of *FLG* with asthma in European populations. This association has been recently confirmed in a cohort of white American children with AD. Although the R501X mutation was rarely seen in African Americans, the few ones with the mutation had an elevated risk for asthma, although the effect was not significant [25].

Conflicting results have been obtained in studies evaluating the role of *FLG* mutations in food allergy [26,27]. A

dose-dependent-relationship between environmental exposure to peanut in early life and development of peanut sensitization and allergy in children that carry one or more *FLG* LOF mutations was observed. In this group of children, small quantities of peanut protein in the environment resulted in peanut sensitization and allergy, a risk that increased with augmented exposure. Such an association was not found in children without *FLG* mutations [28[•]]. A recent study demonstrated that *FLG* LOF carrier infants have a 2.4 times higher likelihood to suffer from food allergies at 2 years of age than *FLG* wild-type infants [29]. No association between three *FLG* mutations and food and aeroallergen sensitizations was observed in adults in a Danish study, however a history of AD was significantly associated with the sensitization to both allergens sources [30]. Differences in environmental allergen exposures can explain the conflicting results of the association/lack of association of *FLG* mutations with food sensitization.

Murine models of filaggrin deficiency: recent advances

Flaky tail mice with a spontaneous recessive mutation (*ft*) arose on the background of the existing recessive hair phenotype, matted (*ma*). The mice have been maintained as a mixed strain for the closely linked loci on chromosome 3. *ma/ma Flg^{ft/ft}* mice have been used as an AD model. They express truncated profilaggrin of 215 kDa due to a single nucleotide deletion at position 5303 in exon 3 (5303delA) in the murine *F/g* and show low skin hydration, increased IgE levels and spontaneous dermatitis. However, the role of the *ma* gene in the epithelial barrier function was unknown. Two studies in 2013 were able to further differentiate between functions of the *F/g* mutation and the matted mutation. The matted phenotype was due to a nonsense mutation in the *Tmem79* gene. *Tmem79* was found to be involved in the lamellar granule secretory system. With the deficiency of *Tmem79* the secretion of several proteins was abnormal, affecting the SC barrier formation and resulting in spontaneous dermatitis. In the light of these findings, the contribution of *Tmem79* mutation should be considered when using the double-mutant flaky tail mice as AD model in the context of filaggrin deficiency [31[•],32^{••}]. Furthermore, mice bearing only the *F/g^{ft}* mutation developed spontaneous AD-like inflammation, which progressed with age to pulmonary inflammation, with relocalization of atopic symptoms from the skin to respiratory organs. This represents a novel murine model for filaggrin deficiency studies [33].

Modulation of filaggrin expression as a therapy for AD

Treatments for AD patients are mainly focused on symptom relief, while mechanism-based therapies are not (yet) available. In particular for patients with primary or secondary filaggrin deficiency and resulting skin barrier

impairment it would be of great importance to create therapies focused on the modulation and restoration of filaggrin expression. In this context, candidate compounds that could potentially promote filaggrin expression have been screened using a 1120-compound library. JTC801, a 4-aminoquinoline derivative, considered as a nonpeptidergic ORL1 receptor antagonist, was able to increase *FLG* mRNA expression in both a human immortalized keratinocyte cell line and normal human epidermal keratinocytes. Using a human skin equivalent model, JTC801 increased expression of filaggrin but not loricrin, *K10*, and *TGM1*, markers for keratinocyte differentiation. JTC801 attenuated atopic skin inflammation in NC/Nga mice *in vivo* [34^{**}]. Another possible therapeutic approach could be the application of filaggrin monomers to restore barrier function. In that sense, a recent study engineered a functional filaggrin monomer linked to a cell-penetrating peptide motif (mFLG + RMR). The construct applied to HEK-293 T cells was internalized in a dose-dependent, time-dependent and RMR-dependent manner. Using a reconstructed human epidermis tissue model, recombinant filaggrin penetrated to pathologically relevant layers. Moreover, in *in vivo* studies using the flaky tail mouse model, the construct was internalized and processed to restore the normal phenotype [35^{*}].

A novel Janus kinase (JAK) inhibitor, JTE-052, induced filaggrin expression and decreased the development of AD-like lesions in mice. When IL-4/IL-13 bind to their receptors, they activate JAKs, a family of tyrosine kinases that transduce cytokine-mediated signals through the JAK-STAT pathway. JTE-052 was able to counteract the effects of IL-4/IL-13 by downregulating genes involved in keratinocyte differentiation. The JAK inhibitor suppressed STAT3 activation, increased levels of filaggrin and NMF in a murine model of AD. Therefore, this JAK inhibitor may have therapeutic implications in AD [36].

Even molecular mechanism of one of the oldest therapies used to reduce inflammation and itch in AD patients, the topical application of coal tar, have been investigated in the context of AD and filaggrin. Coal tar, that contains polycyclic aromatic hydrocarbons (PAHs), activates the aryl hydrocarbon receptor (AHR) signaling pathway, increased epidermal differentiation and stimulated the expression of filaggrin. *AHR* knockdown by siRNA completely abrogated that effect. Coal tar activated AHR/NRF2 signaling pathway, which very likely would prevent oxidative inactivation of PTPN1, the phosphatase responsible for inactivation of STAT6. That results in inhibition of the IL-4/STAT6 signaling pathway, and counteracts Th2 cytokine-mediated downregulation of skin proteins. This was the first study that showed that AHR activation might have beneficial therapeutic effects on skin barrier function [37^{*}]. Studies on the role of AHR regulation in filaggrin expression have been recently

extended on sirtuin 1 (SIRT1), a deacetylase which is known to contribute to cellular regulation. SIRT1 enhanced the activation of AHR through AKT, promoting filaggrin expression. Loss of SIRT1 in a mouse model, increased the susceptibility to epicutaneous allergen challenge and late-onset AD-like skin inflammation [38].

Conclusions

Current scientific advances place filaggrin deficiency in the center of AD pathogenesis, with a clear impact on epidermal functionality and structure. Increased data add new insights to the multiple factors that can downregulate filaggrin and its breakdown products. Moreover, important recent advances expand the understanding of several implications filaggrin deficiency has in the complex AD pathophysiology and in the connection with the risk for other atopic diseases. Better knowledge of the role of filaggrin in AD will provide new directions for therapies based on filaggrin modulation.

Conflict of interest

Nothing declared.

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