The Multifunctional Role of Filaggrin in Allergic Skin Disease

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Abstract

Filaggrin is a major structural protein in the stratum corneum of human epidermis. Mutations in the filaggrin gene are the most significant known risk factor for the development of atopic dermatitis. Mutations in *FLG* also confer risk for the associated allergic diseases of food allergy, asthma, and allergic rhinitis. These discoveries have highlighted the importance of skin barrier function in the pathogenesis of atopic diseases and have motivated a surge in research characterizing the filaggrin deficient skin barrier and its consequences. In this review we discuss the mechanisms through which mutations in this protein contribute to the pathogenesis of atopic dermatitis and associated atopic conditions. We focus on recent human and murine discoveries characterizing the filaggrin deficient epidermis with respect to biophysical, immunological and microbiome abnormalities.

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List of Key words

Filaggrin, atopy, dermatitis, eczema, IL-1β

Abbreviations

AD: atopic dermatitis, AMP: antimicrobial peptide, ASC: apoptosis-associated speck-like protein, CNV: copy number variation, *FLG*: human filaggrin gene, *Flg*: murine filaggrin gene, IgE: immunoglobin E, IsdA: iron-regulated surface determinant A, LEKTI: Lympho-epithelial Kazal-type-related inhibitor, NMF: natural moisturizing factor. NLRP3: nucleotide-binding oligomerisation leucine-rich repeat and pyrin domain containing 3, OR: odds ratio, OVA: ovalbumin, PAC: pyrroliodine-5-carboxylic acid, PBMC: peripheral blood mononucleocyte, SEB: Staphlococcal enterotoxin B, *Staph aureus: Staphlococcus aureus*, SpA: Staphlococcus protein A, SC: stratum corneum, Th: T helper, TEWL: transepidermal water loss, TJ: tight junction, UCA: trans-urocanic acid, US: United States, UK: United Kingdom.

Following standard genetic practice, in this paper *FLG* -/- designates a patient homozygous for null alleles (i.e. 2 null alleles); *FLG* +/- a heterozygote null allele/ wildtype (i.e. one null allele) and *FLG* +/+ a homozygote wildtype (i.e. 0 null alleles). A further abbreviation describes AD patients with *FLG* mutations (*FLG* +/- and *FLG* -/-) as AD_{FLG} and those without *FLG* mutations (i.e. *FLG* +/+) as $AD_{NON\text{-}FLG}$.

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Introduction

Atopic dermatitis (AD) affects approximately 11% of children in the US (1) and up to 25% in the UK. (2, 3) It is the most common chronic inflammatory disease of early childhood (4) and is associated with significant morbidity for the patient and their families. (5) Atopic dermatitis is characterized by an epidermal barrier abnormality, cutaneous inflammation, immune dysregulation with a systemic 'allergic' T helper (Th2) cell response, and frequent *Staphylococcus aureus* colonization.(4) It is often the initial step in the so called 'atopic march', with the subsequent development of allergies, asthma and hay fever.(6) The critical importance of the epithelium in the development of AD and allergic sensitisation has become apparent. Mutations in the *FLG* gene, which codes for the skin barrier protein filaggrin, have been shown to be the most significant risk factor, to date, for developing AD.(4) The specific dynamic interactions between an impaired skin barrier and the immune system remain to be fully elucidated. Here we review recent insights into the role of filaggrin in the pathomechanisms of AD and its associated diseases.

Epidermal Structure and Function: Role of Filaggrin

The epidermis, particularly the outermost stratum corneum (SC) layer, is the first line of defense between the host organism and its environment. The SC also minimizes water loss from the body and protects against both every day and extreme environmental insults. (7) The SC is the end product of a highly organized differentiation process in which keratinocytes in the basal layer of the epidermis progress to form the spinous and granular layers, ultimately forming a tough multilayer of corneocytes rich in intracellular lipids. (7) The SC matrix is an extensively cross-linked lipid protein matrix organized into neutral, lipid enriched, extracellular lamellar bilayers.(8) This hydrophobic extracellular matrix, together with

corneodesmosomes and tight junctions, specialized cohesive intercellular junctions in the stratum corneum and stratum granulosum, forms a very effective barrier.(9, 10)

Filaggrin is a major structural protein in the SC. (4) The role of filaggrin in epidermal structure and function has been reviewed in detail in recent papers. (4, 7, 11) Filaggrin is produced as the precursor pro-protein profilaggrin. Profilaggrin is expressed in terminally differentiating keratinocytes in the outmost layers of the human epidermis and is the major constituent of keratohyalin granules in the stratum granulosum. (7) Profilaggrin consists of multiple filaggrin repeats flanked by an S100-type calcium-binding domain, A and B domains at the N-terminal, and a unique tail sequence at the C terminal [Figure 1a](4)]

During terminal differentiation at the granular to cornified layer transition, profilaggrin is rapidly dephosphorylayed and cleaved by several endoproteases to generate 10,11 or 12 functional filaggrin monomers. (12) Extracellular proteases, such as matriptase, may also influence the expression of filaggrin monomers.(13) Filaggrin monomers aggregate and align keratin bundles, *in vitro*, and are thus postulated to contribute to the mechanical strength and integrity of the SC *in vivo.* (14) [Figure 1a] Ultrastructural studies have shown that filaggrin deficiency results in disorganized keratin filaments, impaired lamellar body loading, and abnormal architecture of the lamellar bilayer.(10) [Figure 1b] It has been proposed that *FLG* N-terminal provides a feedback mechanism that controls epidermal homeostasis.(15) The C-terminal domain's exact function is unclear but it is necessary for profilaggrin to filaggrin processing. Truncated profilaggrin, lacking a C-terminal, results in almost a complete absence of filaggrin.(12)

In the upper layers of the SC, filaggrin monomers are deiminated and degraded by proteases to release their component hygroscopic amino acids and their derivatives.

(16) Filaggrin is a histadine-rich protein, and its major metabolites are the organic acids trans-urocanic acid (trans-UCA) and pyrroliodone-5-carboxylic acid (PCA).(11) Filaggrin breakdown products, together with chloride and sodium ions, lactate, and urea, form 'natural moisturizing factor' (NMF) which contributes to epidermal hydration and barrier function. (11) [Figure 1a] In addition, these organic acid breakdown products help maintain the pH gradient of the epidermis. The acidic pH is key for many functions of the SC; it has an antimicrobial effect, is important for the functional activity of enzymes involved in ceramide metabolism, and modulates the activity of the serine protease cascade required for coordinated epidermal differentiation and cornified cell envelope formation. (11) [Figure 1b]

The Filaggrin gene (*FLG*) is located in the epidermal differentiation complex on chromosome 1q21. (4) Exon 3 of *FLG* is one of the largest exons in the genome and encodes almost the entire profilaggrin protein. Loss-of-function mutations within exon 3 all have a similar biological endpoint; they result in a truncated profilaggrin molecule lacking the C terminus, and hence an absence of filaggrin.(12) There are common size-variant *FLG* alleles in the general population, with 10, 11 or 12 repeats.(17) Therefore, excluding null mutations, the number of filaggrin units in humans varies from 20 to 24.The frequencies of copy number variation (CMV) alleles have been studied in the Irish population; 33.9% had 10 filaggrin repeats, 51.1% had 11 repeats, and 14.6% 12 repeats.(17)

<u>Filaggrin: Disease Associations.</u>

In 2006, *FLG* mutations were shown to be strongly associated with AD in an Irish population, and with AD plus asthma in a Scottish population.(18) This highly significant association has been replicated in over 30 independent studies. (11) Meta

analyses of these data have estimated the odds ratio (OR) of developing AD in association with *FLG*-null genotype to be 4.78(19) and 3.12.(20)

Filaggrin null mutations are seen in less than a third of total AD population.(18, 21) In moderate-to-severe AD, up to 45.7 to 56.6% of cases carry one or more *FLG* null mutations and the population attributable risk fraction has been estimated at between 4.2 and 15.1%.(22) On a population level, therefore, approximately 50% of moderate-severe AD cases may be attributed, at least in part, to *FLG* null mutations, whereas up to 15% of mild to moderate AD may be explained by *FLG* .(22) Among a group of AD patients, attending a tertiary referral clinic, 3% were homozygous for the *FLG* null genotype whereas 20% were heterozygous.(23) A study of an unselected population cohort of children demonstrated that the penetrance of *FLG* null mutations, with respect to flexural AD, was 55.6% for homozygous and compound heterozygous individuals, compared with 16.3% for heterozygotes. (24) Patients who were *FLG* null homozygotes had statistically significantly higher severity scores than heterozygotes and wild type patients.(24)

The profile of AD most strongly associated with *FLG*-null mutations (AD_{FLG}) is that of early onset, severe, persistent disease,(25, 26) and with raised total IgE and allergic sensitization. (27) Furthermore, patients with AD_{FLG} have a higher incidence of skin infections with herpes virus (eczema herpeticum) (28) as well as a greater risk of multiple allergies (19) and asthma (29) than patients with AD without *FLG* mutations ($AD_{NON\text{-}FLG}$). A US longitudinal cohort study suggested that there may be mutation-specific variability in the response to treatment in AD_{FLG} children.(26) Taken together, these studies suggest that patients with AD_{FLG} may have a distinct AD endophenotype and profile of associated disease, compared with individuals with $AD_{NON\text{-}FLG}$.(4) [figure 2]

It has been demonstrated that AD risk in an Irish population is related to filaggrin copy number variation (CNV) in a dose dependent fashion.(17) The lowest CNV genotype (10, 10 filaggrin repeats) carried by 11.5% of the Irish population had an eczema risk of 1.67, independent of *FLG* loss-of-functions mutations. The addition of each additional filaggrin repeat decreased the OR for AD by 0.88.(17) Furthermore, the concentration of filaggrin breakdown products was significantly correlated with filaggrin total CNV.(17) Thus, a modest increase in epidermal filaggrin expression may protective against developing eczema, and upregulation of cutaneous filaggrin expression in at-risk individuals may be a potential therapeutic approach.(11) [figure 3] Recent work suggests that the methylation status of *FLG* may further influence AD risk. (30)

The strong association of *FLG* mutations with AD is one of the most robust genotype-phenotype linkages observed in human complex genetic disorders.(19) However, pathomechanisms other than *FLG* mutations, or *FLG* modifying factors, are involved in AD. A significant number of patients with AD do not have any of the known *FLG* mutations, and conversely, approximately 40% of individuals with *FLG* null alleles do not develop AD.(31) In addition many patients with AD_{FLG} eventually recover from the disease. (29) More work is needed to establish influences, other than *FLG*, on epidermal barrier defects in AD.

The 'atopic march' describes the tendency for AD to precede the stepwise development of food allergies, asthma, and allergic rhinitis. Approximately 70% of patients with severe AD will develop asthma or allergic rhinitis in later life.(6) *FLG* mutations are a genetic risk factor for each of these diseases.(4)

Filaggrin haploinsufficiency confers an overall risk of 1.48 to 1.79 for asthma, but this risk is limited to those who have AD or a history of the disease.(29, 32) AD_{FLG}

have a much greater risk of asthma than $AD_{NON\text{-}FLG}$.(29) Asthma patients with *FLG* mutations have a more difficult disease course and more frequent exacerbations. (33) Therefore, AD is a causal risk factor for asthma in the context of *FLG* mutation, although the mechanism is not fully elucidated.(4)

FLG mutations confer an overall OR of 5.3 for peanut allergy, with a residual OR of 3.8 when corrected for AD. (34) This data suggests a barrier defect that facilitates enhanced exposure of peanut allergen to antigen-presenting cells, even in the absence of AD. An Australian cohort study found that *FLG* mutations were significantly associated with food sensitization, but did not additionally increase the risk of food allergy in 1-year-old infants.(35) These results suggest that the skin barrier dysfunction increases the risk of food sensitization, but other factors may be important in the conversion from food sensitization to allergy.(35) An association with *FLG* and allergic rhinitis has been reported in population studies. (19, 32)

Filaggrin immunostaining is restricted to the cornified epithelium of skin, oral mucosa and nasal vestibule. There is no detectable staining in the epithelium from bronchial biopsies or gastrointestinal epithelium. (32, 36, 37) *FLG* mutations, therefore, are unlikely to affect barrier function and allergen sensitization in the organs where these allergic diseases manifest. It is thought that *FLG* mutations drive allergic disease at distant mucosal sites through enhanced penetration by antigens through a defective skin barrier, with subsequent sensitization and allergen responsiveness.(38)

The prevalence of AD has more than doubled in industrialized countries with no clear cause. (1, 39) Environmental factors are thought to contribute to this rising prevalence. It has been postulated that the impaired skin barrier with *FLG* may potentiate the effects of environmental allergens.(40) Some studies have

investigated putative environmental risk factors for atopic disease genotype with regards to *FLG* status.

Two cohort studies, one from Denmark and the UK and the other from the Netherlands, have shown that cat ownership in early life increases the risk of AD, as an additional interactive effect to the risk associated with *FLG*-null genotype.(40, 41) The Danish-UK study did not, however, demonstrate any correlation between AD severity and specific IgE to cat dander, or *FLG*-null mutations and cat dander IgE,(40) making the mechanism of this association likely to be a host-defense initiation between the microbiome and a defective skin barrier. Another potentially important environmental effect in early life is contact with other children, as this may increase exposure to pathogens and allergens. Two German birth cohort studies have shown that children with *FLG*-null mutations have a significantly higher risk of eczema if they have an older sibling, and attendance at a day-care centre lessened this risk, reducing the odds ratio from 2.34 to 1.7. (42) Epidemiological data can be difficult to interpret in a complex disease such as AD. Different causal pathways between genes and the environment may be important in patients with AD who carry mutations as opposed to those who do not. It would be important, where possible, to stratify for *FLG* in future epidemiological studies of AD.(11)

The significant discoveries that *FLG* mutations are a strong risk factor for developing AD and atopic diseases have validated the key role of skin barrier in these conditions. The result has been a research emphasis on functionally characterizing the skin barrier, as well as identifying pathways connecting epidermal barrier disruption, antigen uptake, and the antigen-specific adaptive immune responses.

<u>Characterization of filaggrin deficient skin barrier function.</u>

The barrier integrity phenotype associated with *FLG* mutations is becoming better understood, with human and murine studies supporting the theory that *FLG* mutations lead to a functional epidermal barrier defect and subsequent allergic sensitization.

FLG genotype has been shown to be the major determinant of NMF in human studies. The SC levels of the filaggrin breakdown products PCA, UCA and histadine, which are major components of NMF, in epidermal tape strips, strongly correlate with *FLG* genotype.(43) O'Regan *et al* demonstrated that *in vivo* Raman microspectroscopic NMF signatures could be used as accurate proxy markers of *FLG* genotype in patients with moderate-to-severe AD, allowing rapid and highly accurate stratification of AD_{FLG}.(44) [figure 3] AD severity itself, however, is associated with a reduction in NMF and the relative importance of epidermal defects and immune dysregulation as key initiating and perpetuating factors in AD pathogenesis require further studies.(11)

Transepidermal water loss (TEWL) at non-lesional sites in AD correlates with disease severity and serum IgE. (45-47) Several studies suggest that non-lesional TEWL in AD is a common end point that is not influenced by *FLG* status. (44, 48, 49) *FLG* mutations were, however, were associated with higher TEWL in clinically normal forearms in a small cohort of 3-month-old infants, which was not dependent on AD status. (50) Further studies are needed to clarify the relationship between TEWL, barrier integrity, *FLG* status and subsequent allergen sensitization.(38)

Mechanistic Insights from Murine Models

Mouse models of filaggrin deficiency have demonstrated barrier impairment with enhanced percutaneous allergen sensitization. (51-54) The spontaneous flaky tail (*ft*) mouse arose on the background of an existing recessive hair phenotype, matted

(*ma*). Flaky tail mice carry a 1-bp-deletional mutation in the murine filaggrin gene (*Flg*). The relative contribution of *flg* and *ma* to the compound phenotype has yet to be fully defined. Flaky tail mice develop spontaneous dermatitis with increased IgE levels. (54) Fallon and colleagues demonstrated that the topical application of the clinically relevant allergen ovalbumin (OVA) to flaky tail (*ft/ft*) mice resulted in cutaneous inflammation and enhanced cutaneous allergen priming with development of allergen-specific antibody responses (51) The mice had a systemic immune response generating OVA specific IgG and IgE, as well as OVA specific Th2 (IL-4, IL-5, IL-13), Th1 (INF-γ), regulatory (IL-10) and TH17 (IL-17) cytokines, indicating a generalized allergen-specific cytokine response that was not solely Th2 skewed. Following sensitization, a further skin barrier defect, as measured by elevated TEWL, was observed, suggesting that the initial heritable barrier defect is exacerbated by allergic sensitization. These data provide experimental evidence that antigen transfer through a defective epidermal barrier is a key mechanism underlying elevated IgE sensitization and initiation of cutaneous inflammation. This suggests that sensitization might also be an early event in filaggrin-deficient humans.(51) Whether early intervention in AD, especially filaggrin deficient AD, would diminish systemic allergy in later life is an interesting research question.

Kawasaki *et al* generated filaggrin-null mice ($Flg^{-/-}$). (52) These mice develop dry scaly skin between 3 and 6 days of life. They have loss of the normal interlace keratin pattern in the epidermis with increased susceptibility to mechanical stress. *In vivo* confocal microscopy showed reduced NMF levels in the $Flg^{-/-}$ mice (52) in keeping with human studies on patients with *FLG* mutations. (44) The loss of NMF as a result of filaggrin deficiency did not lead to decreased SC water content in the $Flg^{-/-}$ mice. This is in contrast to the *ft/ma* mice, which have increased TEWL with loss of SC hydration, consistent with findings with human AD (with and without *FLG*

mutations).(49) *Flg*$^{-/-}$ SC, after hapten application, allowed penetration of protein antigens, which was followed by exaggerated systemic immune responses.(52) It is notable that the SC lipid composition in the *Flg*$^{-/-}$ was aberrant, and this may have additional direct or indirect effects on SC barrier function.(52)

These murine studies support the hypothesis that filaggrin deficiency results in enhanced percutaneous cellular and humoral immune responses, which are important steps in the early phase of AD pathogenesis. This important work characterizing a dysfunctional skin barrier and downstream systemic effects has provided an opportunity to focus on the importance of barrier improvement as a key therapeutic approach in this disease. Tailored emollients such as ceramide-lipid (55) or filaggrin replacement or upregulation are exciting possibilities.(11) In addition, these studies support the notion that a pro-active, rather than reactive approach, to eczema management could have a positive impact on systemic sensitization and the 'atopic march'. Perhaps even a prophylactic approach may be possible, with the regular use of emollients or other topical therapies, immediately after birth in high-risk babies reducing the risk of AD. Large, well-designed, randomized controlled trials will be needed to answer these intriguing questions.

Filaggrin status and immune dysregulation: a complex interaction

Both innate and adaptive immunity contribute to the immunopathology of AD. Innate responses occur rapidly, are efficient at killing pathogens and are involved in regulating the magnitude and the specific outcomes of the adaptive immune response. (56)The cutaneous innate immune system consists of three major components: the physical barrier, which includes the SC and intracellular junctions; the cellular component (antigen presenting cells, keratinocytes, mast cells and

neutrophils); and secretory elements (antimicrobial peptides, cytokines, and chemokines).(56) In patients with AD, the initial exposure to allergens (sensitization phase) induces a systemic “allergic” T helper type 2 (Th2) cell response that is magnified with each subsequent exposure (effector phase). Critical features of the Th2 immune response includes the local production of Th2 cytokines (IL-4, IL-5, and IL-13), bone marrow production, prolonged survival and activation of eosinophils and mast cells, and the production of allergen-specific IgE.(38) Acute AD lesions exhibit Th2-dominant inflammation characterized by dermal infiltration of CD4+ T cells and eosinophils with deposition of eosinophil-derived products and increased skin expression of IL-4,IL-5 and IL-13.(57) A pathogenic role for IL-4 in AD is supported by the observation that keratinocyte-specific overexpression of IL-4 in transgenic mice results in AD-like lesions.(58) Individuals with *FLG* null mutations have been associated with significantly higher frequencies of allergen-specific CD4+ T helper 2 cell responses. (59)

Filaggrin expression is down regulated in AD patients, regardless of *FLG* genotype, likely due to the effect of elevated Th2 cytokines, IL-4 and IL-13.(60) [figure 3] Keratinocytes differentiated in the presence of IL-4, IL-13, as well as already differentiated keratincytes, have significantly downregulated filaggrin expression.(60) These findings support the theory that filaggrin deficiency in many AD patients is acquired because of the Th2 cyotkine milieu.(60) The specific pathways linking epidermal barrier disruption and allergen sensitization are becoming clearer. One accepted hypothesis is that epidermal disruption facilitates skin-resident antigen presenting cells (Langerhan and dendritic cells) in capturing environmental allergens. Furthermore, barrier-disrupted keratinocytes release immune adjuvants that activate and cause maturation of antigen presenting cells and affect their ability to direct

native Th polarization, thereby influencing the character of the Th response. The resulting adaptive immune response further disrupts barrier function. (38)

The cytokine profile in the epidermis in AD_{FLG} is now becoming clearer. The majority of the studies to date, however, are *in vitro* or in murine models. One study has investigated SC cytokines from AD patients stratified by *FLG* status. (61) Here we review recent data examining the interaction between filaggrin status and the immune response.

a) Interleukin-1

IL-1 mediators influence innate immune responses and bridge the innate and adaptive immune systems. Keratinocytes constitutively produce high amounts of IL-1α.(62) (63) In inflammatory states human keratinocytes also produce IL-1β.(64) The release of IL-1 cytokines leads to cutaneous inflammation through the induction of secondary cytokines and the upregulation of endothelial adhesion molecules.(62, 65) The multiple proteases necessary for epidermal homeostasis and cleavage of IL-1 cytokines have optimal activity at pH values that are more alkaline than the SC surface. (66)

Murine studies have indicated the importance of IL-1β and IL-18 for the development of AD. Skin–specific caspase-1-transgenic mice, which over-express human CASP1 in their keratinocytes, when maintained under pathogen free conditions, spontaneously developed chronic dermatitis, accompanied by abnormally elevated skin and serum IL-18 and IL-1β levels.(67) An IL-18 transgenic mouse that exhibited over-secretion of IL-18 from epidermal cells developed AD-like skin eruptions. This phenotype was rescued by knockout of IL-18. (68) Furthermore, an AD mouse model, generated through the daily application of protein A (*Staphlococcus aureus* surface model and virulence factor), had complete

amelioration of the AD-like skin eruptions by either administration of a neutralizing anti-IL-18 antibody or IL-18 gene knockout.(69)

Stratum corneum IL-1α, IL-1β, IL-18 and IL-1RA levels were recently shown to be increased in the uninvolved skin of patients with moderate to severe AD_{FLG} compared with $AD_{NON\text{-}FLG}$. (61) IL-1 cytokine levels were correlated inversely with SC NMF levels. An association was demonstrated between increased pH and decreased NMF levels. Although AD severity influences SC NMF, this was shown to be a minor effect compared with *FLG* status, which was the major determinant of NMF.(61) [Figure 1b] These findings were also observed in a complementary murine study. Filaggrin deficient mice (*ft/ft*) had upregulated expression of IL-1β and IL-1RA in the SC.(61) Thus, it is possible that a reduction in filaggrin and its acidic breakdown products increases pH and serine protease activity contributing to the generation of the active cytokines IL-1α and IL-1β from their inactive pro-proteins, representing the first stage of the cytokine cascade that contributes to AD inflammation.(61) These cytokines have a further inhibitory effect on *FLG* expression. This work suggests that there may be a pre-existing, or enhanced, pro-inflammatory status in the skin of patients with AD_{FLG}. (61) [Figure 1b]

b) The inflammasome

The work on IL-1 in the setting of AD_{FLG} is in consistent with prior studies on the inflammasome in atopy. The innate immune system senses invading pathogens via evolutionary conserved pathogen recognition receptors such as Toll-like receptors (TLRs) and nuleotide-binding oligomerization domain-like receptors (NLRs). NLR members form an intracellular multiprotein complex, the inflammasome.(70) Inflammasomes enable autocataclytic activation of inflammatory caspases, which drive the host immune response by releasing cytokines and alarmins into the

circulation, and by inducing pyroptosis, a proinflammatory cell death mode.(71) The inflammasome activates caspase-1 and ultimately leads to the processing and release of the proinflammatory cytokines IL-1β, IL-18, and IL-33.(70) There is strong evidence that inflammasomes play an important role in skin inflammation.(72)

Research has focused on the nucleotide-binding oligomerisation leucine-rich repeat and pyrin domain containing 3 (NLRP3) inflammasome, which is made up of NLPR3, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and caspase-1.(70) An association has been reported between the NLRP3 inflammasome and susceptibility to food-induced anaphylaxis and aspirin induced asthma.(73) Hemolysins and bacterial lipoproteins from *Staph aureus* can activate the NLRP3 inflammasome. (74, 75) It has been recently shown that house dust mite allergens trigger assembly of the NLRP3 inflammasome, activate caspase-1, and thus stimulates the processing and release of IL-1β and IL-18 from keratinocytes *in vitro*.(76) The release of these cytokines may trigger or exacerbate AD-associated inflammation and be important in the pathogenesis of the disease.(76)

c) TSLP

Thymic stromal lymphoprotein (TSLP) is an IL-7-like cytokine that has a key role in Th2 cell differentiation and in the pathogenesis of allergic inflammation.(77) TSLP is highly expressed in the epidermis from AD subjects and TSLP-activated dendritic cells produced Th2 attracting chemokines, and primed naïve T cells to differentiate into Th2 cells.(78) Increased serum TSLP is an indicator of an epidermal barrier defect in mouse models.(79) Proteases, through proteinase-activated receptor-2 (PAR-2), can induce TSLP expression from keratinocytes or airway epithelial cells.(80, 81) A number of allergens that are clinically relevant in AD, including house dust mite, cockroach, fungi and several pollens, contain proteases that can trigger

epithelial production of TSLP *in vitro*.(82) Epithelial TSLP is also induced through a TLR2-, TLR3- and TLR5-mediated mechanism in response to microbial products.(38) TSLP expression in a reconstituted human epidermal layer was increased under filaggrin knockdown conditions.(83) This work suggests that filaggrin deficiency induces TSLP expression and a resultant Th2 immune reaction. (83) TSLP may be more important in the elicitation phase, rather than the sensitization phase, of AD. (38, 84)

d) Interleukin 33

IL-33 is a novel member of the IL-1 family and is expressed by the cells of barrier tissues. It is recognized as an 'alarmin' or DAMP molecule as it is released during epithelial cell death, is associated with infection or tissue injury, and is induced by microbial ligands through a TLR-mediated pathway.(85) IL-33 activates naive and Th2 lymphocytes, mast cells, and eosinophils to produce Th2 type cytokines. Furthermore, mast cells produce IL-33 in response to IgE dependant activation, and IL-33 amplifies the inflammation resulting from mast cell and basophil activation.(86, 87)

IL-33 is markedly elevated in the serum of patients with asthma and in the skin of patients with AD. (88) Increased levels of IL-33 and its specific receptor, ST2, have been demonstrated in AD skin following allergen or Staphylococcal enterotoxin B (SEB) exposure, as well as in the skin of filaggrin deficient mice.(89) The expression of IL-33 and ST2 were spontaneously upregulated in the skin of 22 week old *ft/ft* mice, and a 10-fold and a 15-fold increased of ST2 and IL33 expression were found, respectively, in 38-week-old *ft/ft* mice compared with 4-week-old *ft/ft* mice. The expression of IL-33 caused by irritant, allergen, or SEB challenge was suppressed in flaky tail (*ft/ft*) mouse skin by topical tacrolimus treatment.(89) This work suggests

435 that keratinocytes of *ft/ft* mice respond to environmental factors and start to produce
436 cytokines related to innate immunity.(89)

437 e) Interleukin 25

438 IL-25 (or IL-17E) is a member of the IL-17 cytokine family that, when over
439 expressed in murine models, results in the production of Th2 cytokines, eosinophilia,
440 and elevated serum IgE. (90) IL-25 is expressed by mouse epithelial cells following
441 allergen stimulation (91) and in the human skin of AD patients.(92) It has been
442 shown that IL-25 inhibits filaggrin synthesis by keratinocytes and therefore IL-25
443 may contribute to barrier dysfunction in AD subjects.(93)

444 f) Interleukin 22

445 IL-22 is another cytokine that could play a critical role in AD. Th22 cells are a skin-
446 homing phenotype and are a potent source of IL-22.(94) Th22 cells, Tc22 and Th17
447 cells, which secrete IL-22, are increased in the peripheral blood of patients with AD
448 and are observed in inflamed skin.(95-98) In addition, cutaneous dendritic cells have
449 been shown to induce a Th22 phenotype in T cells.(99) IL-22 secretion by peripheral
450 blood mononucleocytes (PBMCs) and CD4+ T cells is enhanced by *Staphlococcus*
451 *aureus* exotoxins in AD patients.(100) Exposure to IL-22 cytokine downregulated
452 profillagrin/filaggrin expression in keratinocytes *in vitro* at both mRNA and protein
453 level. An alteration in expression of genes encoding enzymes involved in
454 profilaggrin/filaggrin processing was also observed, suggesting that IL-22 could also
455 affect pathways generating functional filaggrin monomers.(101)

456 G) Interleukin 17

457 IL-17 is another important cytokine in AD. Serum and skin levels of IL-17 are
458 increased in patients with AD compared with healthy controls, and Th17 cells were

found to accumulate at early stages of skin inflammation in AD. (98, 102) Furthermore, IL-17 is chronically present during skin inflammation, especially when exposed to *Staph aureus* or allergens.(102, 103) The filaggrin-deficient flaky tail mouse exhibits Th17-dominated skin inflammation from an early age, even before increased IL- 4 expression.(54)

Recent work has shown that stimulation of keratinocyte cultures with IL-17A results in a significant decrease in profilaggrin mRNA levels and filaggrin protein expression. (104) [figure 2] Several genes encoding proteins were affected by IL-17 suggesting that IL-17A downregulates filaggrin expression at mRNA level both directly and indirectly, by affecting profilaggrin mRNA expression, production of functional filaggrin monomers, and their degredation.(104) IL-17A appears to not only influence filaggrin expression, but also affects the expression of other important components of the epidermal barrier.(104)

The evidence is mounting that filaggrin deficiency plays an important role in the cytokine profile of patients with AD. Cytokines have a further inhibitory effect on filaggrin expression, and thus a positive feedback loop probably exists in this setting. The cytokines involved in the pathogenesis of AD, similar to other inflammatory skin diseases, are multiple and complex and much work will be needed to clarify these pathways. As yet, there is no effective and specific 'biologic' treatment for AD. A greater understanding of such functional mechanisms involved in the disease are needed in order to identify potential therapeutic targets.

Filaggrin deficiency and the microbiome

The skin microbiome, which consists of both commensal and pathogenic bacteria, affects the skin barrier and epithelial innate immune responses. Skin microbes are thought to have a critical role in the development of AD. Atopic dermatitis patients

experience frequent bacterial and viral cutaneous infections. More than 90% of patients with atopic eczema are colonised with *Staphlococcus aureus* (*Staph aureus*), in comparison with 5% of normal subjects. (105) The severity of dermatitis correlates with both colony counts of *Staph aureus* colonized from AD skin, (106) and the presence of superantigen-producing *Staph aureus*.(107, 108)

To survive on skin, bacteria have to overcome acidic conditions, antimicrobial peptides and fatty acids.(109) *Staph aureus* colonization in AD is promoted by host and microbial mechanisms, including the dysfunctional skin barrier and bacterial surface associated proteins that can bind to host adhesive molecules. (109) *Staph aureus* surface associated proteins and virulence factors also contribute to inflammation. (109) Furthermore, high levels of Th2 cytokines inhibit cutaneous antimicrobial peptides (AMPs), further promoting bacterial proliferation.(110)The surface protein Staph protein A (SpA) stimulates cytokine release and subsequent inflammation on airway epithelial cells.(111) In combination with subclinical levels of detergent, SpA has been demonstrated to induce skin inflammation in animal models.(69)

Miajlovic *et al* investigated *Staph aureus* in the presence of UCA and PCA. These filaggrin breakdown products, at physiological concentrations, demonstrated an inhibitory effect on the growth of *Staph aureus*.(109) The increase in SC pH in AD, therefore, may lead to enhanced *Staph aureus* adhesion and muliplication.(109) Furthermore, there was a decreased expression of iron-regulated surface determinant A (IsdA) in the presence of these filaggrin breakdown products that was independant of pH. UCA and PCA appear, therefore, to have a specific antistaphlococcal effect by directly inhibiting this surface protein. IsdA promotes bacterial adhesion to squames and plays a role in *Staph aureus* survivial on the skin.(109) Thus, a reduction in filaggrin breakdown products in AD, either from *FLG*-

null alleles or from Th2 inflammation, may increase expression of *Staphlococcal* IsdA and promote survival of *Staphlococcal aureus*. (109) [figure 4] Therapies that reduced the SC pH could positvely impact on disease severity by minimizing *Staph aureus* colonisation and improving epidermal function.(109) Application of low-pH creams and acidic electrolytic water on epithelial surfaces have been shown to reduce *Staph aureus* colonisation severity of AD. (112, 113)

Approximately 50% of isolated *Staph aureus* isolates from AD patients produce superantigens, including enterotoxin B (SEB). (114) The ability for superantigens to cause stimulation of T cells and macrophages, Langerhans cells, and activated keratinocytes accounts for the majority of their pathological effect.(115) Superantigen production by *Staph aureus* strains is positively correlated with T-cell activation and increased severity of disease in AD.(116) In addition, staphylococcal superantigens induce the production of superantigen-specific IgE in AD patients.(117) Sensitization to superantigen-specific IgE has been correlated with AD severity. (118)

Superantigen enterotoxin B (SEB) is shown to enhance house dust mite induced patch test reactions in patients with AD.(119) Topical SEB superantigen exposure in the skin induces a mixed Th1/Th2 type dermatitis and production of IgE antibodies in a murine model of AD in wild type mice. (120) Epicutaneous exposure of superantigen SEB in mice stimulated a systemic Th17/IL-17 immune environment and enhanced epicutaneous-Ova induced systemic Th2 immune responses.(121) These changes lead to an eosinophil rich and neutrophil predominant lung inflammation and airway hyperresponsiveness. This effect was significantly diminished in when the IL17A gene was knocked out.(121) These data suggest that SEB plays an important role in Ova-induced lung inflammation and airway hyperresponsiveness via an IL-17A-dependent pathway.(121) Superantigen SEB

secreting *Staph aureus*, therefore, could be important for the development of asthma in patients with AD whose skin is often colonized with bacteria.

Recent work has demonstrated that filaggrin expression, as a result of keratinocyte differentation, significantly inhibits *Staph aureus* alpha toxin mediated pathogenicity. Futhermore, alpha toxin was particularly lethal to filaggrin deficient epidermal cells in *ft/ft* mice. Filaggrin's protective effect against alpha toxin was via mediation of sphingomyelinase secretion, an enzyme that reduces the number of alpha toxin binding sites on the cell surface. The impaired host defense against *Staph aureus* alpha toxin, resulting in enhanced cytotoxicty of alpha toxin, potentially further exacerbates the compromised barrier in AD_{FLG}.(122)

These studies suggest that *Staph aureus* plays a key role in AD and asthma pathogenesis, and filaggrin deficient SC may be particularly susceptible to *Staph aureus*. Whether targeting bacterial colonization early in the disease course of AD could halt the development of asthma in patients with AD remains to be investigated.

Conclusion

The pathomechanisms of AD are complex and include both structural abnormalities and immunological dysregulation. With the discovery of the role of *FLG* mutations and copy number variation, the epithelium is now recognized as a critical factor in the development of AD and subsequent allergic sensitization. This has directed the development skin barrier focused therapies. Genetic and environmental influences on filaggrin expression as well as the dynamic, bidirectional crosstalk between the skin barrier and immune system should be further understood with time. New insights into the complex pathophysiology of this disease should allow more targeted

560 treatments and a more individualized approach to treatment as well as a
561 preventative approach in at-risk individuals.
562

Figure legends

Figure 1: The role of filaggrin in the skin and the structural and biophysical consequences of filaggrin deficiency

a) The stratum corneum (SC) is produced by a highly organized differentiation process in which keratinocytes in the basal layer of the epidermis move to the spinous and granular layers.(7)Profilaggrin is the major constituent of keratohyalin granules in the stratum granulosum and is expressed in terminally differentiating keratinocytes in the outmost layers of the human epidermis. Profilaggrin consists of multiple copies of filaggrin, flanked by an S100-type calcium-binding domain, A and B domains at the N-terminal, and a unique tail sequence at the C terminal. During terminal differentiation at the granular to cornified cell transition, profilaggrin is dephosphorylayed and cleaved by several proteases, including caspase-14, to functional filaggrin monomers. Filaggrin monomers aggregate and align keratin bundles, in vitro, in the cornified cell envelope and are thus postulated to contribute to the mechanical strength and integrity of the stratum corneum in vivo. Terminal epidermal differentiation is calcium dependant, and calcium may be involved in the control of profillagrin processing. Absence of the serine protease LEKTI, (encoded by SPINK 5), leads to premature processing of profilaggrin.

In the upper layers of the SC, filaggrin monomers are deiminated and degraded by proteases to release their component hygroscopic amino acids. Peptidylarginine deiminase (PAD) isoforms 1 and 3 are involved in the deimination process. The major metabolites are the organic acids trans-urocanic acid (trans-UCA) and pyrroliodone-5-carboxylic acid (PCA). Filaggrin breakdown products form 'natural moisturizing factor' (NMF) which contributes to epidermal hydration and barrier function, help maintain the pH gradient of the epidermis which is key for many functions of the SC, and possibly plays a role in UV protection.

b) The filaggrin deficient skin barrier has reduced pro-protein in F-type keratohyalin granules. The consequences of this are as yet unknown. Ultrastructually, *FLG* loss-of-function mutations are associated with disorganized keratin filaments, impaired lamellar body loading and abnormal architecture of the lamellar bilayer. There is also reduction in corneodesmosome density and tight junction expression. These factors

may contribute to the dysfunctional skin barrier and enhanced allergen exposure. *FLG* null mutations also result in decreased levels of NMF, reduced SC hydration and elevated transepidermal water loss (TEWL) and clinically dry skin. The acidic pH of the SC is key for many functions; it has an antimicrobial effect, is important for the functional activity of enzymes involved in ceramide metabolism, and modulates the activity of the serine protease cascade required for co-ordinated epidermal differentiation and cornified cell envelope formation. The reduction in filaggrin breakdown amino acids causes an elevation in SC pH. This more alkaline pH enhances protease activity and may contribute to the pro-inflammatory stratum corneum in AD, as well as facilitating adhesion and proliferation of *Staphlococci*.

Figure 2: Comparison of the clinical and biophysical features of AD_{FLG} and $AD_{NON\text{-}FLG}$

Patients with AD and FLG mutations (AD_{FLG}) have a particular AD endophenotype or profile of associated disease and biophysical features. AD_{FLG} patients have palmar hyperlinearity, which is also observed in ichthyosis vulgaris, the Mendelian disease cause by *FLG* mutations. AD_{FLG} patients have more severe, persistent eczema and a higher incidence of infections with herpes virusas well as a greater risk of allergic sensitizationand asthma than patients with AD without *FLG* mutations ($AD_{NON\text{-}FLG}$). The biophysical profile of AD_{FLG} shows an elevated SD pH and production of IL-1β in AD_{FLG} compared with $AD_{NON\text{-}FLG}$.

Figure 3: Known genetic and immunological influences on filaggrin expression.

a) The major determinant of filaggrin expression is *FLG* genotype, with three distinct, but overlapping, populations according to the number of *FLG* loss-of-function mutations. The observed inter-individual variation in NMF within the mutation groups approximates to a normal distribution curve, which reflects additional genetic and environmental modifiers of filaggrin expression.

b) There are common size-variant *FLG* alleles in the population with 10,11 or 12 repeats. Excluding null mutations, the number of filaggrin units in humans, termed filaggrin copy number variation (CNV), varies from 20 to 24. AD risk is related to filaggrin CNV. In keeping with this, the concentration of filaggrin breakdown products (NMF), quantified by HPLC of tape-stripped SC, is statistically significantly correlated to filaggrin copy number variation. Furthermore disease severity drives down filaggrin expression independent of *FLG* mutation status.

C) Filaggrin expression *in vitro* is downregulated in the presence of inflammatory cytokines. Keratinocyte cultures differentiated in the presence of IL-4 and IL-13 exhibit significantly reduced filaggrin gene expression. Exposure to IL-22 cytokine downregulates profillagrin/filaggrin expression in keratinocytes at both mRNA and protein level. Keratinocytes cultured with IL-17A also resulted in a significant decrease in profilaggrin mRNA levels and filaggrin protein expression. IL-17A appears to downregulate filaggrin expression at mRNA level both directly and indirectly.

Patients with AD_{FLG} and AD_{nonFLG} have an acquired defect in filaggrin secondary to the presence of inflammatory cytokines. In the setting of AD_{FLG}, the combination of genetically determined and acquired filaggrin insufficiency may lead to a greater and more prolonged filaggrin downregulation.

Figure 3: Filaggrin deficiency and susceptibility to *Staphlococcus Aureus*

a) *Staph aureus* has a variety of bacterial surface associated proteins that can bind to host adhesive molecules and promote colonization in the dysfunctional skin barrier of AD. These surface associated proteins also contribute to inflammation.

b) Acidification of growth media using physiological concentration of the filaggrin breakdown products UCA and PCA found in healthy skin in

individuals wild type for *FLG*, resulted in reduced expression of secreted and cell wall-associated proteins, including proteins involved in colonization (clumping factor B, fibronectin binding protein A) and immune evasion (protein A).

c) Correction of pH, after the addition of physiological concentrations of filaggrin breakdown products, resulted in restoration of all the surface proteins expression, except for IsdA whose expression was not restored. IsdA promotes adhesion to squamous cells and enhances survival on human skin. The expression of IsdA appears to be directly affected by the presence of UCA and PCA, independent of pH. These *in vitro* studies suggest pathomechanisms, other than pH, through which reduced filaggrin expression may result in enhanced susceptibility to *Staph aureus* colonization.

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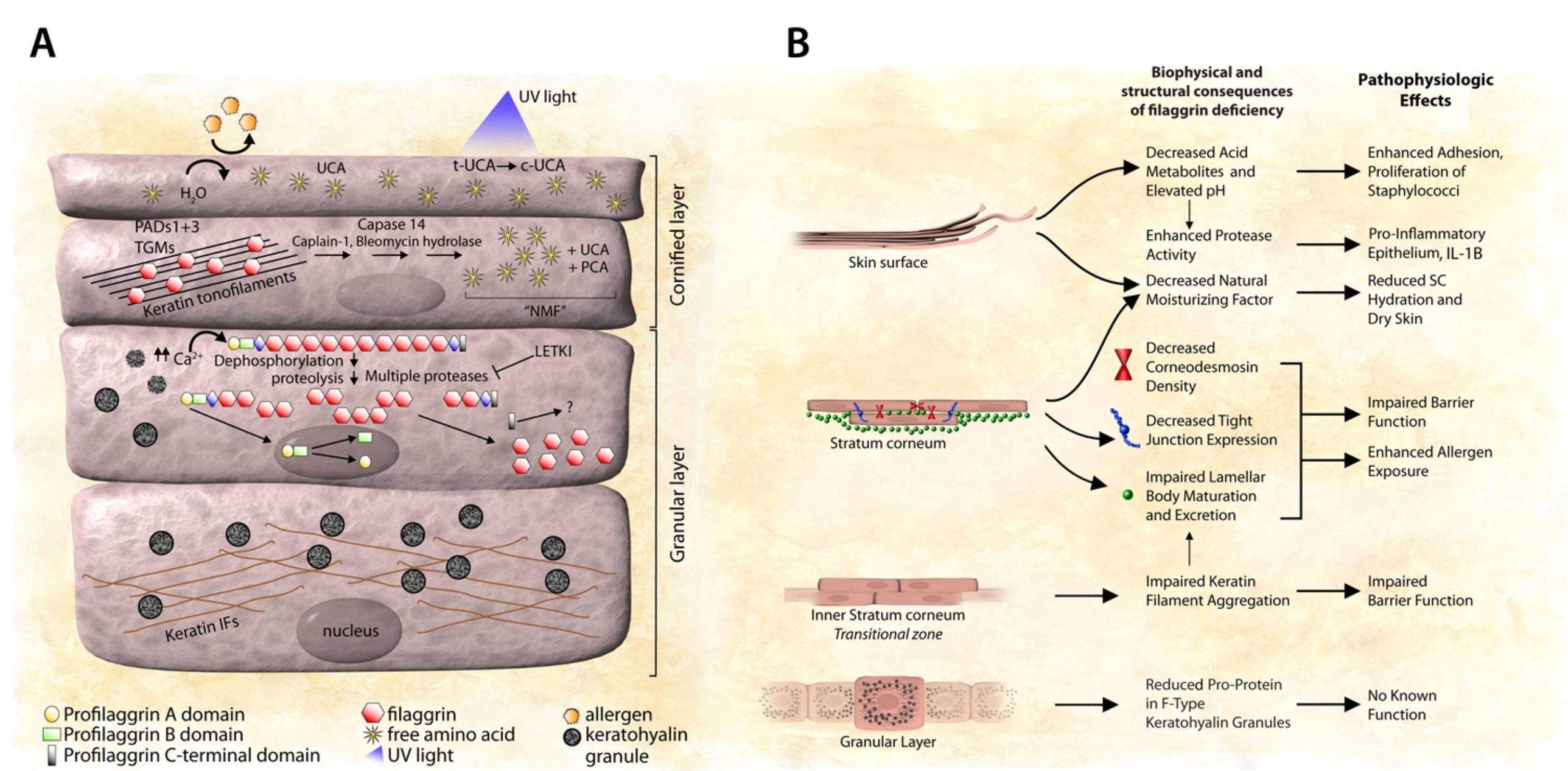
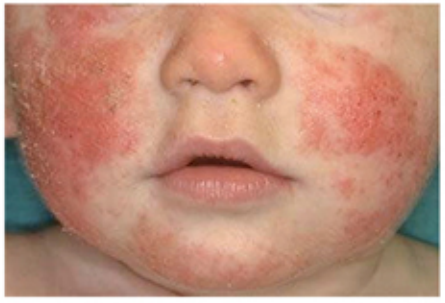
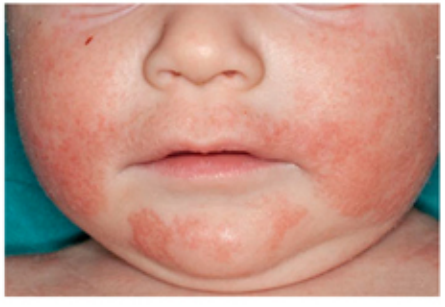

A
UV light
UCA
t-UCA→ c-UCA
H_2O
PADs1+3
TGMs
Capase 14
Caplain-1, Bleomycin hydrolase
+ UCA
+ PCA
Keratin tonofilaments
"NMF"
Cornified layer
Ca^{2+}
Dephosphorylation
proteolysis
Multiple proteases
LETKI
?
Granular layer
Keratin IFs
nucleus
Profilaggrin A domain
Profilaggrin B domain
Profilaggrin C-terminal domain
filaggrin
free amino acid
UV light
allergen
keratohyalin granule
B
Biophysical and structural consequences of filaggrin deficiency
Pathophysiologic Effects
Skin surface
Decreased Acid Metabolites and Elevated pH
Enhanced Adhesion, Proliferation of Staphylococci
Enhanced Protease Activity
Pro-Inflammatory Epithelium, IL-1B
Decreased Natural Moisturizing Factor
Reduced SC Hydration and Dry Skin
Stratum corneum
Decreased Corneodesmosin Density
Decreased Tight Junction Expression
Impaired Barrier Function
Enhanced Allergen Exposure
Impaired Lamellar Body Maturation and Excretion
Inner Stratum corneum
Transitional zone
Impaired Keratin Filament Aggregation
Impaired Barrier Function
Granular Layer
Reduced Pro-Protein in F-Type Keratohyalin Granules
No Known Function

AD_{FLG}

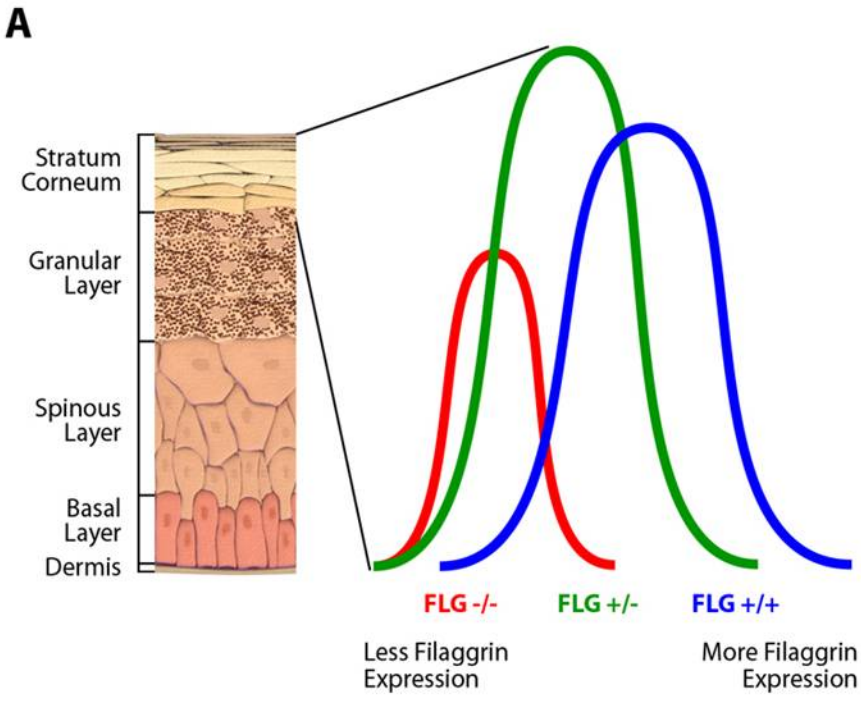


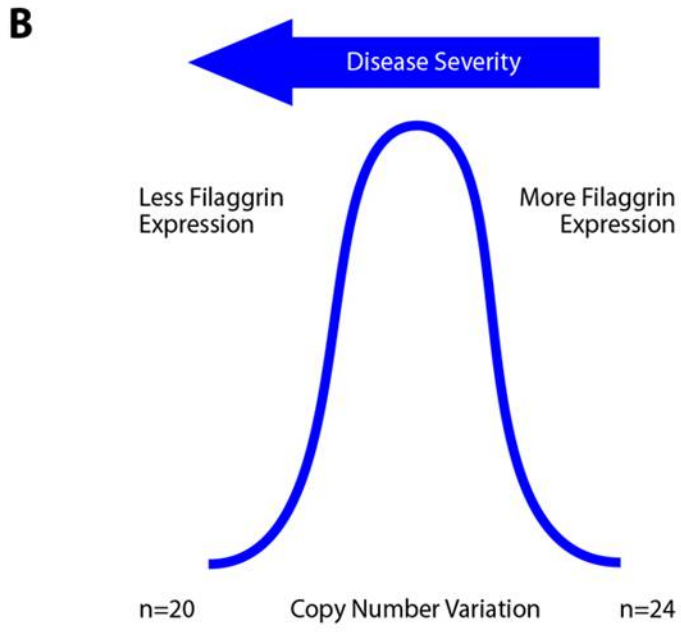
Clinical Features	Biophysical Features
Palmar Hyperlinearity	**Severe Decrease in Natural Moisturizing Factor (NMF)**
More Persistent	**↑pH**
↑Allergic Sensitization	**↑IL-1β**
↑Risk of Asthma	
↑Severity	
↑Eczema Herpeticum	

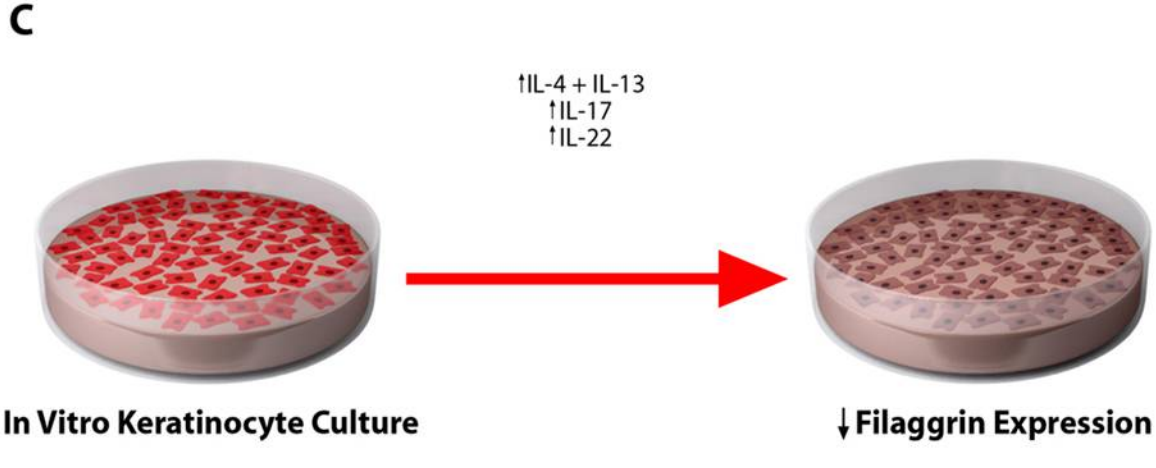
$AD_{NON\text{-}FLG}$



Clinical Features	Biophysical Features
No Palmar Hyperlinearity	Mild Decrease in Natural Moisturizing Factor (NMF)
Less Persistent	pH Lower Compared to AD_{FLG}
Less Allergic Sensitization	IL-1β Low Compared to AD_{FLG}
Lower Risk of Asthma	

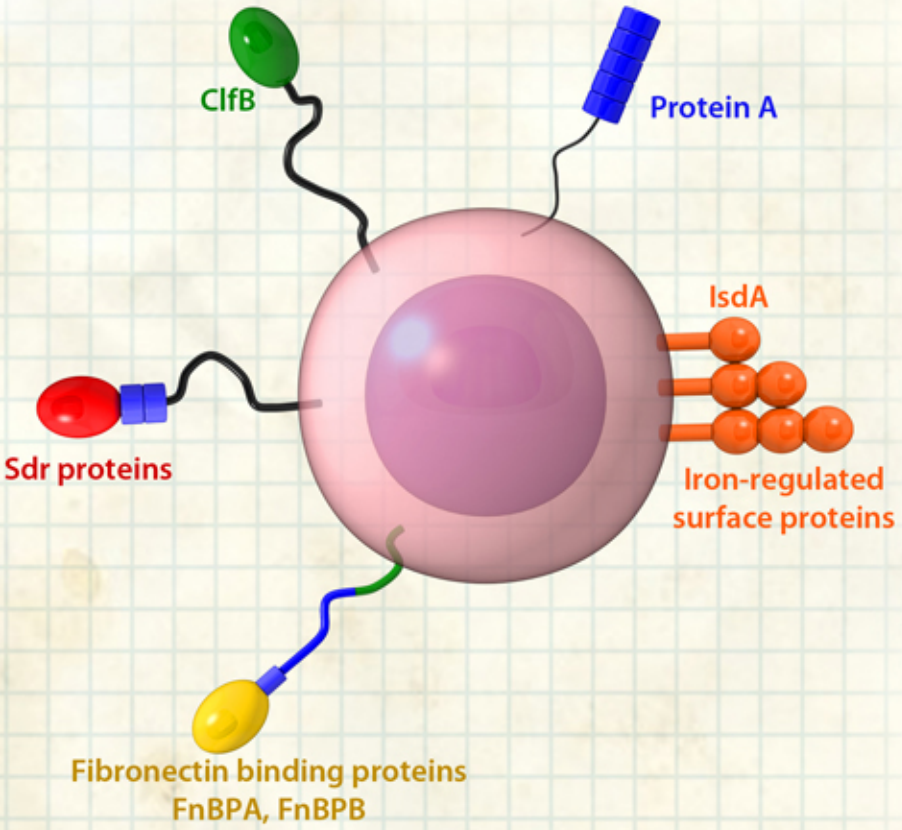

A
Stratum Corneum
Granular Layer
Spinous Layer
Basal Layer
Dermis
FLG -/-
FLG +/-
FLG +/+
Less Filaggrin Expression
More Filaggrin Expression


B
Disease Severity
Less Filaggrin Expression
More Filaggrin Expression
n=20
Copy Number Variation
n=24


C
↑IL-4 + IL-13
↑IL-17
↑IL-22
In Vitro Keratinocyte Culture
↓Filaggrin Expression

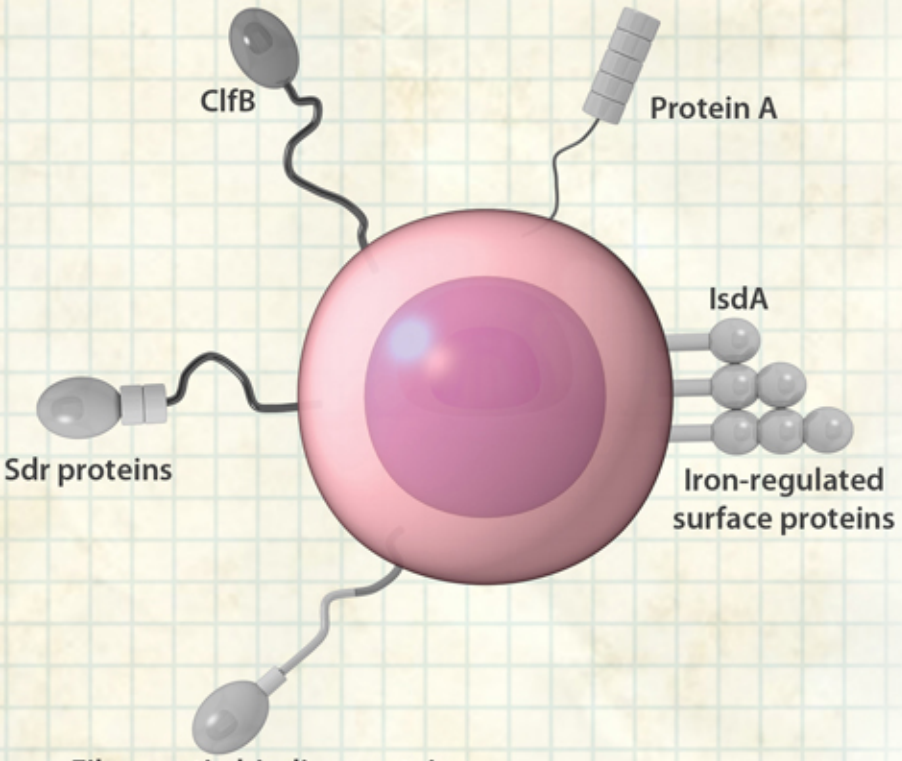
A

Neutral pH
No added filaggrin breakdown products



B

Physiologic concentrations of filaggrin breakdown products PCA and UCA
Not pH corrected



C

Physiologic concentrations of filaggrin breakdown products PCA and UCA
pH corrected