

Filaggrin loss-of-function mutations are associated with enhanced expression of IL-1 cytokines in the stratum corneum of patients with atopic dermatitis and in a murine model of filaggrin deficiency

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Background: Filaggrin (*FLG*) mutations result in reduced stratum corneum (SC) natural moisturizing factor (NMF) components and consequent increased SC pH. Because higher pH activates SC protease activity, we hypothesized an enhanced release of proinflammatory IL-1 cytokines from corneocytes in patients with atopic dermatitis (AD) with *FLG* mutations (*AD_{FLG}*) compared with that seen in patients with AD without these mutations (*AD_{NON-FLG}*).

Objectives: We sought to investigate SC IL-1 cytokine profiles in the uninvolved skin of controls and patients with *AD_{FLG}* versus patients with *AD_{NON-FLG}*. We also sought to examine the same profiles in a murine model of filaggrin deficiency (*Flg^{fl}/Flg^{fl} [Flg^{delAPfal}]* mice).

Methods: One hundred thirty-seven patients were studied. NMF levels were ascertained using confocal Raman spectroscopy; transepidermal water loss and skin surface pH were measured. IL-1 α , IL-1 β , IL-18, IL-1 receptor antagonist (IL-1RA), and IL-8 levels were determined in SC tape strips from 93 patients. All subjects were screened for 9 *FLG* mutations. *Flg^{fl}/Flg^{fl} (Flg^{delAPfal})* mice, separated from *maFlg^{fl}/maFlg^{fl}* (flaky tail) mice, were used for the preparation and culture of primary murine keratinocytes and as a source of murine skin. RT-PCR was performed using primers specific for murine IL-1 α , IL-1 β , and IL-1RA.

Results: SC IL-1 levels were increased in patients with *AD_{FLG}*; these levels were inversely correlated with NMF levels. NMF values were also inversely correlated with skin surface pH. Skin and keratinocytes from *Flg^{fl}/Flg^{fl}* mice had upregulated expression of IL-1 β and IL-1RA mRNA.

Conclusions: *AD_{FLG}* is associated with an increased SC IL-1 cytokine profile; this profile is also seen in a murine homologue of filaggrin deficiency. These findings might have importance in understanding the influence of *FLG* mutations on the inflammasome in the pathogenesis of AD and help individualize therapeutic approaches. (*J Allergy Clin Immunol* 2012;129:1031-9.)

Key words: Atopic dermatitis, confocal Raman spectroscopy, eczema, filaggrin, natural moisturizing factor, pH, transepidermal water loss, IL-1, IL-18, skin barrier, *FLG* gene mutations, *Flg^{fl}/Flg^{fl} (Flg^{delAPfal})* mice

The recent identification of mutations in the gene encoding the key epidermal protein filaggrin (*FLG*) as a remarkably strong and widely replicated risk factor for atopic dermatitis (AD) has led to a new focus on skin barrier deficiency in patients with AD. However, the functional consequences of *FLG* mutations and the downstream mechanisms that underlie immunologic changes in AD skin remain largely unknown.^{1,2}

Before the discovery of *FLG* mutations, Elias and Feingold³ hypothesized a barrier abnormality as a driving force for development of an inflammatory response in patients with AD. This so-called outside-inside hypothesis is in contrast to a more traditional view known as the inside-outside hypothesis, which holds that skin barrier defects in patients with AD are a secondary

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Abbreviations used

AD:	Atopic dermatitis
AD _{FLG} :	AD with <i>FLG</i> mutations
AD _{NON-FLG} :	AD without <i>FLG</i> mutations
<i>FLG</i> :	Filaggrin gene
<i>FLG</i> ^{-/-} :	Patient homozygous for null alleles (ie, 2 null alleles)
<i>FLG</i> ^{+/-} :	Heterozygote null allele/wild-type (ie, 1 null allele)
<i>FLG</i> ^{+/+} :	Homozygote wild-type (ie, 0 null alleles)
IL-1RA:	IL-1 receptor antagonist
NESS:	Nottingham Eczema Severity Score
NMF:	Natural moisturizing factor
SC:	Stratum corneum
TEWL:	Transepidermal water loss

consequence of the inflammatory response to irritants and allergens.⁴ Elias et al⁵⁻⁷ subsequently hypothesized that reduced levels of filaggrin and in particular its acidic derivative urocanic acid lead to increased pH of the stratum corneum (SC), altering the activity of the multiple serine proteases and 2 ceramide-generating enzymes that regulate homeostasis of the SC.⁷ Another important downstream consequence of increased pH and serine protease activity is generation of the active primary cytokines IL-1 α and IL-1 β from their inactive proproteins, representing the first step in the cytokine cascade that has been proposed as a primary contributor to inflammation in patients with AD. Sustained antigen ingress through a defective barrier leading to a T_H2-dominant infiltrate is proposed as a secondary cause of inflammation in patients with AD.

Mediators from the IL-1 gene family control innate immune responses through a number of mechanisms, including promoting the recruitment of leukocytes and regulating synthesis of the extracellular lipid bilayers as the principal barrier of the skin.^{5,8,9} IL-1 mediators also bridge the innate and adaptive immune systems and thus constitute an important function in immune defense.^{10,11} There are 11 members of the IL-1 family of which IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1RA), and IL-18 have been most thoroughly studied. IL-1 α and IL-1 β initiate responses by binding to the IL-1 receptor, which is antagonized by IL-1RA. These elements of the IL-1 system are represented in the epidermis. Keratinocytes constitutively produce high amounts of IL-1 α , and the epidermis contains important quantities of biologically active preformed IL-1 α .^{12,13} In addition, in inflammatory conditions human keratinocytes also produce IL-1 β ¹⁴; however, blood monocytes, tissue macrophages, and dendritic cells are considered the primary sources of IL-1 β .¹⁰ IL-1 α is produced in the cytoplasm as a precursor protein (pro-IL-1 α) and processed into a mature protein by the intracellular calcium-dependent cysteine protease calpain, which has an optimum activity at neutral pH.^{12,15} The precursor of IL-1 β is biologically inactive and must be cleaved into its biologically active form, a process that is largely mediated by caspase-1, although some serine proteases and several other enzymes have been reported to cleave both IL-1 α and IL-1 β .^{10,12,16,17} Caspase-1 is a cysteine protease that also cleaves precursors of IL-18, a cytokine that plays an important role in the pathogenesis of AD.^{18,19} The pH optimum for caspases ranges between 6.5 and 6.8.²⁰ Thus multiple proteases crucial for SC homeostasis and cleavage of IL-1 cytokines have optimal activity at pH values higher than the physiologic outer SC/skin surface layer pH.⁵⁻⁷ Release of IL-1 cytokines leads to

cutaneous inflammation through the induction of secondary cytokines, such as IL-8, and upregulation of endothelial adhesion molecules.^{10,12} Production of IL-1 β has also been linked to the sensitization and initiation phase of contact allergy.^{21,22}

Although little is known about the very early events initiating atopic skin inflammation, it is likely that primary proinflammatory cytokines play an important role. Although several research groups have investigated IL-1 cytokines in the lesional and noninvolved skin of patients with AD,²²⁻²⁴ thus far, no study has focused on cytokine levels in patients with AD in relation to *FLG* genotype. Using Raman spectroscopy, we have recently shown that *FLG* genotype is a major determinant of natural moisturizing factor (NMF) in the SC.^{25,26} In the present study we sought to determine the levels of IL-1 cytokines in the SC of uninvolved skin and to relate these levels to *FLG* genotype, pH, and levels of filaggrin degradation products, which are the constituents of NMF. Furthermore, in a complementary murine study we examined the effects of filaggrin status on IL-1 expression in the skin and isolated keratinocytes.

METHODS

Clinical study: Subjects

One hundred thirty-seven unrelated Irish children with a history of moderate-to-severe AD were recruited from dedicated secondary and tertiary referral AD clinics; the collection used in this study overlaps but is not identical to that used for previous studies.²⁶ Diagnosis and phenotyping of AD was made by experienced pediatric dermatologists. All subjects met the United Kingdom diagnostic criteria.²⁷ Exclusion criteria from the study were patients who had received systemic therapy, such as oral corticosteroids or immunosuppressants, in the preceding 3 months and patients whose ancestry was not exclusively Irish (4/4 grandparents). Detailed phenotypic data were collected and are presented in Table I. The Nottingham Eczema Severity Score (NESS)²⁸ was selected as a measure of chronic disease severity. The study was conducted in accordance with Helsinki Declarations and was approved by the Research Ethics Committee of Our Lady's Children's Hospital, Dublin, Ireland. Full written consent was obtained from all patients or their parents.

Genotyping

All patients were screened for the 9 most common *FLG* mutations found in the Irish population (R501X, Y2092X, 2282del4, R2447X, S3247X, R3419X, 3702X, S1040X, and G1139X) and as previously described.²⁶ These 9 mutations account for greater than 95% of all *FLG* mutations in the Irish population (Irvine and McLean, unpublished data). On the basis of screening for these 9 prevalent mutations, 59% were carriers of 1 or more *FLG* mutations (42% *FLG*^{+/+} [homozygote wild-type; ie, 0 null alleles], 40% *FLG*^{+/-} [heterozygote null allele/wild-type; ie, 1 null allele], and 18% *FLG*^{-/-} [patient homozygous for null alleles; ie, 2 null alleles]).

Determination of cytokines in the SC

The levels of cytokines in the tape strips were measured in the SC of the volar forearm by using a previously described method.²⁹ Briefly, round adhesive tape discs (3.8 cm², D-Squame; CuDerm, Dallas, Tex) were attached to the skin of the forearm. Each tape was pressed onto the volar aspect of the forearm for 10 seconds with standardized force by using a disc pressure applicator (CuDerm).³⁰ The tape strip was gently removed with tweezers and stored in a closed vial at -80°C until analysis. The first strip was discarded because it might have contained dirt and remnants of cosmetic products; the second, third, and fourth tape strips were applied on the same skin spot. The collected 3 tape strips were cut into 2 equal pieces. For the analysis, halves of 3 strips were pooled for the analysis. To determine the amount of soluble protein and cytokines, 2 mL of PBS (Merck, Darmstadt, Germany) with 0.005% Tween-20 (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to

TABLE I. Cohort characteristics according to genotype

<i>FLG</i> genotype†	All mutations combined	Age (y), median (range)	Male sex, no. (%)	NESS, median (range)	TEWL (g/m ² /h), median (range)	Log IgE,* mean (SD)	NMF (AU), median (range)	pH, mean (SD)
Screened (n)	137	137	137	137	99	131	129	58
+/+	57 (41.6%)	8 (3-17)	34 (59.6%)	12 (4-15)	12.6 (5.2-47.4), n = 39	2.9 (1.0), n = 54	1.33 (0.73-1.65), n = 51	4.75 (0.51), n = 20
+/-	55 (40.1%)	7 (1-17)	32 (58.2%)	12 (4-15)	14.05 (5.3-33.0), n = 43	2.8 (0.8), n = 54	0.83 (0.40-1.29), n = 54	4.79 (0.37), n = 26
-/-	25 (18.2%)	8 (3-17)	15 (60.0%)	12 (6-15)	15.9 (8.5-36.1), n = 17	3.1 (0.7), n = 23	0.58 (0.18-0.76), n = 24	5.34 (0.41), n = 12
<i>P</i> value		.84‡	.97§	.36‡	.22‡	.45	<.0001	<.0006¶

AU, Arbitrary unit.

*IgE data were positively skewed. The mean of the log-transformed data is presented.

†+/+ denotes no *FLG* mutation, +/- denotes 1 *FLG* mutation, and -/- denotes 2 *FLG* mutations.

‡Comparison of means among the 3 groups of *FLG* mutations using the Kruskal-Wallis test.

§Comparison of proportions using the χ^2 test for comparison of a 2 × 3 × 3 contingency table.

||Comparison of means among the 3 groups of *FLG* mutations using ANOVA.

¶ANOVA.

each vial, and the vials were left on ice for 30 minutes. Extraction was performed with an ultrasound sonifier equipped with a probe (Salm & Kipp, Breukelen, The Netherlands) for 15 minutes in ice water. The extract was centrifuged (for 1 minute at 15,000g), and supernatant aliquots of 225 μ L were refrozen at -80°C until required for further analysis.

Concentrations of IL-1 α , IL-1 β , IL-1RA, and IL-8 in the SC strips were measured with a Luminex-based multiplex system (Bio-Plex Human Cytokine 27-plex panel and single plexes; Bio-Rad Laboratories, Hercules, Calif) on a Bioplex 100, according to the manufacturer's instructions. Samples were diluted 4-fold. The amount of cytokines was normalized for the protein content, which was determined by using the Micro BCA protein assay kit (Pierce, Rockford, Ill), with the BSA supplied as standard. For statistical analysis, cytokine concentrations of less than the limit of detection were given a value of half the limit of detection. The limit of detection was 0.2 pg/mL for IL-1 α , 0.01 pg/mL for IL-1 β , 1.4 pg/mL for IL-1RA, 0.3 pg/mL for IL-8, and 0.045 pg/mL for IL-18.

Biophysical analysis of the SC

Skin biophysical measurements were performed under standardized conditions (room temperature of 22°C-25°C and humidity levels of 30% to 35%). Before measurements, patients were acclimatized for a minimum of 10 minutes. All measurements were performed by one of 2 investigators (G. M. O'R. and P. M. J. H. K.). Topical therapies, including emollients, were withheld from the measurement sites for 48 hours preceding the study. Transepidermal water loss (TEWL) and pH were measured on nonlesional skin of the extensor forearm (Tewameter 300 and Skin-pH-Meter, PH905; Courage and Khazaka Electronic GmbH, Cologne, Germany).

NMF was measured in the SC of the thenar eminence by using confocal Raman microspectroscopy (model 3510 Skin Composition Analyzer; River Diagnostics, Rotterdam, The Netherlands). The principles of this method and the procedure have been described elsewhere.^{25,26}

In a further 17 adult control subjects and 25 white patients with AD, we determined the levels of IL-1 α by using a specific ELISA kit (Biosource International, Camarillo, Calif). Healthy volunteers had no visible skin abnormalities or history of past or present AD or other skin diseases. All of them were wild-type for the 4 most common *FLG* mutations in the Dutch population (R501X, 2282del4, R2447X, and S3247X). Patients with AD were given diagnoses according to the Hanifin and Rajka criteria^{31,32} and were divided into 2 subgroups according to the presence of *FLG* mutations. We excluded patients who had received systemic therapy, such as corticosteroids and immunosuppressants, or phototherapy in the past 3 months. The test sites, both midvolar arms, had been free of dermatitis for at least 3 months before the experiment. Written informed consent was obtained from all subjects before participation. The study was approved by the Ethics Committee of the Academic Medical Center, Amsterdam, and was conducted according to the principles of the Declaration of Helsinki. Nine of the patients with AD were wild-type for *FLG* mutations (AD_{NON-FLG}), and 12 were heterozygous and 4 were homozygous or compound heterozygous carriers of 4 investigated mutations (AD_{FLG}).

Murine study

Adult (12- to 14-week-old) C57BL/6J or congenic *Flg^{fl}/Flg^{fl}* (*Flg^{delAPfal}*) mice, separated from *maFlg^{fl}/maFlg^{fl}* (flaky tail) mice, were used for the preparation and culture of primary murine keratinocytes.³³ These mice are homozygous for murine *Flg* loss-of-function mutations and express no filaggrin protein.³³ Mice were housed in a pathogen-free facility in individually ventilated and filtered cages under negative pressure (Tecniplast, Northants, United Kingdom). All animal experiments were performed in compliance with Irish Department of Health and Children's regulations and approved by the Trinity College Dublin BioResources Ethical Review Committee.

Murine keratinocyte culture

Isolation of murine primary keratinocytes was performed as previously described.^{34,35} Briefly, the epidermis was separated from the dermis of tail skin after overnight incubation in 0.25% trypsin (Lonza, Basel, Switzerland). Keratinocytes were isolated from the SC with trituration, centrifuged at 150g, and filtered through a 100- μ m sieve (BD Biosciences, San Jose, Calif), followed by further centrifugation. The cells were resuspended in calcium-free KGM-2 media (BulletKit, Lonza) and adjusted to 0.05 mmol/L Ca²⁺, with 50 U/mL penicillin and 50 μ g/mL streptomycin (Gibco, Carlsbad, Calif). Cells were plated on fibronectin/collagen-coated dishes and incubated at 36°C at 7% CO₂. The keratinocytes were cultured for 4 days, at which point the attached keratinocytes were analyzed for expression of cytokine mRNA or induced to differentiate. Cells were cultured in KGM-2 BulletKit media adjusted to 0.5 mmol/L Ca²⁺ and harvested for analysis at 72 hours to induce keratinocytes to undergo terminal differentiation.

RNA isolation and real-time PCR

RNA was isolated from primary keratinocytes, prepared as above, or dorsal skin from mice by using the RNeasy kit (Qiagen, Crawley, United Kingdom) and reverse transcribed with the Quantitect reverse transcription kit incorporating a genomic DNA elimination step (Qiagen). Real-time quantitative PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Paisley, United Kingdom) using predesigned TaqMan gene expression assays specific for murine IL-1 α (Mm00439620_m1), IL-1 β (Mm01336189_m1), and IL-1RA (IL-1rn;Mm00446186_m1). Specific gene expression was normalized to murine glyceraldehyde-3-phosphate dehydrogenase. Fold expression was calculated by using the comparative cycle threshold method of analysis and is presented as relative quantification.³⁶ Data expressed as relative quantification were calculated and compared with glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene.

Statistical analysis

Patients were characterized *a priori* into 3 genotypes (*FLG*^{+/+}, *FLG*^{+/-}, and *FLG*^{-/-}), where *FLG*^{+/+} represents patients with 0 *FLG* mutations, *FLG*^{+/-} represents patients with 1 *FLG* mutation (heterozygotes), and

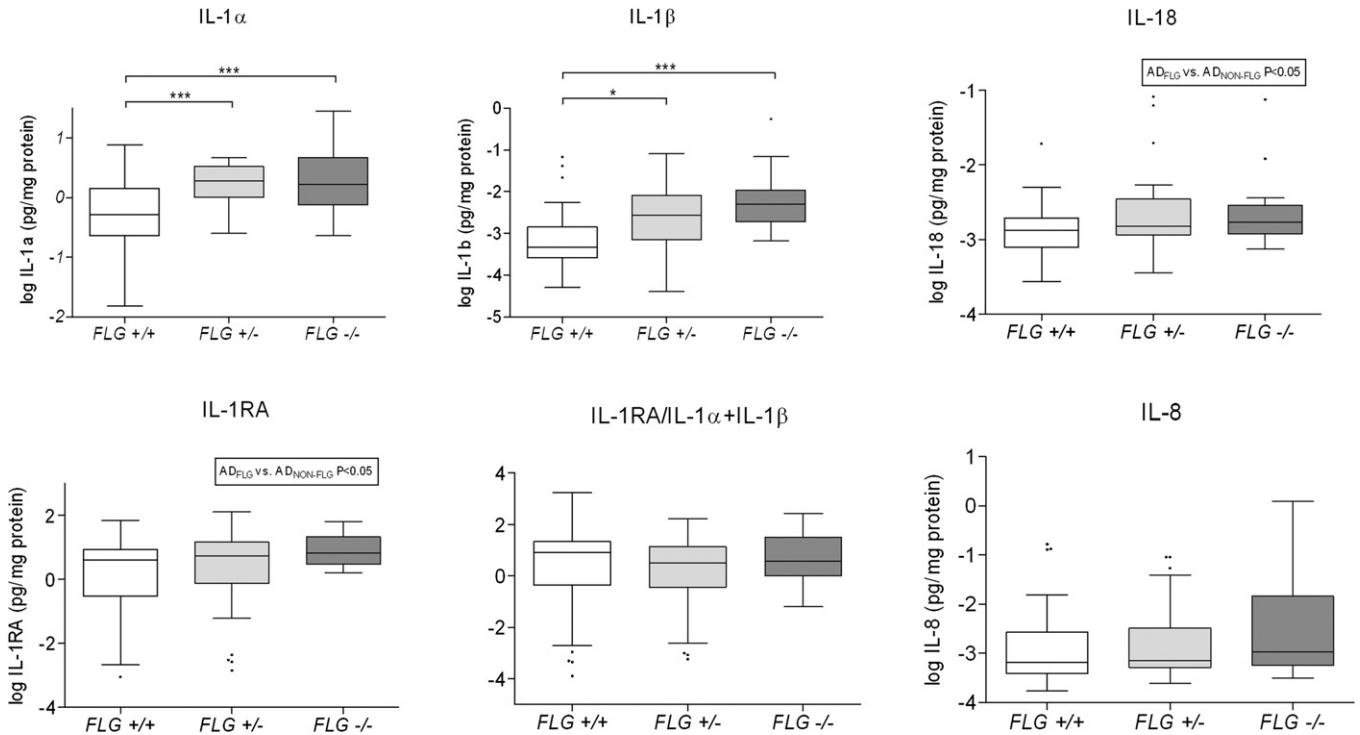


FIG 1. Box-and-whiskers plot cytokine levels in the SC by *FLG* genotype showing the median (midline) and interquartile ranges. * $P < .05$ and *** $P < .001$, as determined by using Kruskal-Wallis and Dunn multiple comparison tests. The differences in IL-18 and IL-1RA levels between the $AD_{NON-FLG}$ and AD_{FLG} groups have been tested by using the 2-sided Mann-Whitney test.

$FLG^{-/-}$ represents patients with 2 *FLG* mutations (homozygotes or compound heterozygotes). For testing of distribution, we used the Shapiro-Wilk test. Data on cytokine levels were log-transformed and presented as medians with interquartile ranges (25th-75th box length). For comparison of differences between the genotype subgroups of patients with AD, ANOVA followed by a *post hoc* Tukey analysis was applied. In case of deviation from normal distribution, we used the Kruskal-Wallis and Dunn multiple comparison tests. P values of less than .05 were considered statistically significant. For the correlation analysis, we used the Spearman rank correlation test in the case of deviation from normal distribution. The Student t test was used to test for statistical differences between wild-type and Flg^{fl}/Flg^{fl} mice in cytokine mRNA expression in isolated skin or keratinocytes. The Prism 5 (GraphPad Software, Inc, San Diego, Calif) and SPSS (17.0; SPSS, Inc, Chicago, Ill) software programs were used for statistical calculations.

RESULTS

Patients' characteristics

Clinical characteristics and summary data of the study cohort, including *FLG* genotype, are outlined in Table I. The 3 *FLG* genotype subgroups had similar disease severity, as assessed by using the NESS. TEWL values, which are one measure of skin barrier function, were not significantly different among the 3 AD subgroups ($P > .05$, Kruskal-Wallis test). Although there was an apparent increase in IgE levels in patients with AD with respect to the number of *FLG* mutations (Table I), there was no significant difference in total IgE levels between the *FLG* subgroups. *FLG* mutations were associated with reduced levels of NMF (Table I). The difference between patients with AD_{FLG} and patients with $AD_{NON-FLG}$ was highly ($P < .001$) significant, as was the difference between the $FLG^{+/+}$ and $FLG^{-/-}$ ($P < .001$) and $FLG^{+/+}$ and $FLG^{+/-}$ ($P < .05$, Kruskal-Wallis test followed by

Dunn Multiple comparison test) subgroups. The amounts of extracellular protein harvested by using tape strips did not significantly differ between patient groups with and without *FLG* mutations (data not shown). Because the study took some time to complete, not all data points were collected from all patients. Patient numbers collected for each analysis are shown in Table I.

Cytokine levels in relation to *FLG* allele status and NMF

Patients with AD_{FLG} had higher amounts of IL-1 α and IL-1 β compared with patients with $AD_{NON-FLG}$ (Fig 1). The differences in IL-1RA and IL-18 levels between the 3 subgroups did not reach significance, as assessed by using Dunn multiple comparison testing. However, a statistically significant difference for both IL-1RA and IL-18 levels was observed between carriers of *FLG* null mutations (AD_{FLG} and $AD_{NON-FLG}$; $P < .05$ and $P = .02$, respectively, Mann-Whitney 2-sided test). Levels of IL-8 and the IL-1RA/IL-1 α plus IL-1 β ratio were not significantly different between the *FLG* subgroups nor between the patients with AD_{FLG} and $AD_{NON-FLG}$.

Correlations between IL-1 cytokines, IL-8 levels, and clinical parameters are further shown in Table II. Levels of IL-18 were associated with those of IL-1 β ; however, neither IL-18 nor IL-1 β levels were correlated with IL-1 α levels. Interestingly, IL-1 β and IL-18 levels, but not IL-1 α levels, were positively correlated with IL-1RA and IL-8 levels (Table II). The IL-1RA/IL-1 α plus IL-1 β ratio was positively correlated with IL-18 and IL-8 levels; disease severity, as measured based on the NESS; TEWL; and IgE levels. We did not observe an association between pH and IL-1 cytokine levels (Table II). Correlations between NMF and IL-1

TABLE II. Correlations between IL-1 cytokine levels, IL-8 levels, and clinical parameters

	IL-1 α	IL-1 β	IL-18 (n = 87)	IL-8 (n = 91)	NESS (n = 92)	TEWL (n = 78)	IgE (n = 86)	pH (n = 40)
IL-1 α		NS	NS	NS	-0.30 (-0.48 to -0.09), <i>P</i> < .005	NS	NS	NS
IL-1 β	NS		0.41 (0.21 to 0.57), <i>P</i> < .005	0.31 (0.10 to 0.49), <i>P</i> < .005	NS	0.32 (0.10 to 0.51), <i>P</i> < .005	NS	NS
IL-18	NS	0.41 (0.21 to 0.57), <i>P</i> < .005		0.38 (0.18 to 0.55), <i>P</i> < .0005	NS	0.33 (0.11 to 0.52), <i>P</i> < .005	NS	NS
IL-1RA	NS	0.50 (0.33 to 0.65), <i>P</i> < .0001	0.50 (0.32 to 0.65), <i>P</i> < .0001	0.32 (0.11 to 0.49), <i>P</i> < .005	0.31 (0.10 to 0.48), <i>P</i> < .005	0.42 (0.21 to 0.60), <i>P</i> = .0001	NS	NS
IL-1RA/IL-1 α plus IL-1 β	-0.64 (-0.75 to -0.49), <i>P</i> = .02	0.33 (0.13 to 0.51), <i>P</i> = .001	0.40 (0.20 to 0.57), <i>P</i> < .0001	0.24 (0.04 to 0.44), <i>P</i> = .02	0.39 (0.19 to 0.55), <i>P</i> = .0002	0.39 (0.19 to 0.55), <i>P</i> < .0001	0.26 (0.04 to 0.45), <i>P</i> < .05	NS

Results are presented as Spearman correlation coefficient values (2-tailed) with 95% CIs and significance. NS, Not significant.

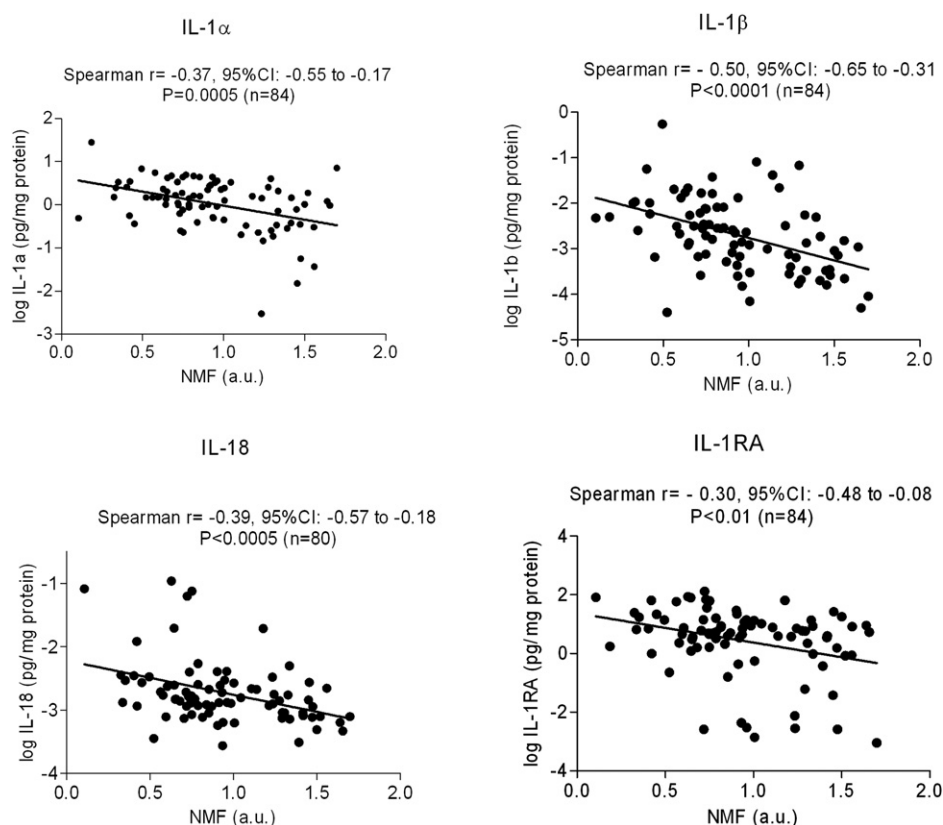


FIG 2. Correlation between levels of cytokines and NMF in the SC of patients with AD. Correlations are unadjusted for *FLG* mutations. *a.u.*, Arbitrary units; *r*, Spearman (2-tailed) correlation coefficient.

cytokine levels are shown in Fig 2 and Table III; the NMF level was inversely associated with levels of all investigated IL-1 cytokines.

In the small additional adult study, levels of IL-1 α in the SC of patients with AD_{FLG} were significantly higher than those in healthy control subjects and patients with AD_{NON-FLG} (see Fig E1 in this article's Online Repository at www.jacionline.org).

SC pH in relation to *FLG* allele status

Homozygous and compound heterozygous carriers (*FLG*^{-/-}) had higher pH values than wild-type (*FLG*^{+/+}) or heterozygous (*FLG*^{+/-}) carriers (Fig 3). Levels of NMF in the SC of the patients with AD were negatively correlated with skin surface pH, as shown in Fig 4. The correlation was more striking in the subgroup of *FLG* mutation carriers (Fig 4, left graph) compared with all subjects (Fig 4, right graph).

TABLE III. Correlations between NMF, cytokine levels, and clinical parameters

	IL-1 α (n = 84)	IL-1 β (n = 84)	IL-18 (n = 80)	IL-1RA (n = 84)	IL-8 (n = 83)	NESS (n = 129)	TEWL (n = 99)	IgE (n = 126)	pH (n = 58)
NMF	-0.37 (-0.55 to -0.17), <i>P</i> = .0005	-0.50 (-0.65 to -0.31), <i>P</i> < .0001	-0.39 (-0.57 to -0.18), <i>P</i> < .0005	-0.30 (-0.48 to -0.08), <i>P</i> < .01	NS	NS	-0.35 (-0.51 to -0.15), <i>P</i> = .0004	NS	-0.35 (-0.56 to -0.09), <i>P</i> < .01

Results are presented as Spearman correlation coefficient values (2-tailed) with 95% CIs and significance. NS, Not significant.

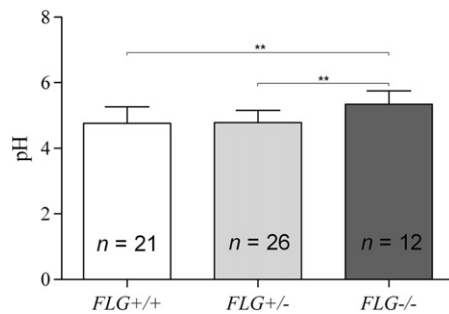


FIG 3. Distribution of pH values according to *FLG* status (mean \pm SD). ***P* < .01, as determined by using the ANOVA *post hoc* Tukey test.

Animal study in *Flg^{ft}/Flg^{ft}* (*Flg^{delAPfal}*) mice

Skin and keratinocytes from *Flg^{ft}/Flg^{ft}* mice have upregulated expression of IL-1 β and IL-1RA mRNA. mRNA expression of IL-1 β and IL-1RA is upregulated in primary epidermal keratinocytes in the proliferative (Fig 5, A) and terminal differentiation (Fig 5, B) states, but there was no significant change in levels of IL-1 α . Dorsal skin from *Flg^{ft}/Flg^{ft}* mice had increased IL-1 β mRNA expression but not IL-1 α expression relative to levels detected in wild-type animals (Fig 5, C).

DISCUSSION

In this study we sought to investigate whether levels of IL-1 cytokines, including IL-1 α , IL-1 β , IL-18, and IL-1RA, are increased in the uninvolved skin of patients with AD with *FLG* loss-of-function mutations. Although we have recently shown that AD severity *per se* influences SC NMF status,³⁷ this is a minor effect compared with the major effect that *FLG* genotype has on NMF.^{26,37} Because AD severity was similar across all 3 *FLG* genotype subgroups, this AD severity effect is unlikely to influence the results presented herein.

We found increased IL-1 α , IL-1 β , IL-18, and IL-1RA expression in patients with AD *FLG* mutations compared with that seen in wild-type patients with AD. In line with these results, IL-1 cytokine levels correlated inversely with NMF levels in the SC. In a complementary murine study we also observed altered mRNA IL-1 expression in the skin and cultured keratinocytes of mice with an *Flg* loss-of-function mutation.

Our findings support the hypothesis by Elias et al⁵⁻⁷ that “reduced levels of filaggrin and in particular its acidic derivatives such as urocanic acid, lead to increased pH of the SC, which may promote activity of serine proteases involved in cleavage of the pro-forms of IL-1 α and IL-1 β .”^{7,16,17} Here we show a correlation between increased pH and decreased NMF levels,

which is consistent with recent data. An increased (less acidic) pH can activate proteases with neutral-to-alkaline pH optima to process inactive forms of IL-1 α , IL-1 β , and IL-18 that are stored in the cytosol of corneocytes.^{16,38-41} Consistent with this view, Hosomi et al⁴² showed increased activity of caspase-1 in the SC of patients with Netherton syndrome, an autosomal recessive inherited disease characterized by features of AD and uninhibited breakdown of filaggrin in the SC. Increased pH in the *FLG^{-/-}* genotype subgroup compared with the *FLG^{+/+}* and *FLG^{+/-}* subgroups and the negative correlation between NMF and pH demonstrated in the present study support the view that NMF contributes to the pH of the SC. This is in good agreement with a recent study by Jungersted et al.⁴³ However, we found no correlation between SC pH and the SC levels of either IL-1 α or IL-1 β , but this might be due to a lack of power in our study to determine this correlation. It should be noted that we measured pH on the skin surface and that this measurement might not be representative of the epidermal compartment, where proforms of IL-1 cytokines are activated. Furthermore, activity of proteases and consequently levels of IL-1 might be influenced also by factors other than pH, such as increased calcium concentration caused by reduced water in the SC. We showed previously that carriers of *FLG* mutations have reduced levels of NMF, which likely contribute to reduced SC water content.^{25,26,30} The activity of serine proteases is calcium dependent, and thus increased calcium concentrations caused by decreased cytosolic water content could favor their activation.⁴⁴⁻⁴⁶ Proteolytic activity can be influenced further by exogenous proteases, such as those derived from house dust mite, cockroach, and *Staphylococcus aureus*. Allergens derived from house dust mites and *S aureus* exhibit cysteine and serine protease activity.⁴⁷⁻⁵¹ Consistent with these findings, Inoue et al⁵² reported increased levels of a caspase-1-mediated IL-18 in the SC of patients with AD; IL-18 production was associated with *S aureus* colonization. Recently, we have shown that the presence of filaggrin breakdown products results in reduced growth rates of *S aureus* and decreased expression of several proteins known to be involved in colonization and inflammation of skin.⁵³ Thus reduced levels of filaggrin degradation products might lead to the increased growth of *S aureus* and consequently to enhanced activity of proteases in the skin.

In the murine study, using *Flg^{ft}/Flg^{ft}* mice, upregulated expression of IL-1 β and IL-1RA mRNA was seen in skin and isolated keratinocytes. No difference in IL-1 α expression was detected. The murine results suggest that loss-of-function mutations in *FLG* are a determinant of increased epidermal IL-1 β expression. In the murine study we measured mRNA, which should not be influenced by SC pH-enhancing protease activity, and thus a

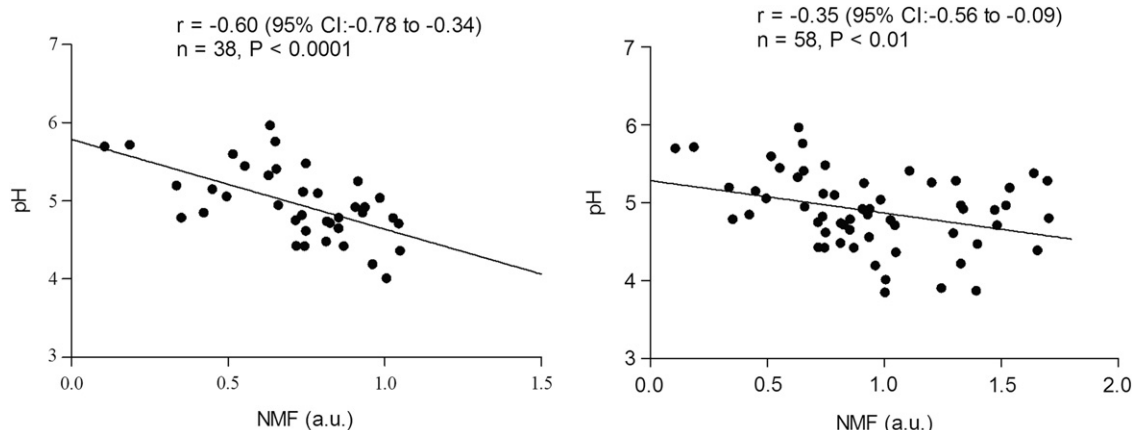


FIG 4. Skin pH values in relation to NMF levels. *Left*, Patients with AD_{FLG}. *Right*, Patients with AD_{FLG} plus patients with AD_{NON-FLG}. a.u., Arbitrary units; *r*, Pearson (2-tailed) correlation coefficient.

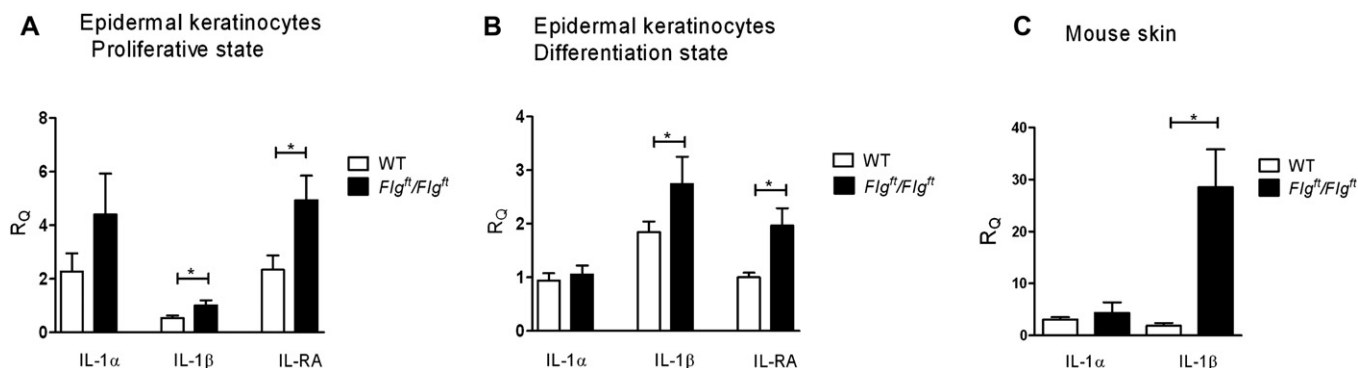


FIG 5. **A** and **B**, Expression of IL-1 α and IL-1 β in keratinocytes and skin from *Flg^{fl}/Flg^{fl}* mice. mRNA expression of IL-1 β and IL-1RA in primary epidermal keratinocytes in the proliferative (Fig 5, **A**) and terminal differentiation (Fig 5, **B**) states. **C**, IL-1 α and IL-1 β mRNA expression in dorsal murine skin. Data are representative of 3 independent experiments (means \pm SEMs). **P* < .05.

pH-driven increase in protease activity is unlikely to entirely explain the increased IL-1 β levels in these mice.

Flaggrin has a key role in aggregation of the keratin filaments in the corneocytes and is likely to play a role in the integrity of the SC through other indirect effects.^{1,2} Barrier disruption itself stimulates keratinocyte proliferation, as well as cytokine and chemokine production, including IL-1 α and IL-1 β .⁵⁴⁻⁵⁶ However, as shown by us and others, the skin barrier in patients with AD is impaired irrespective of *FLG* mutation status,^{26,43,57} and thus barrier dysfunction *sensu latu* would not appear to explain differences in IL-1 cytokine levels between patients with AD with and without *FLG* mutations. Our data suggest that there is an *FLG*-specific effect on the IL-1 cytokine profile. Our small pilot study, which includes healthy adult control subjects (see Fig E1), also supports an *FLG*-specific effect rather than a general AD effect. In this small study control subjects had similar IL-1 α levels to those seen in patients with AD_{NON-FLG}, whereas among patients with AD, those with *FLG* mutations had increased IL-1 α levels.

IL-1 α and IL-1 β are potent proinflammatory mediators, but their activity is counteracted by IL-1RA, levels of which were also increased in patients with AD_{FLG}. The IL-1RA/IL-1 α plus IL-1 β ratio was similar in all 3 *FLG* subgroups. Levels of

IL-1 β and IL-18, but not IL-1 α , were positively correlated with levels of IL-1RA and IL-8. This might indicate that in patients with AD_{FLG}, IL-1 β and IL-18 drives inflammation, although we cannot exclude other underlying proinflammatory stimuli. The nature of this proinflammatory stimulus remains to be determined, but it might be independent of the variation in skin pH. For example, it is known that IL-4 and IL-13, which are overexpressed in unaffected AD skin,⁵⁸⁻⁶⁰ can drive IL-1RA expression. These cytokines, when coexpressed, have a further inhibitory effect on *FLG* expression,⁶¹ and thus a positive feedback loop might be in place.

Taken together, our human and murine data support the concept that there might be a pre-existing enhanced or proinflammatory status in the skin of patients with AD that relates to *FLG* mutations. Additional work is required to clarify the temporal link between *FLG* mutations and the enhanced amounts of these proinflammatory cytokines in human subjects, but the murine work presented herein suggests that it is an early effect. The major findings for IL-1 β are paralleled by those for IL-18, which share the same maturation and secretory mechanism. It would be interesting to investigate whether subjects with *FLG* mutations but without a history of AD also have increased amounts of IL-1 cytokines.

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Key messages

- Among patients with AD, those with AD_{FLG} have a distinct SC cytokine profile from those with AD_{NON-FLG}.
- Similar cytokine profile findings were replicated in a murine homologue of filaggrin deficiency.
- This is the first study to link *FLG* mutations with alterations in IL-1 levels.

REFERENCES

- Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med* 2011;365:1315-27.
- O'Regan GM, Sandilands A, McLean WH, Irvine AD. Filaggrin in atopic dermatitis. *J Allergy Clin Immunol* 2008;122:689-93.
- Elias PM, Feingold KR. Does the tail wag the dog? Role of the barrier in the pathogenesis of inflammatory dermatoses and therapeutic implications. *Arch Dermatol* 2001;137:1079-81.
- Leung DY. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J Allergy Clin Immunol* 2000;105:860-76.
- Elias PM. The skin barrier as an innate immune element. *Semin Immunopathol* 2007;29:3-14.
- Elias PM, Hatano Y, Williams ML. Basis for the barrier abnormality in atopic dermatitis: outside-inside-outside pathogenic mechanisms. *J Allergy Clin Immunol* 2008;121:1337-43.
- Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Curr Opin Allergy Clin Immunol* 2009;9:437-46.
- Barland CO, Zettersten E, Brown BS, Ye J, Elias PM, Ghadially R. Imiquimod-induced interleukin-1 alpha stimulation improves barrier homeostasis in aged murine epidermis. *J Invest Dermatol* 2004;122:330-6.
- Ye J, Garg A, Calhoun C, Feingold KR, Elias PM, Ghadially R. Alterations in cytokine regulation in aged epidermis: implications for permeability barrier homeostasis and inflammation. I. IL-1 gene family. *Exp Dermatol* 2002;11:209-16.
- Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 2009;27:519-50.
- Feldmeyer L, Werner S, French LE, Beer HD. Interleukin-1, inflammasomes and the skin. *Eur J Cell Biol* 2010;89:638-44.
- Murphy JE, Robert C, Kupper TS. Interleukin-1 and cutaneous inflammation: a crucial link between innate and acquired immunity. *J Invest Dermatol* 2000;114:602-8.
- Ansel JC, Luger TA, Lowry D, Perry P, Roop DR, Mountz JD. The expression and modulation of IL-1 alpha in murine keratinocytes. *J Immunol* 1988;140:2274-8.
- Zepter K, Haffner A, Soohoo LF, De Luca D, Tang HP, Fisher P, et al. Induction of biologically active IL-1 beta-converting enzyme and mature IL-1 beta in human keratinocytes by inflammatory and immunologic stimuli. *J Immunol* 1997;159:6203-8.
- Kobayashi Y, Yamamoto K, Saido T, Kawasaki H, Oppenheim J, Matsushima K. Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1-alpha. *Proc Natl Acad Sci U S A* 1990;87:5548-52.
- Nylander-Lundqvist E, Back O, Egelrud T. IL-1 beta activation in human epidermis. *J Immunol* 1996;157:1699-704.
- Nylander-Lundqvist E, Egelrud T. Formation of active IL-1 beta from proIL-1 beta catalyzed by stratum corneum chymotryptic enzyme in vitro. *Acta Derm Venereol* 1997;77:203-6.
- Keller M, Ruegg A, Werner S, Beer HD. Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 2008;132:818-31.
- Nickel W, Rabouille C. Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol* 2009;10:148-55.
- Ishisaka R, Utsumi T, Kanno T, Arita K, Katsumura N, Akiyama J, et al. Participation of a cathepsin L-type protease in the activation of caspase-3. *Cell Struct Funct* 1999;24:465-70.
- Kondo S, Pastore S, Fujisawa H, Shivji GM, McKenzie RC, Dinarello CA, et al. Interleukin-1 receptor antagonist suppresses contact hypersensitivity. *J Invest Dermatol* 1995;105:334-8.
- Junghans V, Gutgesell C, Jung T, Neumann C. Epidermal cytokines IL-1 β , TNF- α , and IL-12 in patients with atopic dermatitis: response to application of house dust mite antigens. *J Invest Dermatol* 1998;111:1184-8.
- Terui T, Hirao T, Sato Y, Uesugi T, Honda M, Iguchi M, et al. An increased ratio of interleukin-1 receptor antagonist to interleukin-1alpha in inflammatory skin diseases. *Exp Dermatol* 1998;7:327-34.
- Jeong CW, Ahn KS, Rho NK, Park JD, Lee DY, Lee JH, et al. Differential in vivo cytokine mRNA expression in lesional skin of intrinsic vs. extrinsic atopic dermatitis patients using semiquantitative RT-PCR. *Clin Exp Allergy* 2003;33:1717-24.
- Kezic S, Kemperman PM, Koster ES, de Jongh CM, Thio HB, Campbell LE, et al. Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum. *J Invest Dermatol* 2008;128:2117-9.
- O'Regan GM, Kemperman PM, Sandilands A, Chen H, Campbell LE, Kroboth K, et al. Raman profiles of the stratum corneum define 3 filaggrin genotype-determined atopic dermatitis endophenotypes. *J Allergy Clin Immunol* 2010;126:574-80.
- Williams HC, Burney PG, Hay RJ, Archer CB, Shipley MJ, Hunter JJ, et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *Br J Dermatol* 1994;131:383-96.
- Emerson RM, Charman CR, Williams HC. The Nottingham Eczema Severity Score: preliminary refinement of the Rajka and Langeland grading. *Br J Dermatol* 2000;142:288-97.
- de Jongh CM, Verberk MM, Spiekstra SW, Gibbs S, Kezic S. Cytokines at different stratum corneum levels in normal and sodium lauryl sulphate-irritated skin. *Skin Res Technol* 2007;13:390-8.
- Kezic S, Kammeyer A, Calkoen F, Fluhr JW, Bos JD. Natural moisturizing factor components in the stratum corneum as biomarkers of filaggrin genotype: evaluation of minimally invasive methods. *Br J Dermatol* 2009;161:1098-104.
- Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol* 1980;92(suppl):44-7.
- Hanifin JM, Thurston M, Omoto M, Cherill M, Tofte SJ, Graeber M, et al. The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. *Exp Dermatol* 2001;10:11-8.
- Fallon PG, Sasaki T, Sandilands A, Campbell LE, Saunders SP, Mangan NE, et al. A homozygous frameshift mutation in the mouse Flg gene facilitates enhanced percutaneous allergen priming. *Nat Genet* 2009;41:602-8.
- Hager B, Bickenbach JR, Fleckman P. Long-term culture of murine epidermal keratinocytes. *J Invest Dermatol* 1999;112:971-6.
- Lichti U, Anders J, Yuspa SH. Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice. *Nat Protoc* 2008;3:799-810.
- Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008;3:1101-8.
- Kezic S, O'Regan GM, Yau N, Sandilands A, Chen H, Campbell LE, et al. Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. *Allergy* 2011;66:934-40.
- Brattsand M, Stefansson K, Lundh C, Haasum Y, Egelrud T. A Proteolytic cascade of kallikreins in the stratum corneum. *J Invest Dermatol* 2005;124:198-203.
- Wiedow O, Meyer-Hoffert U. Neutrophil serine proteases: potential key regulators of cell signalling during inflammation. *J Int Med* 2005;257:319-28.
- Hachem JP, Crumrine D, Fluhr J, Brown BE, Feingold HR, Elias PM. pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. *J Invest Dermatol* 2003;121:345-53.
- Gruber R, Elias PM, Crumrine D, Lin TK, Brandner JM, Hachem JP, et al. Filaggrin genotype in ichthyosis vulgaris predicts abnormalities in epidermal structure and function. *Am J Pathol* 2011;178:2252-63.
- Hosomi N, Fukai K, Nakanishi T, Funaki S, Ishii M. Caspase-1 activity of stratum corneum and serum interleukin-18 level are increased in patients with Netherton syndrome. *Br J Dermatol* 2008;159:744-6.
- Jungersted JM, Scheer H, Mempel M, Baurecht H, Cifuentes L, Høgh JK, et al. Stratum corneum lipids, skin barrier function and filaggrin mutations in patients with atopic eczema. *Allergy* 2010;65:911-8.
- Fairley JA. Calcium metabolism and the pathogenesis of dermatologic disease. *Semin Dermatol* 1991;10:225-31.
- Ovaere P, Lippens S, Vandenabeele P, Declercq W. The emerging roles of serine protease cascades in the epidermis. *Trends Biochem Sci* 2009;34:453-63.
- Hachem JP, Houben E, Crumrine D, Man MQ, Schurer N, Roelandt T, et al. Serine protease signalling of epidermal permeability barrier homeostasis. *J Invest Dermatol* 2006;126:2074-86.
- Roelandt T, Heughebaert C, Hachem JP. Proteolytically active allergens cause barrier breakdown. *J Invest Dermatol* 2008;128:1878-80.
- Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H, et al. Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. *J Immunol* 2006;177:1609-17.

49. Kato T, Takai T, Fujimura T, Matsuoka T, Ogawa K, Murayama A, et al. Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes. *Allergy* 2009;64:1366-74.
50. Hirasawa Y, Takai T, Nakamura T, Mitsuishi K, Gunawan H, Suto H, et al. *Staphylococcus aureus* extracellular protease causes epidermal barrier dysfunction. *J Invest Dermatol* 2010;130:614-7.
51. Jeong SK, Kim HJ, Youm JK, Ahn SK, Choi EH, Sohn MH, et al. Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery. *J Invest Dermatol* 2008;128:1930-9.
52. Inoue Y, Aihara M, Kirino M, Harada I, Komori-Yamaguchi J, Yamaguchi Y, et al. Interleukin-18 is elevated in the horny layer in atopic dermatitis patients and associated with *Staphylococcus aureus* colonisation. *Br J Dermatol* 2011;164:560-7.
53. Miajlovic H, Fallon PP, Irvine AD, Foster TJ. Effect of filaggrin breakdown products on growth of and protein expression by *Staphylococcus aureus*. *J Allergy Clin Immunol* 2010;126:1184-90.
54. Wood LC, Jackson SM, Elias PM, Grunfeld C, Feingold KR. Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J Clin Invest* 1992;90:482-7.
55. Wood LC, Elias PM, Calhoun C, Tsai JC, Grunfeld C, Feingold KR. Barrier disruption stimulates interleukin-1 alpha expression and release from a pre-formed pool in murine epidermis. *J Invest Dermatol* 1996;106:397-403.
56. Nickoloff BJ, Naidu Y. Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. *J Am Acad Dermatol* 1994;30:535-46.
57. Jakasa I, Koster ES, Calkoen F, McLean WHI, Campbell LE, Bos JD, et al. Skin barrier function in healthy subjects and patients with atopic dermatitis in relation to filaggrin loss-of-function mutations. *J Invest Dermatol* 2011;131:540-2.
58. Ong PY, Leung DY. Atopic dermatitis. *Clin Allergy Immunol* 2002;16:355-79.
59. Nomura I, Goleva E, Howell MD, Hamid QA, Ong PY, Hall CF, et al. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 2003;171:3262-9.
60. Leung DY, Bieber T. Atopic dermatitis. *Lancet* 2003;361:151-60.
61. Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, De Benedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 2007;120:150-5.

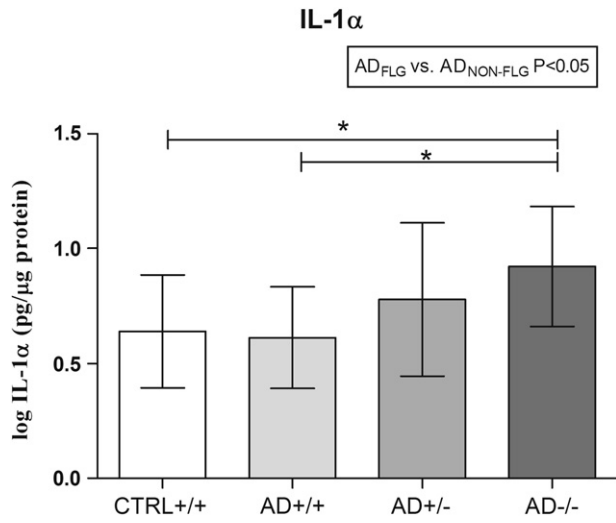


FIG E1. Levels of IL-1 α in the SC of healthy control subjects and patients with AD *FLG* genotype. The differences have been tested by using the Student *t* test ($*P < .05$). Data are presented as mean values \pm SDs. CTRL+/+, Homozygote wild-type subject without AD.