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Authors

Blunder, Stefan
Rühl, Ralph
Moosbrugger-Martinz, Verena
et al.

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Alterations in Epidermal Eicosanoid Metabolism Contribute to Inflammation and Impaired Late Differentiation in *FLG*-Mutated Atopic Dermatitis

Stefan Blunder[1], **Ralph Rühl**[2,3], **Verena Moosbrugger-Martinz**[1], **Christine Krimmel**[1], **Anita Geisler**[1], **Huiting Zhu**[1,4], **Debra Crumrine**[5], **Peter M. Elias**[5], **Robert Gruber**[1,6], **Matthias Schmuth**[1,7], and **Sandrine Dubrac**[1,7]

[1]Department of Dermatology, Venereology and Allergology, Medical University of Innsbruck, Innsbruck, Austria

[2]MTA-DE Public Health Research Group of the Hungarian Academy of Sciences, Faculty of Public Health, University of Debrecen, Debrecen, Hungary

[3]Paprika Bioanalytics, Debrecen, Hungary

[4]Department of Dermatology, Beijing Hospital of Traditional Chinese Medicine Affiliated to Capital Medical University, Beijing, China

[5]Department of Dermatology, University of California, San Francisco, California, USA

[6]Division of Human Genetics, Medical University of Innsbruck, Innsbruck, Austria

Abstract

Loss-of-function mutations in the *FLG* gene cause ichthyosis vulgaris (IV) and represent the major predisposing genetic risk factor for atopic dermatitis (AD). Although both conditions are characterized by epidermal barrier impairment, AD also exhibits signs of inflammation. This work was aimed at delineating the role of *FLG* loss-of-function mutations on eicosanoid metabolism in IV and AD. Using human epidermal equivalents (HEEs) generated with keratinocytes isolated from nonlesional skin of patients with *FLG* wild-type AD (WT/WT), *FLG*-mutated AD (*FLG*/WT), IV (*FLG*/*FLG*), or *FLG* WT control skin, we assessed the potential autocrine role of epidermal-derived eicosanoids in *FLG*-associated versus *FLG*-WT AD pathogenesis. Ultrastructural analyses demonstrated abnormal stratum corneum lipid architecture in AD and IV HEEs, independent of *FLG* genotype. Both AD (*FLG*/WT) and IV (*FLG*/*FLG*) HEEs showed impaired late epidermal differentiation. Only AD (*FLG*/WT) HEEs exhibited significantly increased levels of inflammatory cytokines. Analyses of lipid mediators revealed increased arachidonic acid and 12-lipoxygenase metabolites. Whereas treatment of control HEEs with

Corresponding author: Sandrine Dubrac, Department of Dermatology, Venereology and Allergology, Medical University of Innsbruck, Anichstr 35, 6020 Innsbruck, Austria. sandrine.dubrac@i-med.ac.at.

[7]These authors contributed equally to this work.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.09.034.

arachidonic acid increased expression of inflammatory cytokines, 12-hydroxy-eicosatetraenoic acid attenuated expression of late differentiation markers. Thus, *FLG* mutations lead to alterations in epidermal eicosanoid metabolism that could serve as an autocrine trigger of inflammation and impaired late epidermal differentiation in AD.

INTRODUCTION

The discovery that loss-of-function mutations in the human *filaggrin* (*FLG*) gene cause ichthyosis vulgaris (IV) and predispose to atopic dermatitis (AD) has focused considerable attention on the role of FLG in epidermal barrier function (Gruber et al., 2007; Palmer et al., 2006; Rodriguez et al., 2009; Smith et al., 2006). Various models of IV and AD have been developed to study the role of FLG in skin barrier function and AD pathogenesis. *FLG* knockdown was carried out in organotypic skin cultures (Kuchler et al., 2011; Mildner et al., 2010; Pendaries et al., 2014; van Drongelen et al., 2013; Vavrova et al., 2014). Furthermore, skin cultures supplemented with T-helper type 2 cytokines were used to recapitulate AD in vitro (Danso et al., 2014; Kamsteeg et al., 2011). However, the role of FLG in skin barrier function in IV, a noninflammatory skin condition, and in AD, a disease with a strong inflammatory component, has not been fully elucidated using these models.

By analyzing skin samples from patients, an abnormal composition of stratum corneum (SC) lipids has been reported in AD. Levels of long-chain ceramides and long-chain free-fatty acids decline, whereas ceramides and free-fatty acids with short-chain length increase in both lesional and nonlesional AD skin, independent of *FLG* mutations (Janssens et al., 2012; van Smeden et al., 2014b). In contrast, information about the role of bioactive lipid mediators in AD, with and without *FLG* mutations, and in IV are incomplete, despite the apparently important role of bioactive polyunsaturated fatty acid (PUFA)-derived mediators in skin physiology (Kendall et al., 2015; Krieg and Furstenberger, 2014; Munoz-Garcia et al., 2014). Eicosanoids, the most prominent PUFA derivatives, arise from 20-carbon PUFAs, such as arachidonic (AA; 20:4, ω6), eicosapentaenoic (EPA; 20:5, ω3), and dihomo-gamma-linolenic acid (20:3, ω6) through oxidation via cyclooxygenase, lipoxygenase (LOX), and cytochrome P450 enzymes. They are rapidly synthesized in response to various stimuli, acting locally in an autocrine and/or paracrine manner (Kendall and Nicolaou, 2013). Earlier studies reported increased levels of AA and its bioactive lipid-mediator-metabolites leukotriene B4 and prostaglandin E2 in lesions of patients with AD (Fogh et al., 1989; Ruzicka et al., 1986; Schafer and Kragballe, 1991). Although a critical role of leukotriene B4 in the pathogenesis of AD-like symptoms was shown in a murine model (Oyoshi et al., 2012), the contribution of other eicosanoids to AD remains to be investigated and correlated with *FLG* mutation status. Hence, it seems reasonable that skin lipid profiling in both AD with *FLG* mutations and IV could unravel additional pathogenic mechanisms that favor the development of AD.

In this study, we (i) establish human epidermal equivalents (HEEs), generated with keratinocytes from patients with *FLG* wild-type (WT) AD [AD (WT/WT)], *FLG* heterozygous AD [AD (*FLG*/WT)], *FLG* compound heterozygous or homozygous IV [IV (*FLG*/*FLG*)], and from *FLG* WT control subjects [ctrl (WT/WT)]; (ii) assess the validity of

these patient keratinocyte-derived HEEs as model systems for human skin; and (iii) evaluate the link between *FLG* mutations and the composition of bioactive lipid mediators.

RESULTS

Structural abnormalities in AD (WT/WT), AD (*FLG*/WT), and IV (*FLG*/*FLG*) HEEs recapitulate IV/AD in vivo

We obtained skin biopsies from the nonlesional, non-UV exposed trunk from 12 patients with AD without *FLG* mutations (WT/WT), 3 patients with AD with heterozygous *FLG* mutations (*FLG*/WT), 3 patients with IV with compound heterozygous or homozygous *FLG* mutations (*FLG*/*FLG*), and 14 healthy control (ctrl) subjects without *FLG* mutations (WT/WT). HEEs were generated using isolated keratinocytes and cultured at a humidity of 40–60% (Sun et al., 2015). Histologic analyses showed intact epidermal differentiation in HEEs from all four groups (Figure 1a). IV HEEs exhibited a complete absence of keratohyalin granules in the stratum granulosum (Figure 1a and b, Supplementary Table S1 on-line). Ultrastructural analyses by transmission electron microscopy confirmed lack of keratohyalin granules in IV HEEs. In AD (*FLG*/WT) HEEs, the size and numbers of keratohyalin granules were reduced or normal as compared with ctrl HEEs (Figure 1b, Supplementary Table S1). HEEs from all four groups showed intact corneodesmosomes and regular appearing cornified envelopes (Figure 1b and c) (Oji et al., 2010). To explore the SC lamellar bilayer organization, we used the ruthenium tetroxide postfixation (Gruber et al., 2011). Ctrl HEEs exhibited regular SC lamellar bilayer architecture (Figure 1c). In contrast, SC from AD (WT/WT), AD (*FLG*/WT), and IV HEEs showed disorganized lamellar bilayer structures, that is, a disruption of mature lamellar bilayer organization by incompletely processed lamellar material (Figure 1c). In contrast to ctrl HEEs, there was premature secretion of lamellar body (LB) contents into the extracellular spaces of the stratum granulosum (Figure 1b), and secretion appeared inhomogeneous at the stratum granulosum-SC interface (Figure 1d). Compared with ctrl HEEs, AD (WT/WT), AD (*FLG*/WT), and IV HEEs displayed aberrant LB internal structures (Supplementary Table S1). LB entombment, that is, entrapment of nonsecreted LB contents within the corneocytes, was seen in approximately 50% of AD and IV HEEs (Figure 1c and d). Together, these ultrastructural analyses confirm that HEEs generated from AD and IV keratinocytes recapitulate the structural abnormalities of IV and AD human skin (Gruber et al., 2011; Gruber and Schmuth, 2015; Werner et al., 1987).

Permeability to Lucifer yellow (LY) is not compromised in IV and AD HEEs

To assess barrier permeability properties in HEEs, we used the LY assay. We applied 200 μl of hydrophilic LY dye for a time period of 2 hours onto the SC of fully differentiated HEEs at day 12. Supplementary Figure S1 online shows that LY dye did not permeate into the SC of any HEE.

Alterations of late epidermal differentiation in AD and IV HEEs mimic AD and IV

Compared with healthy controls, *FLG* mRNA levels were not altered in AD (WT/WT) HEEs, but were reduced by 60% in AD (*FLG*/WT) HEEs (Figure 2a, Supplementary Table S2 on-line). IV HEEs exhibited a 90% reduction of *FLG* mRNA levels (Figure 2a).

Accordingly, FLG protein levels were strikingly reduced in AD (*FLG*/WT) HEEs and absent in IV HEEs. Notably, FLG protein was also decreased in AD (WT/WT) HEEs (Figure 2b). In contrast, in both *FLG* WT and mutated AD HEEs, LOR expression did not change at either the mRNA or protein levels when compared with ctrl HEEs (Figure 2). IV HEEs showed reduced *LOR* mRNA levels, but no changes at the protein level (Figure 2a and b). As depicted in Figure 2a and Supplementary Table S2, *HRNR* mRNA levels increased in AD (WT/WT) and AD (*FLG*/WT) HEEs when compared with ctrl HEEs, but declined in IV HEEs. HRNR protein levels were strikingly reduced in AD (*FLG*/WT) and in IV HEEs (Figure 2b). Protein and mRNA levels of early epidermal differentiation markers including TGM1, *KRT1*, and KRT10 remained unchanged in AD and IV HEEs (Figure 2). These data demonstrate that HEEs generated with keratinocytes of patients with AD and IV show similar FLG expression patterns as in patient skin (Gruber et al., 2011; Jensen et al., 2004; Pellerin et al., 2013; Suarez-Farinas et al., 2011; van den Bogaard et al., 2013). Furthermore, our findings suggest that a decrease in HRNR parallels *FLG* loss-of-function mutations.

AD HEEs exhibit increased expression of inflammatory cytokines

Next, we evaluated the impact of *FLG* mutations in AD and IV on the expression of cytokines that are known to be produced by keratinocytes in response to skin barrier disruption and/or have been implicated in AD (Czarnowicki et al., 2014; Thyssen and Kezic, 2014). We found a 1.7-fold and a 4.4-fold increase of *IL1B* mRNA levels in AD (WT/WT) and AD (*FLG*/WT) HEEs, respectively, when compared with ctrl HEEs (Figure 2a, Supplementary Table S2). Similarly, *TNFA* and *TARC* mRNA levels were increased 4.2- and 2.7-fold in AD (*FLG*/WT) HEEs versus ctrl HEEs (Figure 2a). In contrast, epidermal mRNA levels of the epithelial alarmins, *IL-33*, and *TSLP* were not altered (Figure 2a), and *IL-25* remained undetectable (data not shown). IV HEEs did not exhibit alterations in levels of inflammatory mediators when compared with crtl HEEs (Figure 2a). These findings demonstrate that AD but not IV HEE displays increased levels of inflammatory mediators.

AD and IV HEEs exhibit changes in ω6- and ω3-PUFA composition

As HEEs generated with patient keratinocytes recapitulate many features of human epidermis of patients with AD and IV (Supplementary Table S3 online), we next used these models to unravel potential new pathways involved in the pathogenesis of the two diseases. PUFAs and their products have been suggested to be mediators in inflammatory skin diseases (Kendall and Nicolaou, 2013). Therefore, we assessed bioactive lipid profiles by liquid chromatography mass spectrometry in AD (WT/WT) HEEs, AD (*FLG*/WT), IV, and in ctrl HEEs. First, we quantified ω6- and ω3-PUFAs. Although ω6- and ω3-PUFA composition displayed changes in both AD and IV HEEs when compared with ctrl HEEs (Figure 3, Supplementary Table S4 online), overall ω6- and ω3-PUFA proportions remained similar in all groups (Supplementary Figure S2 online). Furthermore, the proportions of ω6-PUFAs dihomo-gamma-linolenic acid and AA were increased by 4% (dihomo-gamma-linolenic acid: ctrl: 11.9% ± 0.8%; AD (*FLG*/WT): 15.9% ± 0.6%; $P = 0.02$) and by 15% (AA: ctrl: 39.4% ± 3.3%; AD (*FLG*/WT): 54.4% ± 0.9%; $P = 0.07$) in AD (*FLG*/WT) HEEs when compared with ctrl HEEs (Supplementary Figure S3 online). These data suggest that an imbalance in the metabolic synthesis of ω6-PUFAs may be associated with AD in patients harboring *FLG* mutation, which could be explained by a potential increase in

phospholipase A_2 (PLA_2). We found that mRNA levels of three epidermal PLA_2 tended to be increased in AD (*FLG*/WT) HEEs (Figure S7); however, this increase was not significant. Among ω3-PUFAs, docosapentaenoic acid levels were higher in AD (WT/WT), AD (*FLG*/WT), and IV HEEs when compared with crtl HEEs (Figure 3, Supplementary Table S4 online). In contrast, EPA and docosahexaenoic acid concentrations were only increased in AD (WT/WT) and AD (*FLG*/WT) HEEs, respectively, when compared with ctrl HEEs (Figure 3, Supplementary Table S4). Thus, one might speculate that EPA-, docosahexaenoic-, and docosapentaenoic acid-derived anti-inflammatory lipids such as resolvins and protectins that are termed specialized proresolving mediators may contribute to dampening local inflammation (Basil and Levy, 2016; Morita et al., 2013; Schwab et al., 2007; Serhan et al., 2011). Yet, our analysis did not reveal changes in levels of the docosahexaenoic-derived specialized pro-resolving mediators resolvins D1 and D2, neuroprotectin D1, and maresin (Supplementary Table S5 online). Because we did not assay additional specialized proresolving mediators derived from docoahexaenoic, docosapenaenoic acid and EPA, we cannot entirely exclude a role of these metabolites in AD and IV.

12-LOX metabolism is potentially enhanced in *FLG*-mutated AD HEEs

Because AA levels increased in AD (*FLG*/WT) HEEs (Figures 3 and 4, Supplementary Table S4), we next measured levels of eicosanoids in HEEs using liquid chromatography mass spectrometry methodology. Among the AA-hydroxy-fatty acid metabolites, the 12-LOX metabolite 12-hydroxy-eicosatetraenoic acid (HETE) predominated in all HEE groups (Supplementary Figure S4a and b online). Furthermore, the 12-LOX product, 12-hydroxy-eicosapentaenoic acid (HEPE), was the predominant eicosanoid arising from EPA (Supplementary Figure S4c and d). We next compared changes in eicosanoid composition using a pathway-oriented approach. *FLG*-mutated AD HEEs exhibited a 207% increase of 12-LOX metabolites when compared with ctrl HEEs (Figure 4, Supplementary Table S6 online). Specifically, levels of 12-HETE and 12-HEPE were significantly increased (Figure 4b). While 12-HETE represented 43.3% of all AA-derived metabolites in ctrl HEEs, its levels increased to 77% in AD (*FLG*/WT) HEEs ($P = 0.05$) (Supplementary Figure S4a, b, and e). Compared with ctrl HEEs, the 12-HEPE fraction among all EPA products increased by nearly 20% ($P = 0.06$) in AD (*FLG*/WT) (Supplementary Figure S4c, d, and f).These data point to increased conversion of AA and EPA to 12-HETE and 12-HEPE, likely resulting from enhanced 12-LOX metabolism. Interestingly, 15-HETE and 15-HEPE levels significantly increased in IV HEEs versus ctrl HEEs (Figure 4c), in line with a tendency of elevated 15-LOX metabolite levels ($P = 0.08$) in these HEEs (Figures 3 and 4, Supplementary Tables S4 and S6). Of note, several prostaglandins, such as prostaglandin D_2 and prostaglandin E_2, were detectable in all HEEs, whereas levels of other metabolites, such as thromboxane B_2 and prostaglandine J_2, were below detection limits (<0.2 ng/g) in some samples. Similarly, levels of leukotriene B4 and its metabolites were below the detection limit of the methodology in many samples (Supplementary Table S4). These results suggest that 5-LOX-derived products found in AD skin likely arise from infiltrating immune cells (Kendall and Nicolaou, 2013; Werz, 2002). Accordingly, levels of the 5-LOX product 5-HETE were usually very low or under the detection limit in all examined samples (Supplementary Table S4). In summary, our findings demonstrate potentially augmented 12-

LOX metabolism, resulting in increased 12-HETE and 12-HEPE levels in AD (*FLG*/WT) HEEs.

Treatment of control HEEs with AA or 12-HETE partially recapitulates the phenotype of AD (*FLG*/WT) HEEs

We next tested the effects of AA, 12-HETE, and 12-HEPE on ctrl HEEs. First, we performed dose-response experiments to determine optimal AA, 12-HETE, and 12-HEPE concentrations (Supplementary Figure S5 online). Treatment with AA at a concentration of 50 μM led to a significant increase of *IL1B* and *TARC* mRNA levels in ctrl HEEs, without impacting HEE morphology (Figure 5a and b). In contrast, the expression of late epidermal differentiation markers, such as FLG, HRNR, and LOR, and early differentiation markers including TGM1, KRT1, and KRT10 did not change after AA treatment (Figure 5a–c). Furthermore, treatment of ctrl HEEs with 0.05 μM 12-HETE resulted in a downregulation of *HRNR* and *LOR* mRNA levels (Figure 5d). In line with gene expression data, HRNR protein was strikingly reduced in 12-HETE-treated ctrl HEEs (Figure 5d–f). Despite reduced *LOR* mRNA levels and a trend toward decreased *FLG* gene expression in 12-HETE-treated HEEs, LOR and FLG protein expression did not change when compared with vehicle-treated ctrl HEEs, respectively (Figure 5d–f). Interestingly, 12-HETE treatment led to a decrease of *TNFA* mRNA levels. 12-HETE changed neither gene and protein expression of early differentiation markers *TGM1*, KRT1, and KRT10 nor the morphology of HEEs (Figure 5d–f). Of note, treatment of ctrl HEEs with 12-HEPE at a concentration of 0.5 μM diminished *FLG* and *LOR* mRNA levels, yet it did not alter FLG and LOR protein expression (Supplementary Figure S6 online). Expression levels of other epidermal differentiation markers and of inflammatory mediators were not altered by 12-HEPE treatment (Supplementary Figure S6). In summary, these data show that 12-HETE and 12-HEPE attenuate the expression of various keratinocyte differentiation markers. Yet only treatment with 12-HETE leads to decreased levels of HRNR. In contrast, AA treatment induces the expression of a specific set of inflammatory mediators.

DISCUSSION

The current study describes HEEs generated with patient keratinocytes as an in vitro model for both AD and IV. We report here that AD (WT/WT), AD (*FLG*/WT), and IV HEEs closely recapitulate many epidermal features observed in both diseases as summarized in Supplementary Table S3. In line with data from human skin biopsies, ultrastructural analyses of AD (WT/WT), AD (*FLG*/WT), and IV HEEs show significant alterations in the SC lipid architecture, that is, disturbed loading of LB content, premature LB secretion, and abnormal postsecretory lipid organization resulting in defective SC lipid bilayer structure (Figure 1b–d, Supplementary Table S1) (Gruber et al., 2011; Gruber and Schmuth, 2015; Werner et al., 1987). Notably, these findings do not correlate with *FLG* genotype in AD HEEs (Supplementary Tables S1 and S3). These data suggest that a common denominator, which might be linked to abnormal keratinocyte differentiation, is responsible for LB alterations in both AD and IV, independent of *FLG* genotype. Analyses of epidermal differentiation markers further confirm the close resemblance of HEEs generated with AD and IV keratinocytes with patient skin (Figure 2, Supplementary Table S3). FLG and LOR

expression patterns in AD and IV HEEs largely concur with data from human skin (Supplementary Table S3) (Gruber et al., 2011; Jensen et al., 2004; Nirunsuksiri et al., 1995; Pellerin et al., 2013). Similarly, HRNR protein levels were reduced in AD (*FLG*/WT) and IV HEEs (Pellerin et al., 2013). Yet, in contrast to human skin biopsies, HRNR was not decreased in AD (WT/WT) HEEs. This discrepancy can likely be explained by our HEEs, which are devoid of immune cells, and therefore lack cytokines such as IL-4, IL-13, and IL-25 that were shown to diminish HRNR (Pellerin et al., 2013). Instead, we found increased *IL1B*, *TARC*, and *TNFA* mRNA levels in *FLG*-mutated AD HEEs, similar to earlier findings in human AD skin (Supplementary Table S3) (Kezic et al., 2012; Suarez-Farinas et al., 2011; Szegedi et al., 2015). In IV HEEs inflammatory cytokine levels remained unchanged, underscoring the noninflammatory character of this disease (Figure 2a) (Kypriotou et al., 2013).

Mutations in epidermal structural proteins are often associated with impaired cutaneous barrier function (Elias et al., 2010). However, in this current data set, assessment of epidermal barrier competence by means of hydrophilic LY dye assay did not reveal increased LY barrier permeation depending on *FLG* mutation status (Supplementary Figure S1). In contrast, increased LY penetration in small interfering RNA- and shRNA-mediated *FLG*-knocked down organotypic skin cultures has been observed (Mildner et al., 2010; Pendaries et al., 2014). These conflicting data can be attributed to differences in model systems, that is, patient keratinocytes versus RNA interference techniques (Mildner et al., 2010; Pendaries et al., 2014; van Drongelen et al., 2013). Because we did not assess barrier competence using lipophilic compounds, we cannot comment on permeability for lipophilic substances. Prior work did not report impaired barrier function for the lipophilic substances butylPABA and testosterone in stably *FLG*-knocked down organotypic skin cultures (Honzke et al., 2016; van Drongelen et al., 2013).

Besides fulfilling important structural functions (van Smeden et al., 2014a), epidermal lipids also serve as important bioactive signaling molecules in the epidermis (Hammarstrom et al., 1979; Kendall and Nicolaou, 2013; Kendall et al., 2015; Ruzicka et al., 1986). It has previously been reported that AA levels increase in lesional AD (Schafer and Kragballe, 1991). Accordingly, we found higher amounts of free AA in AD (*FLG*/WT) HEEs, when compared with ctrl HEEs (Figures 3 and 4b, Supplementary Table S4). Free AA can originate from various sources including increased release from phospholipids via IL-1β-induced PLA_2 activity and enhanced breakdown of linoleic acid (Kendall and Nicolaou, 2013; Marcelo and Dunham, 1993; Murakami et al., 2015). We here report significantly increased *IL1B* expression and decreased proportions of linoleic acid associated with significantly increased amounts of AA in AD (*FLG*/WT) HEEs. It is possible that in AD HEEs, both increased metabolism of linoleic acid and PLA_2 activity via IL-1β contribute to increasing AA, which is supported by our results on PLA_2 mRNA levels (Supplementary Figure S7 online). Thus, corticosteroids exert their anti-inflammatory functions in AD therapy not only via effects on immune cells but also by inhibiting the release of AA through inhibition of PLA_2 activity as well (Vane and Botting, 1987). Similarly, the calcineurin-inhibitor cyclosporin A, a drug used in systemic AD treatment, diminishes the T-cell response in AD and decreases both IL-1β and AA concentrations in mouse epidermis (Gupta et al., 1989). We show here that treatment with AA increases *IL1B* expression in HEEs

generated with ctrl keratinocytes (Figure 5a). Therefore, it is possible that a mutual regulation exists between AA and IL-1β. The fact that AA levels increase in AD HEEs and that AA induces an inflammatory phenotype in HEEs strongly suggests a pathomechanistic role of AA in AD (Figure 6) (Schafer and Kragballe, 1991).

Previous reports demonstrate a decrease in 12-HETE keratinocyte-binding sites after UVB and cyclosporin A treatment, two regimens exerting therapeutic effects in AD (Arenberger et al., 1991; Kemeny et al., 1991). We report increased levels of 12-HETE and 12-HEPE in AD (*FLG*/WT) HEEs. 12-HETE exerts chemoattractant effects that cause dermal influx of immune cells and lead to epidermal spongiosis and hyperplasia, histological features observed in AD skin lesions (Chan et al., 1985; Dowd et al., 1985; Ford-Hutchinson et al., 1980; Goetzl et al., 1977; Ruzicka and Burg, 1987; Waldman et al., 1989). Hence, enhanced 12-LOX metabolism in keratinocytes leading to increased 12-HETE levels might contribute to AD development in patients harboring *FLG* mutations. Accordingly, we have shown that 12-HETE treatment of ctrl HEEs partly recapitulates the phenotype of AD (*FLG*/WT) HEEs by substantially reducing HRNR mRNA and protein levels in ctrl HEEs (Figure 5c and e). Thus, treatment of ctrl HEEs with 12-HETE or AA exerts distinct effects replicating several key features observed in AD (*FLG*/WT) HEEs and stressing the role of 12-HETE and AA in AD development and/or persistence. Furthermore, we found that 15-HETE levels are only upregulated in IV HEEs when compared with crtl HEEs (Figure 4c). 15-HETE inhibits T-cell proliferation and reduces synthesis of leukotriene B4 by leukocytes (Bailey et al., 1982; Camp and Fincham, 1985; Chen et al., 2003; Gualde et al., 1985; Hsi et al., 2001; Vanderhoek et al., 1980; Waldman et al., 1989). Moreover, 15-HETE exerts anti-inflammatory effects; for example, it ameliorates psoriasis when injected into skin (Fogh et al., 1988). Hence, 15-HETE via its inhibitory effects on skin-infiltrating immune cells could impede the transition of IV to AD.

In summary, we conclude that *FLG* mutations lead to alterations in epidermal eicosanoid composition in AD that are not observed in IV. In AD, *FLG* mutations increase AA and 12-HETE in keratinocytes that trigger inflammation and impair late epidermal differentiation (Figure 6), similar to what has been proposed previously for T-helper type 2 cytokines in support of the outside-inside back-to-outside concept.

MATERIALS AND METHODS

Human subjects

The study was approved by the Ethics Committee of the Medical University of Innsbruck and conducted in accordance with the Declaration of Helsinki principles. All study subjects gave written informed consent and participated voluntarily. Biopsies were taken from the nonlesional, non-UV-exposed trunk skin of adult Caucasian patients with AD without *FLG* mutations (WT/WT) (n = 12), adult Caucasian patients with AD with heterozygous *FLG* mutations (*FLG*/WT) (n = 3), adult Caucasian patients with IV with compound heterozygous and homozygous *FLG* mutations (*FLG*/*FLG*) (n = 3), and adult Caucasian healthy control subjects without *FLG* mutations (*WT*/*WT*) (n = 14). Study subjects were screened for common European *FLG* variants c.1501C>T, c.2282_2285delCAGT, c.7339C>T, and c.9740C>A as described in the Supplementary Materials online. All *FLG*-mutated patients

with AD were heterozygous for c.2282del4. Patients with IV were compound heterozygous for R501X/2282del4 (n = 1) and for R501X/R2447X (n = 1) and homozygous for 2282del4/2282del4 (n = 1). None of the patients used emollients or any other topical formulations for at least 5 days before a skin biopsy was performed. Patients undergoing treatment with phototherapy or systemic immunosuppressants were excluded from the study.

Keratinocyte isolation

Methods are described in the Supplementary Materials.

Generation of HEEs

HEEs were generated as described previously (Sun et al., 2015). For further details see Supplementary Materials.

RNA isolation and reverse transcriptase-PCR, immunohistochemistry, LY assay, and transmission electron microscopy

Methods are described in the Supplementary Materials.

HPLC-ESI-MS-MS analysis of free-fatty acids and eicosanoids and docosanoids

The HPLC-ESI-MS-MS method was performed as described previously (Ruhl, 2006). For further details see Supplementary Materials.

Statistical analysis

Data are presented, if not other specified, as mean ± standard error of the mean. Statistical significance was determined between AD (WT/WT*)*, AD (*FLG*/WT), or IV (*FLG*/*FLG*) HEEs and ctrl HEEs using Student's unpaired two-tailed *t*-test with significance determined as a *P* value < 0.05. For further details see Supplementary Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	arachidonic acid
AD	atopic dermatitis
ctrl	control
EPA	eicosapentaenoic acid

FLG	filaggrin
HEE	human epidermal equivalent
HEPE	hydroxy-eicosapentaenoic acid
HETE	hydroxy-eicosatetraenoic acid
HRNR	hornerin
IL1B	interleukin-1 beta
IV	ichthyosis vulgaris
KRT1	keratin 1
LB	lamellar body
LOR	loricrin
LOX	lipoxygenase
LY	Lucifer yellow
PLA_2	phospholipase A_2
PUFA	poly-unsaturated fatty acid
SC	stratum corneum
WT	wild type

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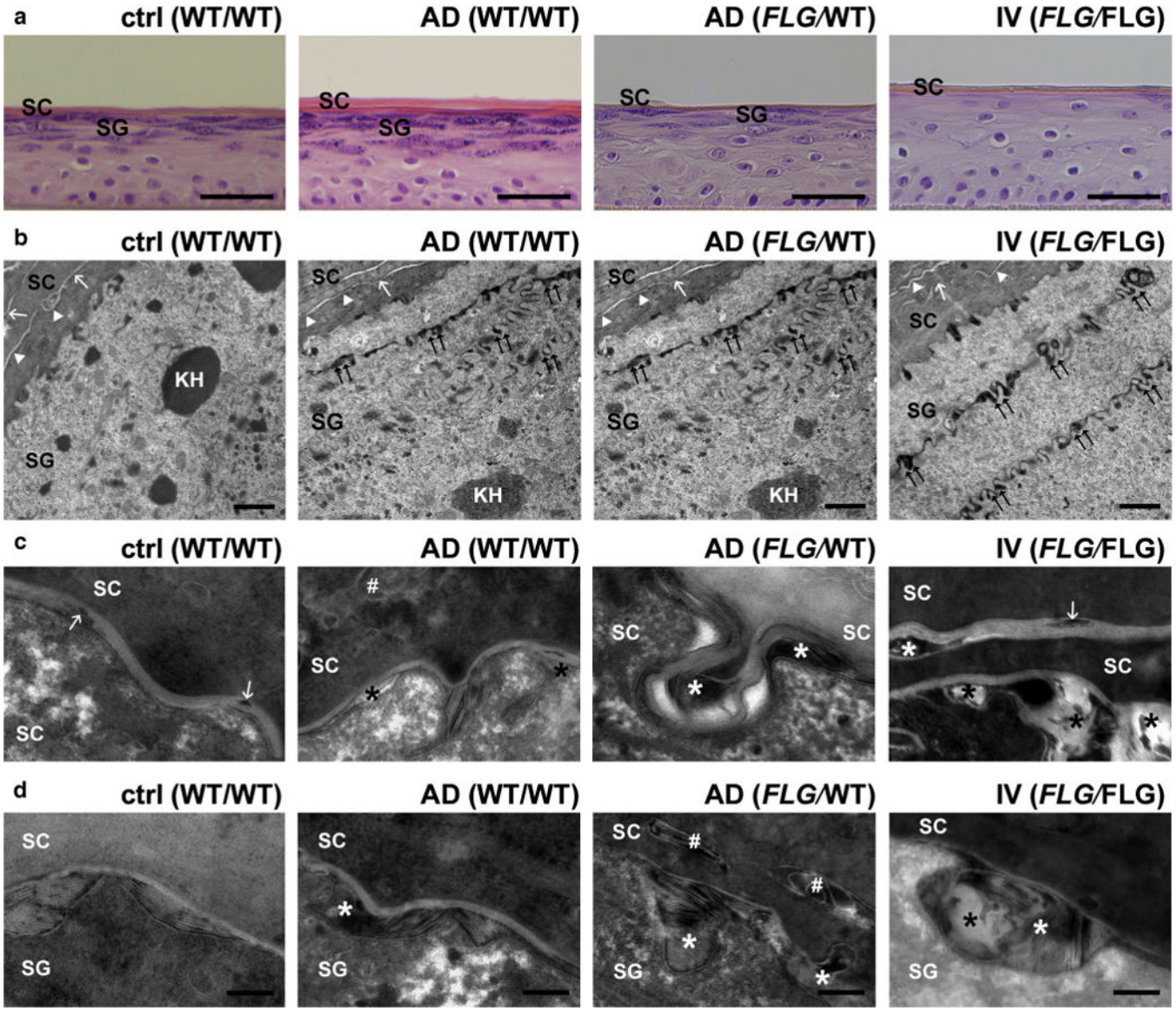

Figure 1. Morphology of AD and IV HEEs

HEEs generated with keratinocytes from healthy donors [ctrl (WT/WT)], from patients with AD (WT/WT), AD (*FLG*/WT), and IV (*FLG*/*FLG*), were analyzed. Representative images are shown. (**a**) H&E images. Bar = 50 μm. ctrl (WT/WT), n = 7; AD (WT/WT), n = 8; AD (FLG/WT), n = 3, and IV (FLG/FLG), n = 3. (**b**) Premature secretion of LB contents (double arrows) in AD and IV versus ctrl HEEs. Reduction and lack of KHGs in AD (*FLG*/WT) and IV HEEs. Intact CDs (arrows) and regular appearing CEs (arrowheads) in all groups. Bar = 1μm. (**c**) Disorganized lamellar bilayers with incompletely processed lamellar material (asterisks) in patient HEEs versus regular lamellar bilayer architecture in ctrl HEEs. Bar = 100 nm. (**d**) Inhomogeneous secretion areas (asterisks) at the SG-SC interface in AD and IV versus ctrl HEEs. LB entombment marked with hashtags. Bar = 100 nm. RuO_4 postfixation (**c, d**). (**b, c, d**) ctrl (WT/WT), n = 3; AD (WT/WT), n = 4; AD (FLG/WT), n = 2, and IV (FLG/FLG), n = 3. AD, atopic dermatitis; CD, corneodesmosomes; CE, cornified envelope;

ctrl, control; FLG, filaggrin; H&E, hematoxylin and eosin; HEE, human epidermal equivalent; IV, ichthyosis vulgaris; KHG, keratohyalin granules; LB, lamellar body; RuO_4, ruthenium tetroxide; SC, stratum corneum; SG, stratum granulosum.

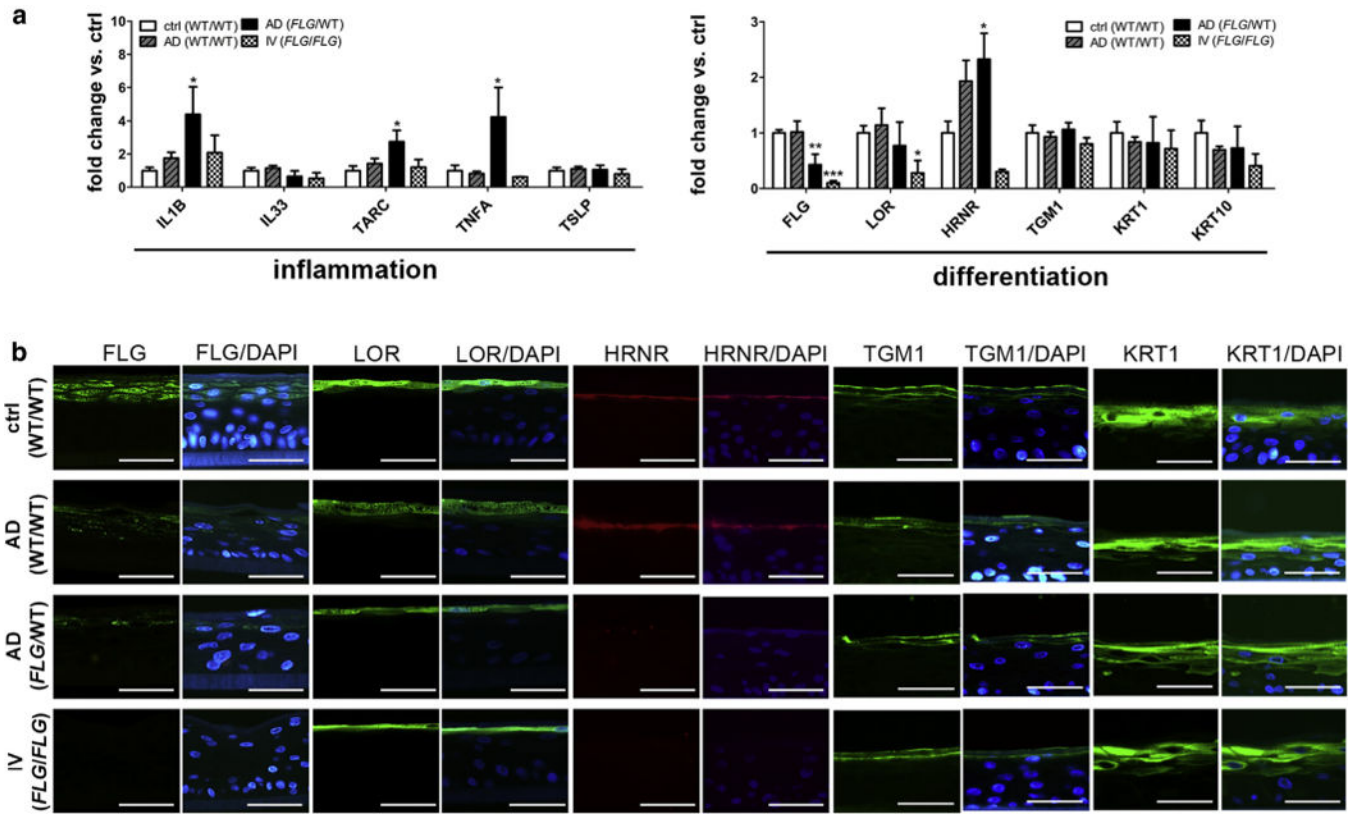

Figure 2. Differentiation and inflammation in AD and IV HEEs

HEEs generated with keratinocytes from healthy donors [ctrl (WT/WT), n = 7], and from patients with AD (WT/WT), n = 8, AD (*FLG*/WT), n = 3, and IV (*FLG*/*FLG*), n = 3, were analyzed. (**a**) mRNA levels of indicated differentiation- and inflammation-related genes as assessed by RT-PCR. Data were analyzed using a Student's *t*-test between ctrl and other groups. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. (**b**) Immunofluorescence staining of FLG, LOR, HRNR, TGM1, and KRT1. n = 3 per group. Representative images are shown. Bar = 50 μm. AD, atopic dermatitis; ctrl, control; FLG, filaggrin; HEE, human epidermal equivalent; HRNR, hornerin; IV, ichthyosis vulgaris; KRT1, keratin 1; LOR, loricrin; TGM1, transglutaminase 1; WT, wild type.

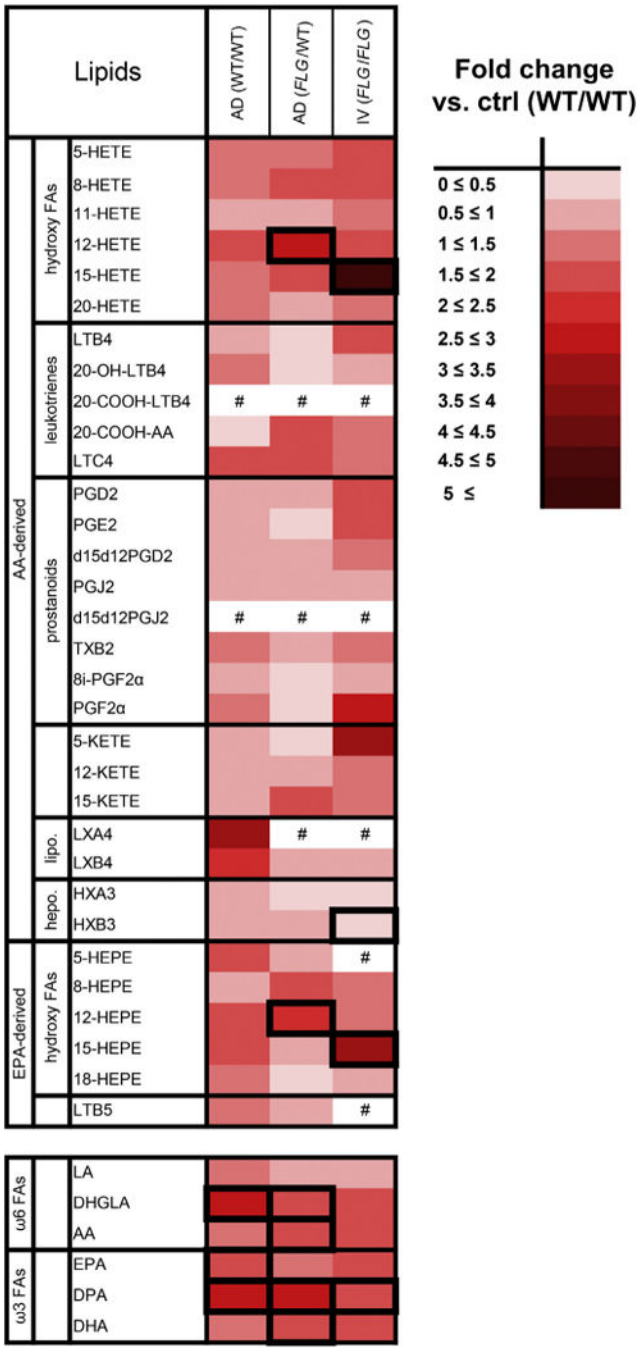

Figure 3. PUFAs and eicosanoids in AD and IV HEEs

Relative levels of AA- and EPA-derived eicosanoids (hydroxy-fatty acids, leukotrienes, prostaglandins, thromboxane, lipoxins, and hepoxilins) and of ω3- and ω6-PUFAs were measured by LC-MS and are displayed in a heatmap showing relative changes. Data are presented in fold change versus mean value of lipid concentrations in ctrl HEEs. Ctrl (WT/WT) (n = 10), AD (WT/WT) (n = 8), AD (*FLG*/WT) (n = 3), IV (*FLG*/*FLG*) (n = 3) HEEs. #, all samples of the indicated group were below our detection limit. Significant values were marked by a black frame. Data were analyzed using a Student's *t*-test versus

ctrl, $P \leq 0.05$. AA, arachidonic acid; AD, atopic dermatitis; ctrl, control; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FLG, filaggrin; HEE, human epidermal equivalent; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HX, hepoxilin; IV, ichthyosis vulgaris; LC-MS, liquid chromatography mass spectrometry; LTB, leukotriene B; LX, lipoxin; PUFA, poly-unsaturated fatty acid; TX, thromboxane; WT, wild type.

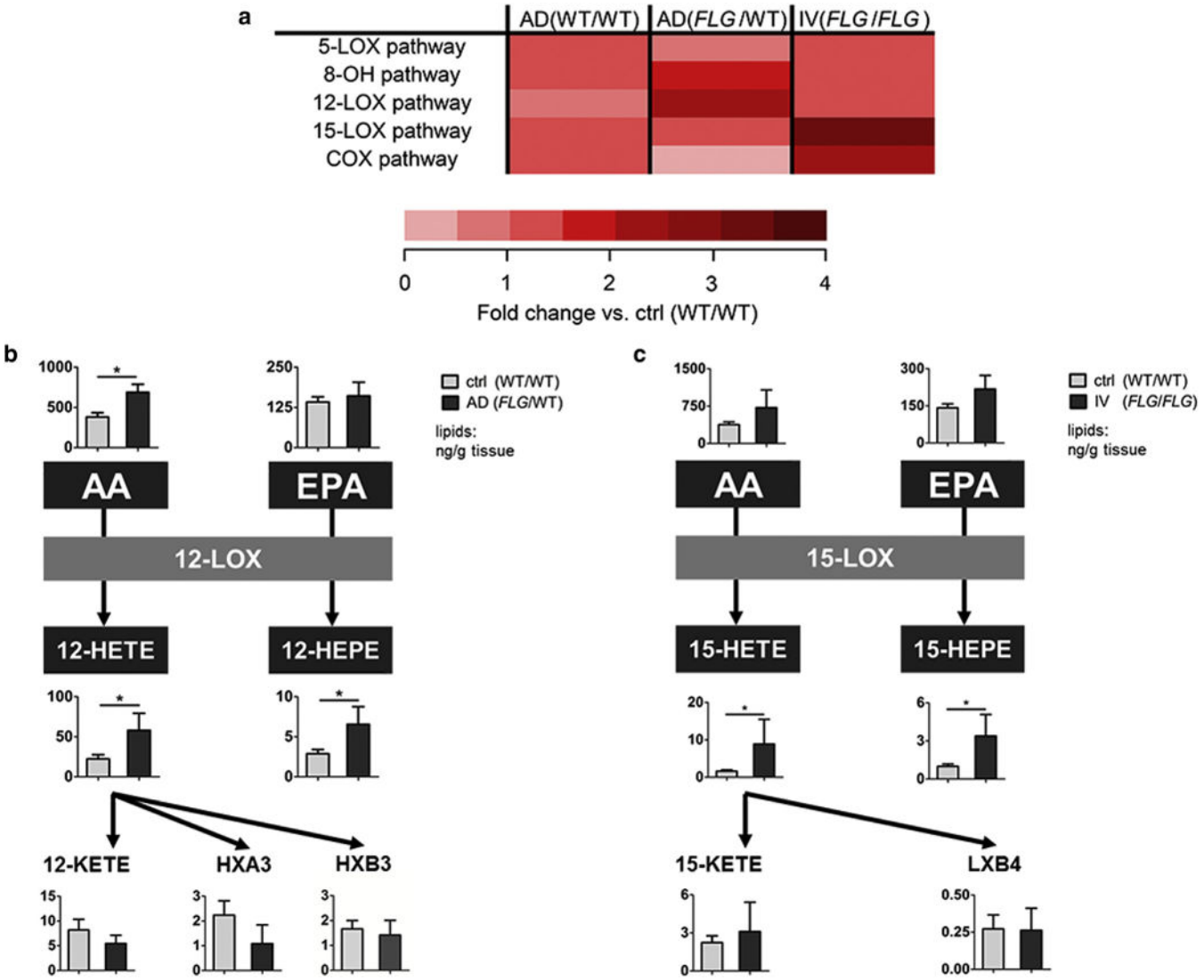

Figure 4. 12-LOX pathway metabolites are increased in *FLG*-mutated AD HEEs

(**a**) Heatmap displaying relative changes of 5-LOX, 12-LOX, 15-LOX, COX, and 8-OH pathway metabolites in AD (WT/WT) (n = 8), AD (*FLG*/WT) (n = 3), and IV (*FLG*/*FLG*) (n = 3) HEEs. Data are presented in fold change versus mean value of the sum of respective pathway metabolite concentrations in ctrl HEEs (n = 10). (**b**) 12-LOX pathway scheme displaying 12-HETE and 12-HEPE generated by 12-LOX from AA and EPA. (**c**) 15-LOX pathway scheme displaying 15-HETE and 15-HEPE generated by 15-LOX from AA and EPA. Data were analyzed using a Student's *t*-test versus ctrl. $*P \leq 0.05$. AA, arachidonic acid; AD, atopic dermatitis; COX, cyclooxygenase; EPA, eicosapentaenoic acid; FLG, filaggrin; HEE, human epidermal equivalent; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HX, hepoxilin; IV, ichthyosis vulgaris; KETE, keto-eicosatetraenoic; LOX, lipoxygenase; LX, lipoxin; 8-OH, 8-hydroxylation pathway; WT, wild type.

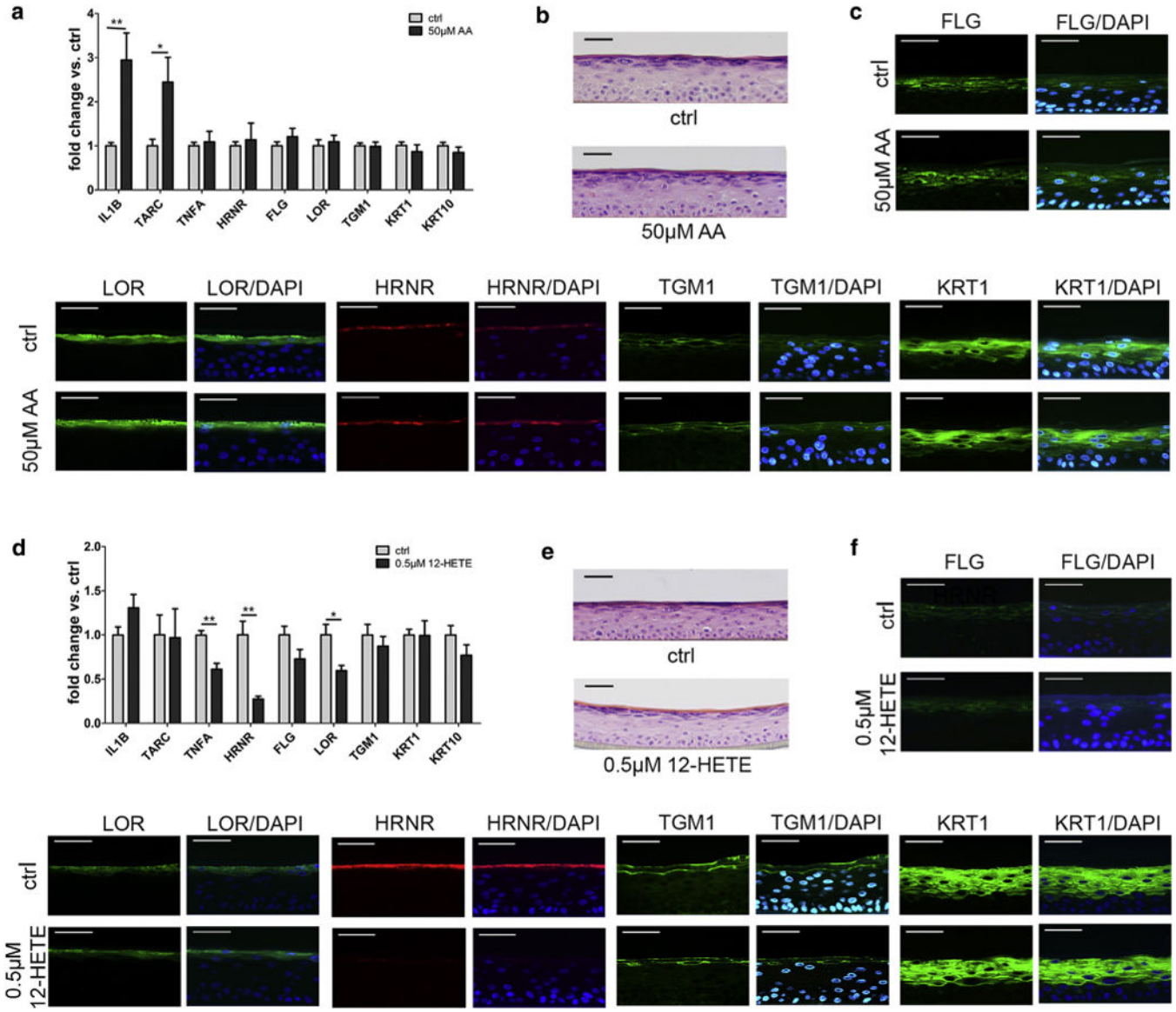

Figure 5. AA and 12-HETE treatment recapitulates characteristics of AD (*FLG*/WT) HEEs

Ctrl (WT/WT) HEEs were treated with 50 μM of AA or vehicle: (**a**) mRNA levels of indicated genes were determined by RT-PCR. Combined results from three independent experiments are shown. (**b**) Representative images of H&E staining. Bar = 50 μm. (**c**) Representative images of immunofluorescence staining for filaggrin, loricrin, hornerin, transglutaminase 1, and keratin 1. Bar = 50 μm; Ctrl (WT/WT) HEEs were treated with 0.5 μM of 12-HETE or vehicle: (**d**) mRNA levels of indicated genes were determined by RT-PCR. Combined results from two independent experiments are shown. (**e**) Representative images of H&E staining. Bar = 50 μm. (**f**) Representative images of immunofluorescence staining for filaggrin, loricrin, hornerin, transglutaminase 1, and keratin 1. Bar = 50 μm; Data were analyzed using a Student's *t*-test. $*P \leq 0.05$; $**P \leq 0.01$. AA, arachidonic acid; AD, atopic dermatitis; ctrl, control; FLG, filaggrin; H&E, hematoxylin and eosin; HEE, human epidermal equivalent; HETE, hydroxyeicosatetraenoic acid; HRNR, hornerin; IL1B,

interleukin-1 beta; KRT1, keratin 1; LOR, loricrin; TARC, thymus activation regulated cytokine; TGM1, transglutaminase 1; TNFA, tumor necrosis factor alpha; WT, wild type.

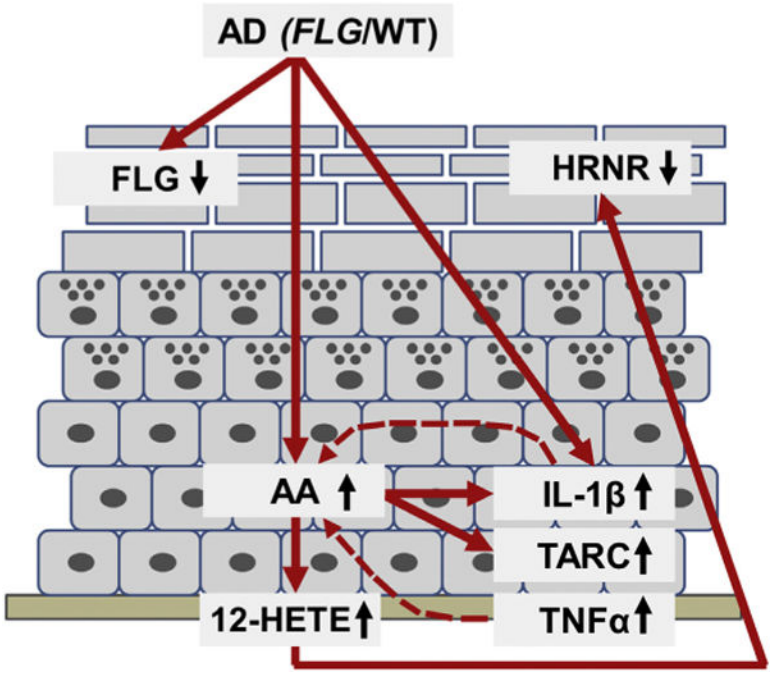

Figure 6. Role of eicosanoids in *FLG*-mutated AD

FLG loss-of-function mutations in AD lead to attenuated FLG expression, enhanced expression of inflammatory cytokines, and increased AA levels. AA triggers inflammation by upregulating *IL1B* and *TARC*. Conversely, IL-1β and TNFα increase AA concentrations in keratinocytes as demonstrated by Sjursen et al. (2000) (dotted line). Enhanced 12-LOX metabolism potentially leads to an increased conversion of AA into 12-HETE in *FLG*-mutated AD. As a result, increased levels of 12-HETE impair late epidermal differentiation. AA, arachidonic acid; AD, atopic dermatitis; FLG, filaggrin; HETE, hydroxy-eicosatetraenoic acid; HRNR, hornerin; IL1B, interleukin-1 beta; LOX, lipoxygenase; TARC, thymus activation regulated cytokine; TNFα, tumor necrosis factor α; WT, wild type.