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FILAGGRIN DEFICIENCY CONFERS A PARACELLULAR BARRIER ABNORMALITY THAT REDUCES INFLAMMATORY THRESHOLDS TO IRRITANTS AND HAPTENS

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Abstract

Background—Mutations in filaggrin (*FLG*) are associated with atopic dermatitis (AD), and are presumed to provoke a barrier abnormality. Yet, additional acquired stressors may be necessary, since the same mutations can result in a non-inflammatory disorder, ichthyosis vulgaris.

Objective—We examined here whether FLG deficiency alone suffices to produce a barrier abnormality; the basis for the putative abnormality; and its pro-inflammatory consequences.

Methods—Using the flaky-tail (ft/ft) mouse, which lacks processed flg due to a frame-shift mutation in profilaggrin that mimics some mutations in human AD, we assessed whether FLG deficiency provokes a barrier abnormality; further localized the defect; identified its subcellular basis; and assessed thresholds to irritant and hapten-induced dermatitis.

Results—Flaky-tail mice exhibit low-grade inflammation, with increased bidirectional, paracellular permeability of water-soluble xenobiotes due to impaired lamellar body secretion and altered stratum corneum extracellular membranes. This barrier abnormality correlates with reduced inflammatory thresholds to both topical irritants and haptens. Moreover, when exposed repeatedly to topical haptens, at doses that produce no inflammation in +/+ mice, ft/ft mice develop a severe AD-like dermatosis, with a further deterioration in barrier function and features of a th2

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immunophenotype (increased CRTH + inflammation, elevated serum IgE levels, and reduced antimicrobial peptide [mBD3] expression).

Conclusions—FLG deficiency alone provokes a paracellular barrier abnormality in mice that reduces inflammatory thresholds to topical irritants/haptens, likely accounting for enhanced antigen penetration in FLG-associated AD.

Keywords

Atopic dermatitis; barrier function; contact dermatitis; filaggrin; flaky tail mouse; lamellar body

INTRODUCTION

Atopic dermatitis (AD) is a chronic, relapsing, inflammatory skin condition, that displays an associated abnormality in permeability barrier function 1–4. Until recently, AD was viewed as a primary immunologic disorder in which sustained allergen exposure in susceptible hosts leads to TH2-dominant inflammation, which then secondarily provokes defects in barrier function ('inside-outside' paradigm) [rev in 5]. However, recent genetic studies have shown a strong association between AD and several loss-of-function mutations in the gene encoding the stratum corneum structural protein, filaggrin (FLG) 6, 7. Moreover, there is a dose-response relationship between FLG-deficiency and disease severity, such that patients with double-allele or compound heterozygote mutations in FLG display more-severe and earlier-onset AD, as well as an increased propensity for AD to persist into adulthood 8–11. This rapidly-growing body of work has led to a paradigm shift in conceptions of AD pathogenesis, with increasing weight being placed on the role of a primary barrier abnormality that then precipitates downstream immunologic abnormalities ("outside-inside" paradigm) [1,12]. According to this concept, TH2-dominant inflammation results from sustained epicutaneous access of haptens through a defective skin barrier 13,14. TH2 cytokines then down-regulate expression of TH1 cytokines [5], epidermal antimicrobial peptide expression 15, 16, and epidermal structural proteins [rev. in 1, 2], completing an "outside-inside-(back to) outside" paradigm for AD 2, 17.

FLG is an intracellular protein, with important roles in stratum corneum hydration [18] and as a structural component of the corneocyte cytosol [19]. However, normal permeability barrier function is not regulated by the corneocyte, but rather by the lipid-enriched, extracellular matrix of the stratum corneum [20, 21]. Indeed, in all inflammatory dermatoses studied to date, including those associated with inherited disorders of corneocyte proteins, the barrier abnormality has been linked to accelerated paracellular water loss [22, 23]. Thus, the new-found link between FLG and AD leaves unanswered whether FLG-deficiency alone provokes a barrier abnormality in the epidermis, and if so, the subcellular basis for such an abnormality. While it has been widely hypothesized that a FLG-associated barrier defect facilitates hapten ingress [12, 13], this relationship was only recently demonstrated [24]. Yet, the mechanism whereby FLG deficiency compromises barrier function, thereby provoking inflammation, remains unknown. Pertinently, *FLG* mutations that occur in AD also occur in the inherited, non-inflammatory condition, ichthyosis vulgaris [25, 26], raising questions about whether FLG deficiency alone suffices to provoke inflammation, or whether additional acquired stressors are required to elicit AD [1, 2].

The above questions are difficult to address within the context of human AD, where multiple genetic and environmental factors intertwine to provoke disease pathogenesis. Thus, we employed a pre-existing model, the flaky tail (ft/ft) mouse, which exhibits very reduced levels of flg [27]. Flaky tail mice possess a homozygous frameshift mutation in *profilaggrin* that prevents the processing of profilaggrin into flg 24, such that ft/ft mice simulate double allele,

loss-of-function mutations in certain human AD patients that also result in reduced levels of processed FLG protein 6, 28. The ft/ft mutation was shown previously to yield a phenotype of diffuse, mild flaking, analogous to human ichthyosis vulgaris 27. Utilizing the ft/ft model, we demonstrate that ft/ft mice display: 1) abnormal barrier function and low-grade inflammation at baseline, confirming recent observations of Fallon, et al. [24]; and 2) enhanced bidirectional, paracellular penetration of water-soluble tracers. We demonstrate further that: 1) Increased penetration through the stratum corneum can be attributed to structural defects in the lamellar body secretory system; 2) flg deficiency alone reduces inflammatory thresholds to both irritants and allergens; and 3) ft/ft mice develop a hapten-induced, AD-like dermatosis at lower challenge doses than do +/+ mice, mirroring recent work in ft/ft mice exposed to a complete antigen [24]. Thus, flg deficiency yields a paracellular barrier abnormality that likely also favors elicitation of AD by allowing enhanced/sustained hapten access.

METHODS (SECTION MOVED FROM AFTER DISCUSSION SECTION)

Materials and animals

Studies were conducted in maft/maft/J(ft/ft) mice (new designation: a/a ma ft/ma ft/JSun JR#9078), and age/sex-matched C57Bl/6J+/+ mice (The Jackson Lab, Bar Harbor, ME), housed under pathogen-free conditions. Flaky tail mice are compound mutants at two loci, both matted (ma/ma) and flaky tail (ft/ft). Matted confers a stable, lifelong, and readily-identifiable phenotype of subtle hair coarseness that was introduced by Jackson to facilitate identification of mutants without reliance on genotyping [29]. Young ft/ft and +/+ mice were between 6–12 weeks of age, while old mice were 50–52 weeks of age. Ethanol, acetone and lanthanum nitrate were purchased from Fisher Scientific (Fairlane, NJ). Oxazolone (Ox) and calcium green were purchased from Sigma Chemical Co. (St Louis, MO). Affinity-purified, rabbit anti-mouse antibodies to both proflg and flg were purchased from BabCo (Richmond, CA). Rabbit anti-mouse antibody against the prostaglandin D2 receptor, CRTH2/DP2, which is expressed solely on th2-bearing lymphocytes and mast cells, was purchased from Cayman Chemical (Ann Arbor, MI). Rabbit anti-mouse antibody against CD3 was purchased from BD Biosciences (San Jose, CA).

Experimental protocols and functional studies

All animal procedures were approved by the Animal Studies Subcommittee (IACUC) of the San Francisco Veterans Administration Medical Center and performed in accordance with their guidelines. Basal stratum corneum hydration and surface pH were measured (CM 825/PH 900, Courage & Khazaka, Germany) on shaved back skin in ft/ft and +/+ mice (n = 4–6 in all experimental groups, except where noted in figure legends). Transepidermal water loss (TEWL) measurements were taken under basal conditions, using an electrolytic water analyzer (Meeco, Warrington, PA), as well as 2 and 4 hours after a tape stripping-induced, 3-fold increase in TEWL. Barrier recovery was calculated as percent barrier recovery from '0' levels, immediately after acute disruption [30–37].

Irritant and acute allergic contact dermatitis were induced in ft/ft and +/+ mice using TPA and Ox, respectively, as described previously [38]. TPA (either 0.3% or 0.1% in acetone) was applied once to the inner and outer surface of the ear. Challenge doses of Ox (either 0.02% or 0.5% in acetone) were applied once following initial Ox sensitization (2% Ox). In all cases, ear thickness was measured 2 hrs post-treatment.

To induce an AD-like dermatosis in mice, ft/ft and +/+ mice were sensitized with 2% Ox, as above. One week later, shaved areas on the flanks of ft/ft and +/+ mice were treated every other day topically with 60 μl of 0.5% or 0.02% Ox for an additional 3 weeks (10 challenges). Other groups of ft/ft and +/+ mice were treated with ethanol alone as the vehicle control group (n =

10–12 in each group). At the end of treatments, basal TEWL and stratum corneum hydration were measured again.

Immunohistochemistry

Changes in overall morphology were visualized after hematoxylin and eosin staining of 5 μm paraffin-embedded tissue samples. Both the number of epidermal nucleated cell layers and the density of the inflammatory infiltrate were measured in 20–30 randomly-chosen fields from ft/ft and +/+ mice before and after Ox challenge doses, as above. Immunohistochemical assessment of changes in profilaggrin/flg, CTRH2, and CD3 were performed, as described previously [39]. Briefly, 5 μm paraffin sections were incubated with the primary antibodies overnight at 4°C. After washing, sections were incubated with the secondary antibody for 30 min. Staining was detected with ABC-peroxidase kit from Vector Lab. Frozen sections were examined with a Zeiss fluorescence confocal microscope (Jena, Germany), and digital images were captured with AxioVision software (Carl Zeiss Vision, Munich, Germany). A similar sequence was employed to assess two antimicrobial peptides in 5 μm frozen sections [primary antibodies to mBD3 from Alpha Diagnostics; mouse cathelicidin (CAMP) antibody was a gift from Dr. Richard Gallo (UCSD)].

Ultrastructural Lipase Detection

Ultrastructural cytochemistry of lipase activity has been employed as a content marker for epidermal lamellar bodies, allowing assessment of the efficacy of organelle secretion [40]. Briefly, samples were incubated in 5% Tween 85 (1 ml) in 0.2M HEPES buffer (2.5 ml), 2.5% sodium taurocholate (2 ml) and 10% calcium chloride (1 ml) in 25 ml distilled water (pH 7.4). Microwave incubations were carried out twice for 30 sec at 2450 MHz, and the water bath was changed between the pulses, in order to maintain temperatures below 40°C [41]. After incubation in the same medium for an additional 30 min at 37°C, all samples were incubated further in 0.1M cacodylate buffer containing 0.2% Tween 85 with our without 200 μm of the lipase inhibitor, tetrahydrolipstatin, as we have described 42.

Tracer Penetration

To assess the outward permeation of water-soluble markers, freshly-obtained explants of back skin from untreated ft/ft and +/+ mice were exposed dermis-side-downward (30 min-2 hrs) to the low-molecular weight tracer, colloidal lanthanum nitrate (4%) in Karnovsky's solution. To assess permeation from the outside, we placed 3 +/+ control and 3 ft/ft mice in Petri dishes, while under general anesthesia, with one side of each mouse immersed in 4% colloidal lanthanum, in a 37°C incubator for 2 hr. Lanthanum nitrate was prepared in 4% w/v sucrose in 0.05 M Tris buffer, pH 7.5. After immersion, biopsy samples were taken and processed as below. In parallel studies, a 20 μM solution of calcium green 5N (Invitrogen) in nanopure water was applied on the stratum corneum side of ft/ft and wt mice for 10 and 75 min. Ten mm biopsies were mounted immediately on microscope coverslips, washed in nanopure water, and imaged in a Zeiss 510 meta NLO (Zeiss Germany) at the Live Cell Analysis Core Facility at UCSF. An optically-pumped (Coherent) mode-locked Mira 900 Ti:Saph laser (Coherent), was used as the two-photon excitation source at a wavelength of 800 nm. A 500–550 nm band pass filter was placed in the detection path in order to minimize sample autofluorescence. Under these experimental conditions, we estimated the contribution of autofluorescence to the total signal to be <5%. Z-stacks of 235×235×20 μm^3 images were acquired, beginning with the outer surface of the stratum corneum at z increments of 1 μm using a 40× oil immersion objective with a 1.3 numerical aperture (Zeiss, Germany). Matlab software (The Math Works) was used to quantitate image intensity at each z-stack, and to calculate mean penetration depths.

Electron Microscopy

Aldehyde-fixed biopsies were post-fixed with either 0.25% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide, as described previously [43]. Ultrathin sections were examined using an electron microscope (Zeiss 10A, Carl Zeiss, Thornwood, NY) operated at 60 kV. Images were captured using Digital Micrograph 3.10.0 software from Gatan, Inc. (Pleasanton, CA).

Serum IgE

Blood samples were collected from ft/ft and wt mice under basal conditions, and after prior Ox sensitization, followed by repeated treatments with either Ox or vehicle. Serum IgE concentrations were determined with a mouse IgE ELISA quantitation kit from Bethyl Laboratories (Montgomery, TX), following instructions provided by the manufacturer.

In these, and all other studies, statistical analyses were performed using a Student's t test to compare differences between two groups, with a further ANOVA analysis when three or more groups were compared.

RESULTS

Filaggrin Deficiency Results in Abnormal Barrier Function

Prior studies have shown that ft/ft mice display markedly-reduced F-type keratohyalin granules, with an absence of flg [24], due to a recently-identified, frame-shift mutation in *profilaggrin* [24]. Using a consensus antibody that recognizes both profilaggrin and flg, we found intense, positive immunostaining restricted to the stratum granulosum (granular layer), with no residual staining of flg in the stratum corneum in ft/ft mice (suppl Fig. 1, green staining). In contrast, +/+ epidermis displayed immunostaining in the granular layer, as well as diffuse staining of the stratum corneum. Thus, the mutation in *profilaggrin* in ft/ft mice results in reduced flg in the SC, analogous to some humans with FLG-associated AD [6, 28].

We next assessed epidermal functional parameters in ft/ft and wild-type (+/+) mice, using biophysical instrumentation. Permeability barrier homeostasis, assessed as the kinetics of transepidermal water loss (TEWL) levels after acute disruption [44], but basal barrier function declines with aging in +/+ aged (52–54 wk) mice, as previously described 44 (Fig. 1A). Though within the range of normal ($\leq$ 2.5 mg/cm^2/hr), basal TEWL levels increase significantly (by ≈20–30%) in 52–54 week old ft/ft mice compared to age-matched +/+ controls (Fig. 1A). As occurs in human AD [e.g., 30], the kinetics of barrier recovery after acute barrier abrogation accelerate in old ft/ft vs. +/+ mice (Fig. 1B). Such accelerated barrier recovery also occurs in other chronic inflammatory dermatoses, and is attributed to ongoing signaling of repair mechanisms, which are induced by barrier abrogation [e.g., 31, 32]. Finally, stratum corneum hydration, assessed as changes in electrical capacitance, does not differ in young ft/ft vs. +/+ mice (Fig. 1C), but stratum corneum hydration is significantly lower in young ft/ft mice, with a further decline in older ft/ft mice (Fig. 1C), again mirroring known changes in human AD [33, 45].

Since basal TEWL levels lie within the normal range in ft/ft mice, we next assessed barrier function in these mice under basal conditions by an alternate approach (i.e., ultrastructural visualization of tracer perfusion). The high resolution of this method also allowed us to determine simultaneously whether the barrier abnormality can be attributed to a defective corneocyte or to enhanced paracellular transport. In +/+ mice, the water-soluble, low molecular weight, electron-dense tracer, lanthanum nitrate, does not breach the stratum granulosum-stratum corneum interface (Fig. 2A). In contrast, tracer not only breaches this layer, but also extends several layers outward into the stratum corneum in ft/ft mice (Fig. 2B). Notably, despite

the *intracellular* localization of flg, the tracer remains largely confined to the stratum corneum *extracellular* spaces in ft/ft mice (Fig. 2B, arrows). Together, these results demonstrate that flg-deficiency leads to alterations in basal barrier function via a defect in the stratum corneum extracellular matrix.

Enhanced Uptake of Epicutaneously-Applied, Water-Soluble Tracers via the Extracellular Route

Normal stratum corneum does not allow entry of water-soluble xenobiotes [46]. Therefore, we next determined whether epicutaneously-applied, water-soluble tracers enter the stratum corneum of flg-deficient mice, and by which route. Lanthanum nitrate tracer was applied topically to the flanks of ft/ft and +/+ mice for 30 min to two hrs, while under general anesthesia. As shown previously [46], lanthanum fails to breach the stratum corneum of +/+ mice (Fig. 3A). In contrast, tracer permeates 3–5 layers deep into the stratum corneum of ft/ft mice by 2 hrs (Fig. 3B&C). Notably, the tracer localizes solely within the extracellular spaces, indicating again that penetration occurs via the paracellular route (Fig. 3C, arrows). In parallel studies, we visualized the permeation of the low-molecular weight, water-soluble fluorophore, calcium green, by dual photon, confocal microscopy. Differences in the extent of penetration can be seen as early as 10 min after application of tracer to ft/ft skin, with further penetration evident by 75 min in ft/ft mice (Fig. 3D&E). In contrast, very little of the fluorophore can be detected beneath the outer stratum corneum of +/+ mice at 75 min. Together, these results demonstrate that the stratum corneum of flg-deficient mice is more permeable to water-soluble tracers, and that penetration of an electron-dense, water-soluble tracer occurs via the same paracellular pathway that is utilized by water itself, exiting the skin.

Structural Basis for Altered Permeability in Flaky Tail Mice

We next assessed the structural basis for the extracellular barrier abnormality in ft/ft mice. By standard transmission electron microscopy, ft/ft mice display not only the previouslyreported paucity of F-type keratohyalin granules in the stratum granulosum [cited in 19], but also a previously-undescribed abnormality in lamellar body secretion (Fig. 4). Lamellar body formation appears normal (neither lamellar body density nor organelle contents differ in ft/ft vs. +/+ mice). Yet, substantial numbers of lamellar bodies appear to be retained within terminally-differentiating keratinocytes in ft/ft mice (Fig. 4A vs. B). These unsecreted organelles then become entombed within the cytosol of nascent corneocytes, instead of being secreted *in toto* into the extracellular matrix, as occurs in +/+ mice (Fig. 4C vs. D). Partial entombment (failed secretion) of lamellar bodies could also be demonstrated by an alternate method: identification of the fate of a lamellar body content marker (acid lipase) by ultrastructural cytochemistry [32, 40]. In +/+ epidermis, enzyme activity localizes both to lamellar body contents (suppl Fig. 2A), and within the stratum corneum, solely to the extracellular domains (suppl Fig. 2B). Flaky tail mice reveal a similar localization of enzyme activity to lamellar bodies, but evidence of reduced secretion; i.e., lower amounts of initially-secreted enzyme at the stratum granulosum-stratum corneum interface, as well as sparse (reduced) enzyme product in the stratum corneum interstices (suppl Fig. 2C, arrows). In contrast to +/+ mice, the cytosol of many ft/ft corneocytes contains abundant enzyme activity, with enzyme activity localizing near retained lamellar arrays (suppl Fig. 2D), further evidence for entombment of lamellar body contents.

We next assessed the consequences of impaired lamellar body secretion and enhanced paracellular transport in ft/ft mice. As a result of impaired lamellar body secretion, the quantities of extracellular lamellar bilayers correspondingly decline in the stratum corneum of ft/ft mice (Fig. 4C vs. D). Moreover, the extracellular processing of newly-secreted lamellar body contents into ‘mature’ lamellar bilayers appears to be impaired or delayed in ft/ft mice (Fig. 4C vs. D). Together, these results suggest that either profilaggrin accumulation or flg

deficiency impedes lamellar body secretion, leading to an abnormal stratum corneum extracellular matrix in ft/ft mice.

Filaggrin Deficiency Alters Thresholds for Irritant and Acute Allergic Contact Dermatitis

We next assessed whether the barrier abnormality in ft/ft mutant mice results in an increased propensity to develop cutaneous inflammation. Flaky tail skin displays low-grade inflammation under basal conditions, indicated by the presence of increased numbers of inflammatory cells under basal conditions (Figs. 5B, E&F; histologic images that correspond to these quantitative data are shown in supplementary Fig. 3C vs. A). Moreover, most, but not all ft/ft mice display elevated serum IgE levels, even under basal conditions (Fig. 5G). Together, these results suggest that ft/ft mice display low-grade inflammation, even under basal conditions.

The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), elicits irritant contact dermatitis when applied topically to mouse ears, with the severity of the inflammatory response correlating with an increase in ear thickness two (2) hours after topical applications [34, 47]. TPA was first applied at a standard concentration of 0.3% to the ears of ft/ft and +/+ mice. Although inflammation appeared greater in ft/ft mice, the differences in ear thickness in ft/ft vs. +/+ mice do not achieve statistical significance (Fig. 6A). However, at a lower dose of TPA (0.1%) that provokes only marginal inflammation in +/+ mice, ft/ft mice display a marked inflammatory response, with a significant increase in ear thickness (Fig. 5A;$p < 0.001$; parallel histologic differences are shown in suppl Fig. 4). Together, these results demonstrate that ft/ft mice display an enhanced propensity to develop irritant contact dermatitis.

We next assessed whether ft/ft mice display an altered threshold to the development of acute allergic contact dermatitis. For these studies, we utilized a single challenge dose (2%) of the universal hapten, oxazolone (Ox), followed one week later by topical application to the ear of either a single challenge dose at a concentration (0.5%) which produces a prominent dermatitis after 24–48 hrs [47, 48], or a much lower hapten concentration (0.02%), which does not produce visible inflammation in +/+ mice. As with the topical irritant (c.f., Fig. 5A), dermatitis appears more exaggerated in ft/ft mice, after challenges with the standard (0.5%) hapten dose, but again the results do not achieve statistical significance (Fig. 5B). At the lower challenge dose of Ox (0.02%), dermatitis does not develop in +/+ mice, while in contrast, substantial inflammation is evident in similarly-challenged ft/ft mice (Fig. 5B; $p<0.0001$ vs. +/+; parallel histologic differences are shown in suppl Fig. 4). These results show that ft/ft mice display a reduced threshold for the development of hapten-induced, acute allergic contact dermatitis.

Filaggrin Deficiency Predisposes To the Development of an AD-Like Dermatosis in Mice

We previously showed that Ox sensitization, followed by repeated (10×) hapten challenges over 20–21 days produces a dermatosis with certain structural, biochemical, and immunologic features of human AD [49]. In the next set of studies, we used reduced challenge doses of Ox (10 challenges with 0.02% vs. the standard 0.5% concentration) in ft/ft and +/+ mice. Whereas +/+ mice display minimal signs of inflammation, ft/ft mice reveal erythema, hyperkeratosis, epidermal hyperplasia, and histologically-prominent inflammation at the lower hapten concentration (Figs. 6E&F; suppl Fig. 3). Moreover, while virtually no $CD3^+$ lymphocytes are detected in +/+ mouse skin, a moderately-high density of $CD3^+$ cells can be seen in ft/ft mice repeatedly challenged with low-dose Ox (Fig. 6D). Flaky tail mice also display numerous prostaglandin D2 receptor (CRTH2)-positive cells, consistent with a th2-dominant immunophenotype, as well as much higher serum I_GE levels than do comparably-treated +/+ mice (Figs. 7A&B). Finally, ft/ft mice, repeatedly challenged with low-dose Ox, display reduced immunostaining for mBD3 (mouse homologue of hBD2 [suppl Fig. 5D]), but staining for cathelicidin antimicrobial peptide (CAMP = mouse homologue of LL37) instead appears

to increase (suppl Fig. 5C). Together, these results indicate that flg deficiency predisposes to development of a hapten-stimulated AD-like dermatosis in mice.

More Severe Barrier Abnormality in Filaggrin-Deficient Mice with AD

In normal mice, repeated challenges with 0.5% Ox result in a progressively more-severe barrier abnormality 49, consistent with a proposed 'outside-inside-outside' pathogenic cycle in AD [1, 2]. We next assessed whether repeated, sub-threshold (0.02%) doses of Ox would provoke functional abnormalities in ft/ft mice that occur only at higher doses in +/+ mice. While stratum corneum hydration does not decline in such Ox-challenged mice (Fig. 3F), ft/ft mice develop a severe permeability barrier abnormality at sub-threshold doses of hapten (Fig. 3G), evidenced by an acceleration of TEWL levels to levels produced by full-challenge hapten doses in +/+ or normal mice (c.f., 49). These results show that development of an AD-like dermatosis in flg-deficient mice is paralleled by a further exacerbation of the permeability barrier abnormality.

DISCUSSION

Human atopic dermatitis (AD) is increasingly viewed as a primary barrier disorder, based upon recent data from multiple populations that have demonstrated an association with mutations in *FLG*, the gene encoding the stratum corneum structural protein, filaggrin (FLG). Yet, while these molecular genetic studies have shifted views of AD pathogenesis towards a new 'outside-to-inside' pathogenic paradigm [1, 2], they leave unanswered mechanistic questions of how FLG-deficiency leads not only to a barrier abnormality, but also to an inflammatory phenotype in AD. Because such questions are difficult to address in the complex, multifactorial environment of human disease, we addressed these issues in an animal model of flg deficiency, the flaky tail (ft/ft) mouse. Recently, these mice were shown to carry a frameshift mutation with one base pair deletion (5303delA) in exon3 of *flg* [24], which mimics two common, distal mutations in human AD (i.e., R2447X in repeat 7 and 1033del4 in repeat 10) [6, 28].

In our examination of ft/ft mice, we first confirm that flg deficiency alone suffices to induce a barrier abnormality and low-grade inflammation under basal conditions, as shown recently for ichthyosis vulgaris 50. We also provide subcellular pathogenic insights, showing that despite its intracellular localization, flg deficiency results in enhanced paracellular permeability to both intradermally and epicutaneously applied water-soluble tracers, which do not penetrate +/+ stratum corneum. Movement of these low-molecular weight, water-soluble tracers through the stratum corneum interstices likely reflects the pathway for hapten ingress in AD, since similarly-sized, water-soluble antigens, such as nickel, more frequently induce allergic contact dermatitis in humans with AD [51]. However, since our ft/ft mice were not on a homogenous C578BL6 background, but also express a hair phenotype (matted), we cannot eliminate the possibility that some of our observations could have been influenced by the concurrent *matted* mutation. Nevertheless, the recent work by Fallon, et al [24] shows enhanced antigen ingress in mice with the same *flg* mutation, but no matted in their background. Thus, flg deficiency alone likely explains the barrier abnormality that we demonstrate here in flaky tail mice.

Localizing the barrier defect in FLG deficiency to the extracellular matrix is an important first step in understanding AD pathogenesis. Yet, a key question remains unanswered: how can a defect in an *intracellular* protein, such as FLG, yield a more-porous *extracellular* pathway? FLG has two putative functions: 1) mediation of stratum corneum hydration through humidity-dependent proteolysis of FLG into its constituent amino acids and their deiminated products (e.g., histidine → trans-urocanic acid)[18, 52]; and 2) keratin intermediate filament compaction within the corneocyte cytosol [19, 53]. Examination of either of these roles does not immediately suggest a mechanism by which FLG deficiency could lead to increased *extracellular*

permeability. Potential clues are provided by other inherited defects of intracellular corneocyte proteins that also provoke increased paracellular permeability, but by divergent mechanisms [54]. In one example, in K1/10-deficient epidermolytic hyperkeratosis, dominant-negative pairing of the mutant protein disrupts the cytoskeleton, impairing lamellar body secretion 32. In a second, alternate mechanism, both loricrin keratoderma and transglutaminase 1-deficient lamellar ichthyosis yield an abnormal corneocyte scaffold that disrupts the supramolecular organization of the extracellular lamellar bilayers 36, 37. A third mechanism, mutations in the V1 subdomain of K1 that produce palmar plantar keratoderma, provokes detachment of keratin filaments from the cornified envelope 55. This defect could link an abnormally-collapsed corneocyte to an extracellular defect, yet frozen sections of flg-deficient stratum corneum display corneocytes of normal shape and dimensions (Elias, unpublished observations). Our ultrastructural studies on ft/ft mice show a partial failure of lamellar body secretion, as shown previously for non-genotyped human AD 56. Likewise, our preliminary studies in genotyped ichthyosis vulgaris +/− AD patients show a similar blockade in lamellar body secretion 50. Thus, analogous to K1/10-deficient epidermolytic hyperkeratosis 32, unprocessed profilaggrin might impede lamellar body secretion (op. cit.) (suppl. Fig. 2). This mechanism likely also pertains to human AD associated with distal *FLG* mutations, in which residual profilaggrin expression is seen, but processed FLG is reduced [28]. Yet, it is not clear how this mechanism might apply to those cases of AD caused by more proximal *FLG* mutations, which instead decrease both profilaggrin and FLG. A fourth mechanism, which could apply to both categories of FLG mutations, could be linked to decreased generation of acidic metabolites of FLG [1, 2] suppl. Fig. 6). Deficiency in these polycarboxylic acids could result in a net increase in the pH of the stratum corneum, which in turn could activate neutral-pH-dependent kallikreins, with a variety of negative, downstream consequences for the barrier [1,2]. Such a pH-dependent mechanism could also lead to deactivation of two key ceramide-generating enzymes, acidic sphingomyelinase and β-glucocerebrosidase (suppl. Fig. 6)57. This mechanism may not, however, be operative in ft/ft mice, because the surface pH of these mice remains normal, perhaps due to compensatory upregulation of alternate, endogenous acidifying mechanisms [rev. in 58], such as solute carrier family 9 (sodium-hydrogen exchanger, 1 (slc9a) (old symbol NHE1) 59 and/or secretory phospholipase A_2 [60]. Nonetheless, surface pH measurements do not always reflect pH alterations within specific microdomains of the stratum corneum [59]. We did not specifically examine such potential, compensatory mechanisms in this study.

While our studies demonstrate the increased movement of topically-applied, water soluble compounds through the stratum corneum in ft/ft mice, the movement of water from inside to outside through the stratum corneum (TEWL) is not markedly altered in young ft/ft mice, becoming only slightly elevated in older ft/ft mice in comparison to older +/+ mice. The development of a barrier abnormality with aging of ft/ft mice could reflect the added stress from a steeper water gradient across the SC of older animals reflecting the observed decrease in SC hydration (Fig. 1C). Alternatively or additionally, it could reflect age-related differences in permeability barrier homeostasis 44, 61. While the explanation for the discordance in TEWL rates vs. xenobiote penetration is not clear, it could reflect differences in the thresholds for the barriers to water loss vs. xenobiote ingress/egress, respectively. Pertinently, to increase TEWL levels by tape stripping, multiple layers of the stratum corneum must be removed [62]. Thus, there appears to be substantial redundancy, with only a relatively small amount of stratum corneum required to maintain a normal TEWL. Conversely, the structural basis for inhibiting water movement from inside to outside (i.e., TEWL) could differ from the structural basis for inhibiting the egress or ingress of somewhat larger, water-soluble compounds. Since there are several possible mechanisms that could explain how FLG deficiency leads to a marked abnormality in xenobiote permeation, without altering water loss, additional studies will be required to distinguish among these alternatives.

Having established that FLG deficiency results in an extracellular barrier defect, we next addressed a question of key functional significance; namely, does this barrier defect suffice to alter thresholds to the development of inflammation from epicutaneously-applied irritants and haptens? While *FLG* mutations are associated with the inflammatory disease, AD (op. cit.), the relationship between FLG deficiency and inflammation is obscured by the association of the same *FLG* mutations with the supposedly noninflammatory disorder of cornification, ichthyosis vulgaris (IV). Additional stressors to the barrier could be required to provoke human AD [1, 2], because human skin displays an inherently more competent barrier [63–65] (see below). Nevertheless, the presumption that IV is non-inflammatory should now be re-examined, in light of our observations, and those of Fallon, et al. [24], which show that flg deficiency alone results in epidermal hyperplasia, low-grade inflammation, and elevated IgE levels in otherwise normal mice.

In order to ascertain whether FLG deficiency alone leads to increased susceptibility to inflammatory dermatoses, we employed previously described methods for eliciting irritant and acute allergic contact dermatitis [38, 47], as well as an AD-like mouse model [49], in ft/ft vs. +/+ mice. Our results show that FLG deficiency alone lowers thresholds to inflammation following epicutaneous applications of either irritants or haptens. Most importantly, we demonstrate development of an AD-like phenotype following applications of reduced concentrations of haptens, which do not produce th2-dominant inflammation in +/+ mice. The AD-like phenotype included increased CRTH2-positive cells, decreased immunostaining for mBD3 (the murine homologue of hBD2), and elevated serum IgE levels. Similarly, antigen-challenged mice, with the same *flg* mutation as ft/ft mice, also display multiple features of th2 inflammation [24]. Interestingly, these flg-deficient mice reportedly did not develop significantly higher IgE levels[24], likely reflecting a less severe phenotype due to differences in bioavailability of our hapten vs. the complete antigen employed by Fallon, et al. [24]. Finally, the apparent reduction in mBD3 expression could reflect downstream down-regulation by th2 cytokines [16].

Notably, inflammation can be induced in flg-deficient mice, without a requirement for additional, acquired stressors, such as reduced ambient humidity, high pH surfactants, and/or increased psychological stress, conditions that are known to both further compromise barrier function, and to precipitate AD in humans [1–3]. This leaves unanswered, however, the question of why many IV patients, including some with double-allele *FLG* mutations, do not display a concomitant AD phenotype [25, 26]. Two alternative or perhaps coincident explanations could explain this apparent paradox. First, it is possible that all patients with *FLG* mutations have the potential to develop AD, based upon their inherited barrier defect alone, but repeated epicutaneous deposition of allergens, sufficient to provoke TH2-dominant inflammation, might not occur in some IV patients. Second, in mice, unlike humans, development of inflammation might not require the superimposition of additional acquired stressors, because as noted above, mouse skin displays reduced barrier competence in comparison to human skin [63–65]. This inherent difference could also explain why hapten-induced, acute allergic contact dermatitis converts to an AD-like phenotype following repeated challenges in mice, a transition that is not known to occur in humans.

In summary, we show here that FLG deficiency, as occurs in many cases of human AD, suffices to provoke a barrier abnormality, which in turn allows enhanced permeation of water-soluble tracers via the paracellular route. The paracellular defect can be attributed to abnormal extracellular lamellar bilayers, resulting from compromised lamellar body secretion. These structural abnormalities allow enhanced irritant/hapten ingress, resulting in reduced inflammatory thresholds, providing a mechanistic link between FLG deficiency and development of AD. Finally, these studies substantiate and explain the new, ‘outside-to-inside’ paradigm of AD pathogenesis.

KEY MESSAGES

- Filaggrin deficiency alone provokes a permeability barrier abnormality (no further acquired stressors are necessary).
- The barrier abnormality localizes to the extracellular spaces of the stratum corneum, where if facilitates bidirectional, paracellular percutaneous transport of water-soluble xenobiotes.
- The paracellular abnormality can be ascribed to impaired secretion of epidermal lamellar bodies.
- The barrier abnormality correlates with reduced inflammatory thresholds to topical irritants and haptens.
- Repeated, low dose hapten applications that fail to elicit inflammation in wild-type mice, provoke inflammation with certain th2 features in filaggrin-deficient mice, worsening the barrier abnormality, thereby completing an ‘outside-inside-(back to) outside’ pathogenic cycle in murine AD.

Short Summary of Clinical Implications

FLG deficiency (in human AD) provokes a paracellular barrier abnormality, caused by abnormal extracellular lamellar bilayers, resulting from compromised lamellar body secretion. These structural abnormalities reduce inflammatory thresholds to irritants/haptens, providing a mechanistic link between FLG deficiency and development of AD, explaining the new, ‘outside-to-inside’ paradigm of AD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AD	atopic dermatitis
BD	βdefensin
CAMP	cathelicidin antimicrobial peptide
flg (mouse) or FLG (human)	filaggrin
ft/ft	flaky tail
Krt	keratin
Ox	oxazolone
TEWL	transepidermal water loss
TM	targeted mutant
TPA	12-O-tetradecanoylphorbol-13-acetate
+/+	wild-type

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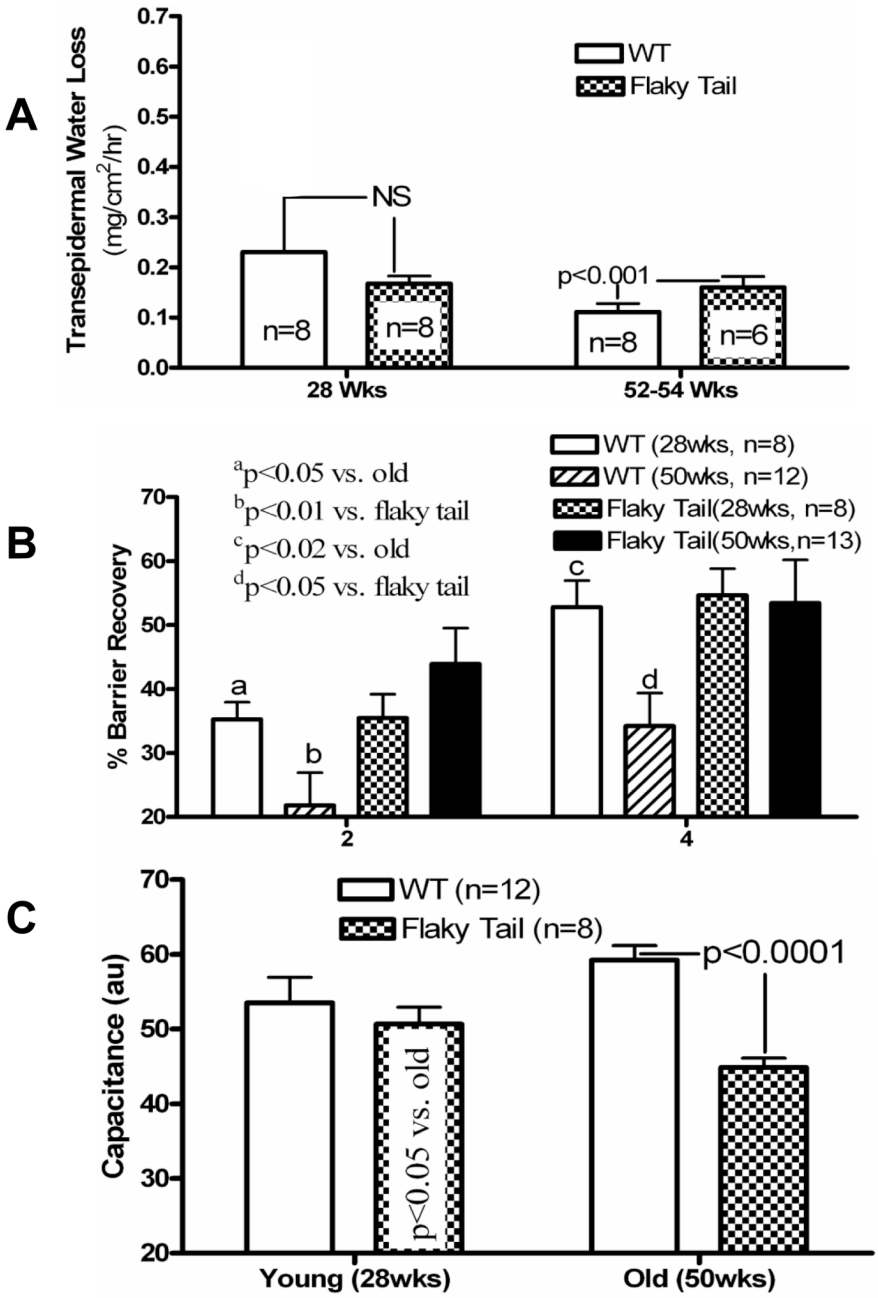

Figure 1. Abnormal Barrier Function and Stratum Corneum Hydration in Flaky Tail Mice

A: Basal transepidermal water loss (TEWL) was assessed with an electrolytic analyzer as mg/cm^2/hr, and expressed as (mean) ± SEM. B: Barrier disruption was achieved with repeated tape stripping until TEWL levels ≥ 3× normal; then % recovery from an initial '0' value, was measured 2 and 4 hrs after stripping, and expressed as mean +/−SEM. C: Stratum corneum hydration was measured as electrical capacitance on shaved back skin in flaky tail and wild-type mice, and shown as changes in arbitrary units (AU) +/− SEM (n=8–13, as indicated).

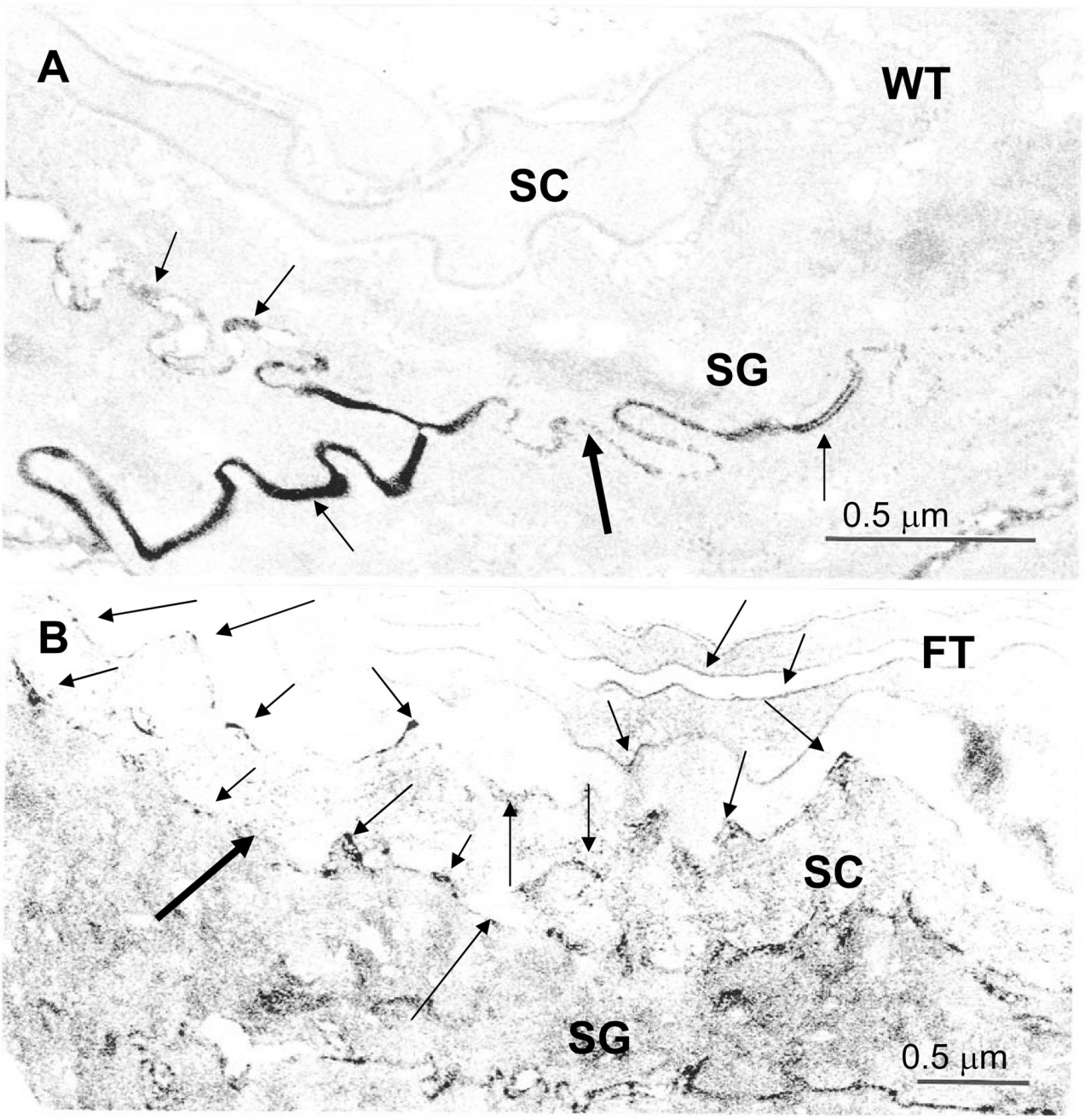

Figure 2. Abnormal Paracellular Permeability Barrier to a Subcutaneous, Water-Soluble Tracer in Flaky Tail Mice

Explants of back skin from sex-matched, 12 wk old, wild-type (WT) and flaky tail (FT) mice (n = 3 each) were immersed dermis-side-downward on a solution containing 4% colloidal lanthanum nitrate (pH 7.5) for 30 min to 2 hr, followed by fixation in Karnovsky's solution (see Methods). Colloidal lanthanum travels outward through the extracellular spaces, but does not reach the stratum corneum (SC) in WT skin (A, arrows). In contrast, in FT skin, lanthanum tracer extends into the lower SC, primarily via the extracellular spaces (B, arrows), suggesting impaired 'inside-to-outside' barrier function. SG = stratum granulosum; A+B, osmium tetroxide post-fixation; Mag bars = 0.5 μm.

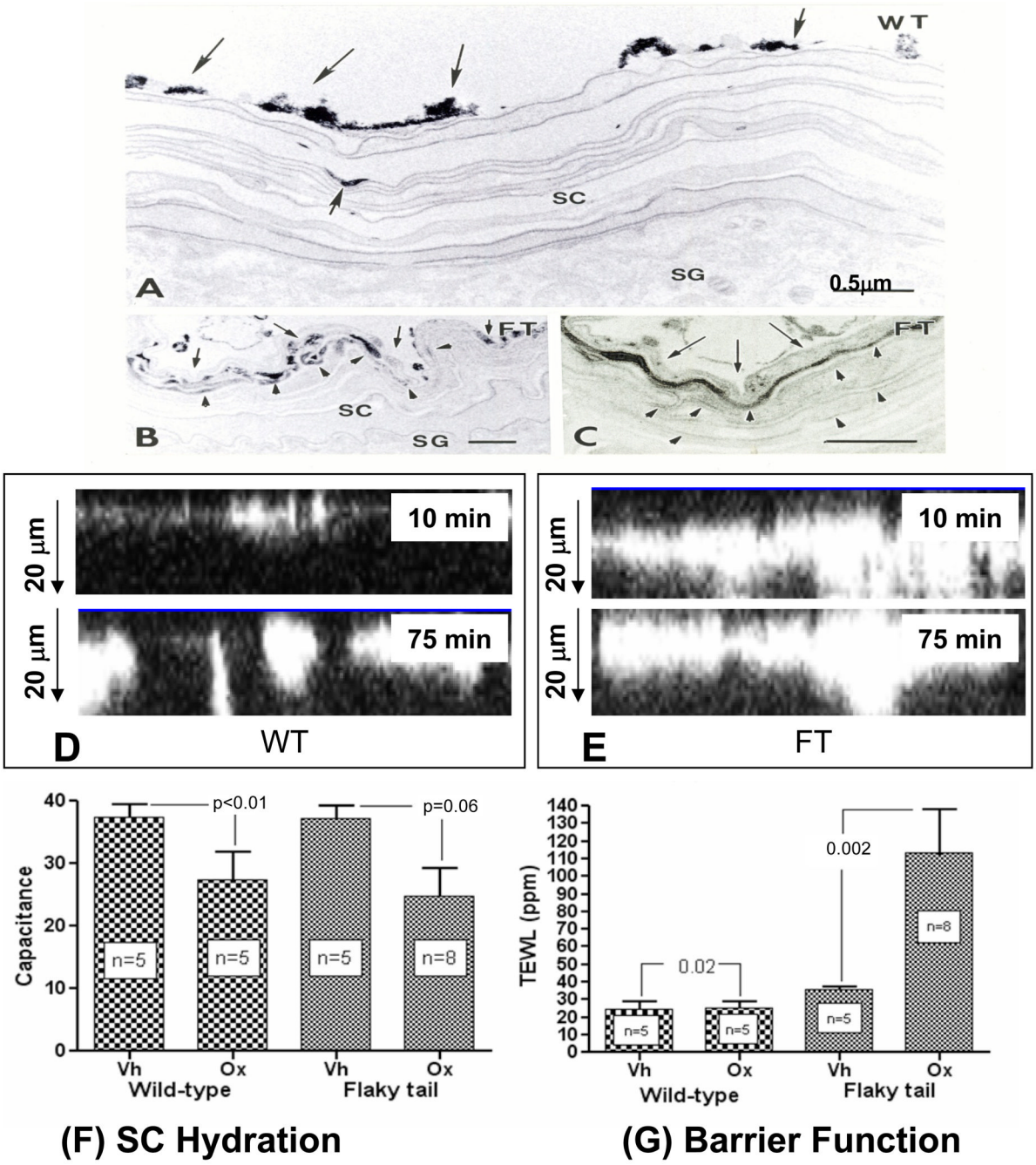

Figure 3. Epicutaneous Tracer Penetrates Flaky Tail Stratum Corneum via the Extracellular Spaces

A–C: Flanks of flaky tail (FT) and wild-type (WT) mice (n = 3 each) were immersed in 4% lanthanum nitrate solution for 30 min to 2 hrs, followed by aldehyde fixation. After 2 hrs, little or no tracer entered the stratum corneum (SC) of WT mice (A, single small arrow). In contrast, abundant tracer reaches ≥4 layers into the SC in FT mice (B, arrowheads), which on higher magnification [C] can be seen to localize to extracellular domains (C, arrowheads). D+E: Calcium green was applied to freshly-obtained explants from ft/ft and wt mice (n = 3 each), and penetration was assessed by dual-photon confocal microscopy, along the 'z' axis after 10 and 75 min. Note much deeper 'outside-to-inside' penetration of calcium green in ft/ft mice at

both time points. Flaky tail (FT) mice, repeatedly-challenged with low-dose Ox, display a more severe barrier abnormality (G), but no further decline in stratum corneum (SC) hydration in comparison to wild-type (WT) mice (F). A–C: Osmium tetroxide post-fixation; mag bar = 0.5 μm.

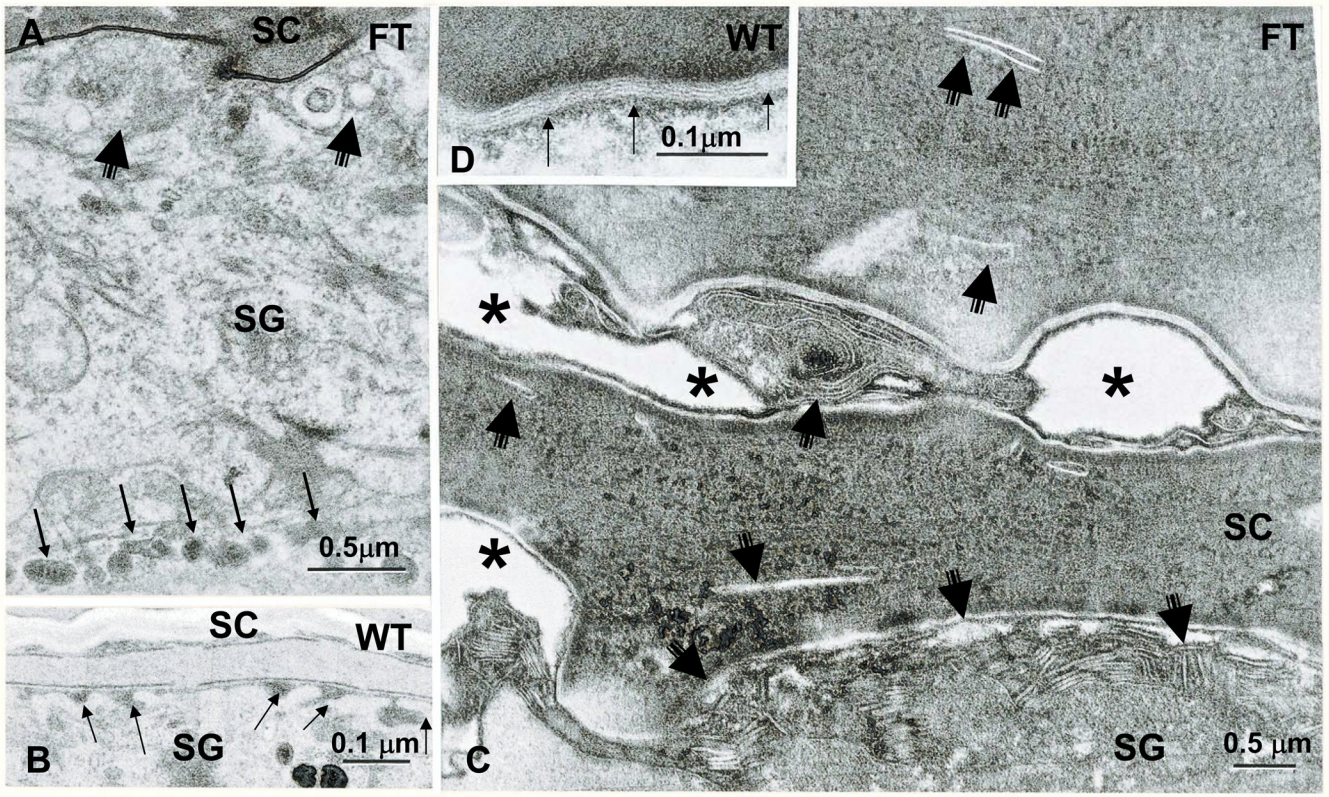

Figure 4. Abnormalities in the Lamellar Body Secretory System in Young Flaky Tail Mice

Partial failure of lamellar body exocytosis is evident in flaky tail (FT) epidermis (A, C). Note lamellar bodies lined up in peripheral cytosol (A, multiple thin arrows); decreased secreted material at stratum granulosum (SG)-stratum corneum (SC) interface (A&C, short, fat arrows); decreased numbers of lamellar bilayers (C): delayed maturation of lamellar bilayers (C); and entombed lamellar body contents within the corneocyte cytosol (C, short, thin arrows). B&D: Normal lamellar body secretion (B, arrows) and extracellular lamellar bilayers in wild-type (WT) epidermis (D, thin arrows) A&B, osmium tetroxide post-fixation; C&D, ruthenium tetroxide post-fixation. Mag bars = A&C, 0.5 μm; B&D, 0.1 μm.

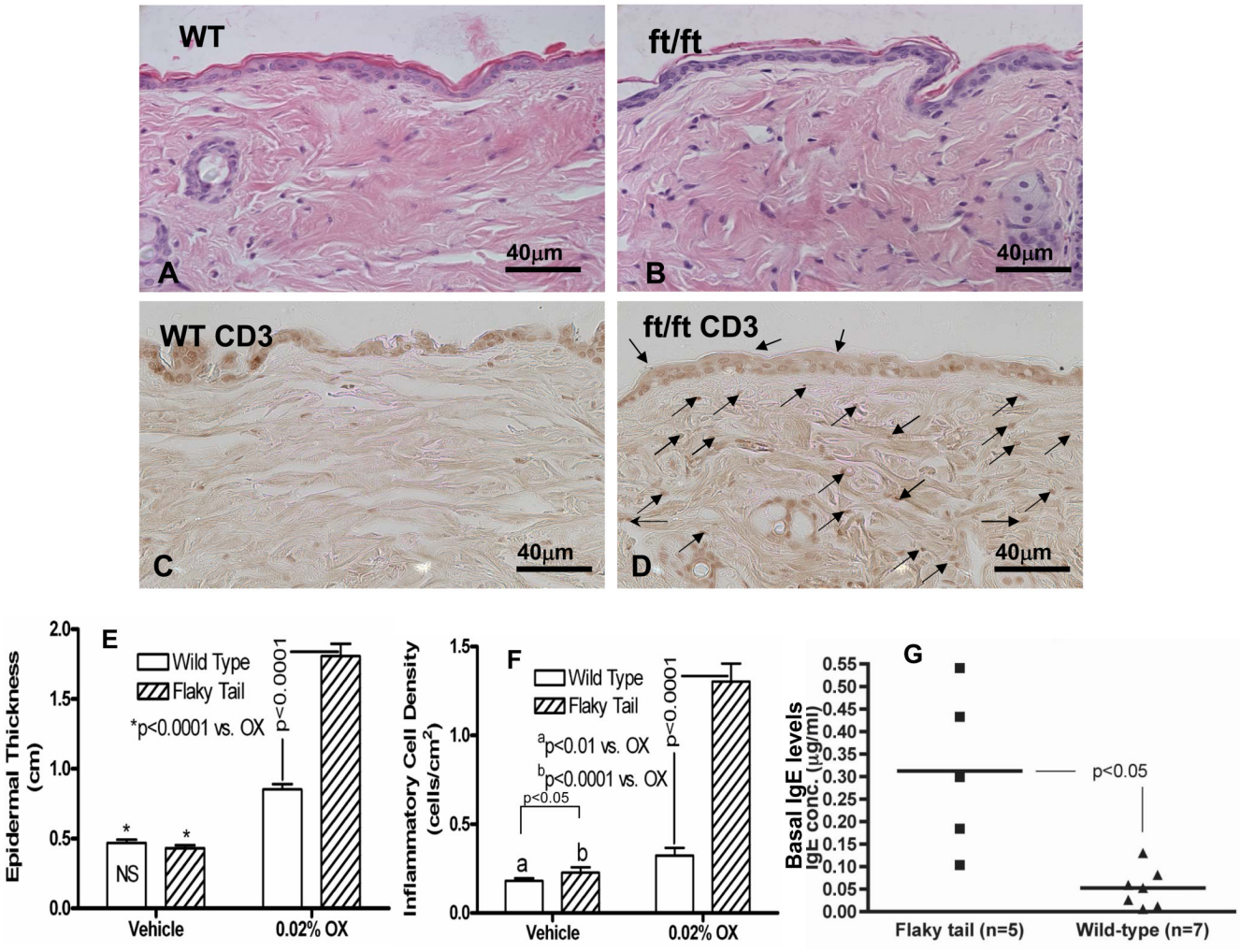

Figure 5. Reduced Threshold for Development of AD-Like Inflammation in Response to Repeated Ox Challenges in Flaky Tail Mice

Flaky tail (FT) and wild-type (WT) mice were sensitized to Ox, and then repeatedly challenged with a subthreshold concentration of Ox (0.02%) every other day to a shaved area of back skin for a total of 10 challenges. Under basal conditions, FT mice display low-grade inflammation (F, see also suppl. Fig. 3C vs. A). FT skin displayed much more prominent erythema, scaling and excoriations than does WT mouse skin (see text), a change that is associated with a modest inflammatory infiltrate (B vs. A & suppl. Fig. 3C), that was enriched in $CD3^+$ dermal lymphocytes (D vs. C). Quantitative changes in epidermal hyperplasia and inflammation are shown in E&F. G: Serum IgE levels are significantly elevated in most flaky tail mice, even under basal conditions. Mag bars = 40 μm.

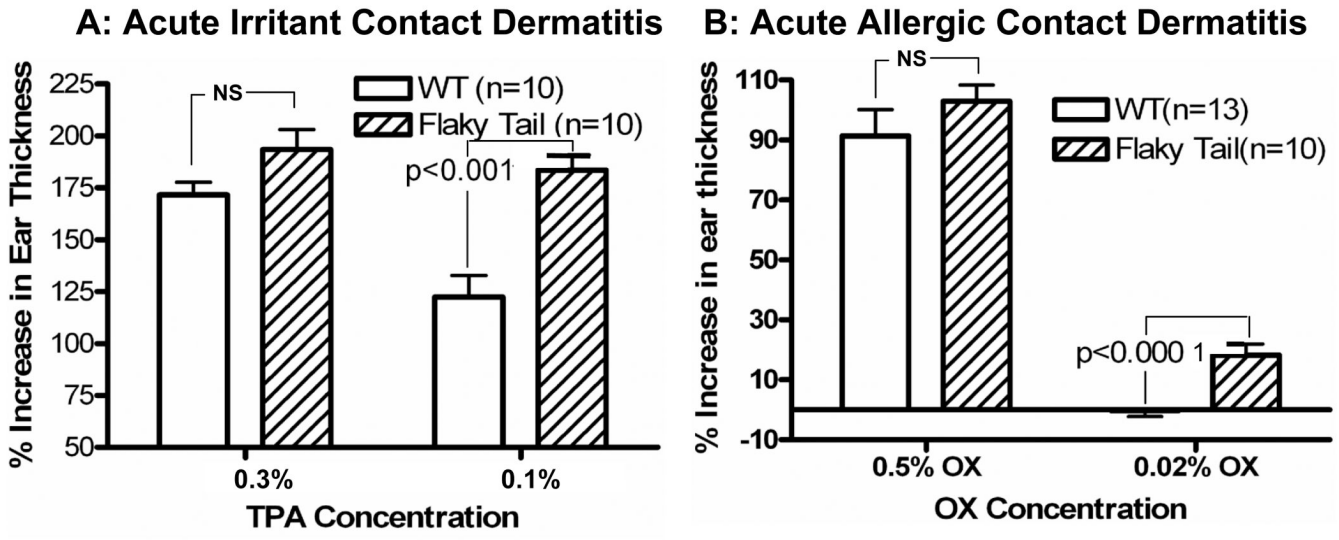

Figure 6. Altered Sensitivity to Irritant/Hapten-Induced Allergic Contact Dermatitis in Flaky Tail Mice

In young, wild-type mice, TPA induces irritant contact dermatitis, while Ox induces allergic contact dermatitis following a single, full-strength challenge dose [38]. While higher concentrations produced comparable changes in young wide-type (WT) and flaky tail mice, lower doses of either TPA (A) or Ox (B) induce inflammation only in flaky tail mice. Changes in ear thickness correlate with severity of inflammation (see suppl Fig. 4).

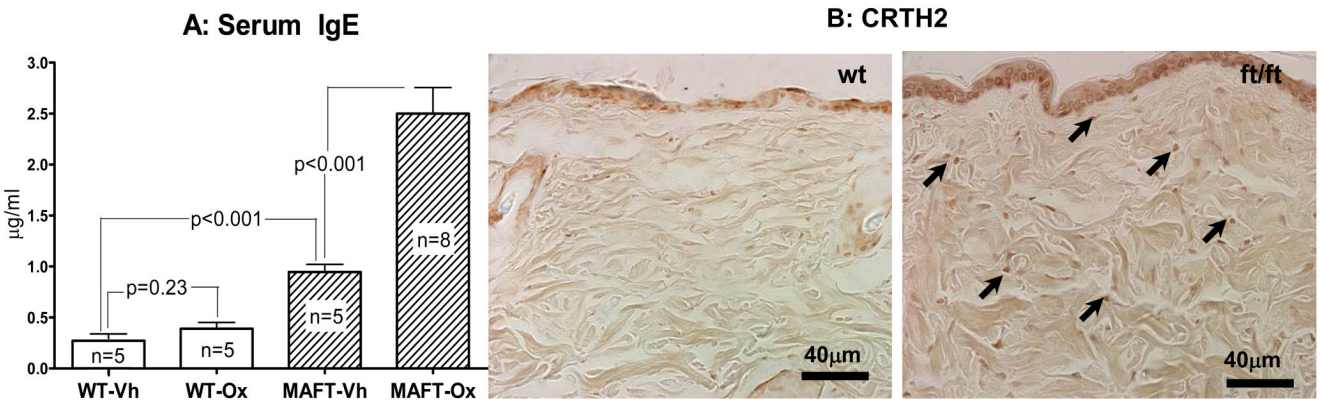

Figure 7. Flaky Tail Mice Demonstrate Increased Th2 Response to Repeated Low-Dose Ox Challenges

Serum was collected from young flaky tail (FT) and wild-type (WT) mice before and after Ox sensitization, and either ten low-dose Ox (0.02%) or vehicle treatments, followed by assessment of IgE levels by ELISA. While no difference from baseline was seen between vehicle and Ox-treated WT mice, a large increase was observed in FT mice (B). Likewise, low-dose Ox-challenged FT mice demonstrate increased staining for CRTH2-bearing cells (A, arrows) in Ox-treated, FT mice. Mag bars = 40 μm.