Influence of Th2 Cytokines on the Cornified Envelope, Tight Junction Proteins, and β-Defensins in Filaggrin-Deficient Skin Equivalents

Stefan Hönzke1, Leonie Wallmeyer1, Anja Ostrowski2, Moritz Radbruch2, Lars Mundhenk2, Monika Schäfer-Korting1 and Sarah Hedtrich1

Atopic dermatitis is a chronic skin condition with complex etiology. It is characterized by skin barrier defects and T helper type 2 (Th2)-polarized inflammation. Although mutations in the filaggrin gene are known to be prominent genetic risk factors for the development of atopic dermatitis, the interdependency between these and an altered cytokine milieu is not fully understood. In this study, we evaluated the direct effects of filaggrin deficiency on the cornified envelope, tight junction proteins, and innate immune response, and report the effects of Th2 cytokines in normal and filaggrin-deficient skin equivalents. Supplementation with IL-4 and IL-13 led to distinct histologic changes and significantly increased skin surface pH, both of which were enhanced in filaggrin knockdown skin equivalents. We detected a compensatory up-regulation of involucrin and occludin in filaggrin-deficient skin that was dramatically disturbed when simultaneous inflammation occurred. Furthermore, we found that a lack of filaggrin triggered an up-regulation of human β-defensin 2 via an unknown mechanism, which was abolished by Th2 cytokine supplementation. Taken together, these results indicate that defects in the epidermal barrier, skin permeability, and cutaneous innate immune response are not primarily linked to filaggrin deficiency but are rather secondarily induced by Th2 inflammation.


INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with a 20% prevalence in children and 2–9% prevalence in adults (Odiambo et al., 2009). The manifestation of AD is triggered by structural and immunologic dysfunctions in human skin as well as by environmental factors; however, their interdependencies and the overall pathologic mechanism are still not fully understood. One hallmark of AD is an imbalance in the T helper type 1/T helper type 2 (Th1/Th2) immune response and IgE-driven inflammation (Kezic et al., 2014). Moreover, disturbed skin barrier function such as that caused by mutations in the filaggrin gene (FLG) significantly increases the risk for AD and atopic diathesis (Brown and McLean, 2009; Palmer et al., 2006; Zheng et al., 2011). The FLG gene is mutated in 10–50% of AD patients (Kim and Leung, 2012). It encodes the skin barrier protein filagrin, which is important for the maintenance of epidermal homeostasis (Brown and McLean, 2012; Presland et al., 2004). Multiple studies in AD patients and on skin equivalents have revealed that a lack of filaggrin disturbs epidermal maturation and keratinocyte differentiation (Küchler et al., 2011; Mildner et al., 2010; Pendari et al., 2014), alters skin lipid composition and organization (Vávrová et al., 2014), reduces natural moisturizing factors (Janssens et al., 2012; Kezic et al., 2012), and increases skin permeability (Gruber et al., 2011). Nevertheless, filaggrin expression is down-regulated in all AD patients irrespective of FLG status, most likely as a downstream effect of the Th2-derived cytokines IL-4 and IL-13 (Howell et al., 2009).

In addition to barrier defects in the stratum corneum, tight junctions (TJs) in the stratum granulosum are affected in AD patients (Brandner et al., 2015; Kübelak and Boddupally, 2014). TJs are another important epidermal barrier, formed by complexes of adhesive and scaffolding proteins that control the paracellular passage of water, ions, and solutes (Niessen, 2007). Expression of claudin-1, a major transmembrane protein in epidermal TJ, is reduced in AD patients and is inversely correlated with levels of circulating eosinophils and serum IgE (De Benedetto et al., 2011). Furthermore, decreased expression of occludin and zonula occludens-1 has also been detected in the epidermis of filaggrin-deficient patients, which might be one factor contributing to the skin barrier impairment in these subjects (Gruber et al., 2011). Although these findings indicate an impaired TJ barrier, it is still ambiguous if TJ dysfunction is a primary effect or secondary to inflammatory processes.
Another common feature of AD is an impaired cutaneous innate immune response, resulting in an increased risk of microbial colonization and subsequent recurrent infections (Bieber, 2008; Zöllner et al., 2000). FLG mutations in AD patients are associated with an increased risk of bacterial infection compared to wild-type FLG carriers (Cai et al., 2012). This can be explained by lower levels of filaggrin breakdown products, which normally efficiently inhibit bacterial growth (Miajlovic et al., 2010). Moreover, reduced levels of antimicrobial peptides (AMPs) further enhance the risk of skin infections (De Benedetto et al., 2009). The role of AMPs in atopic skin is complex and has been the subject to much discussion. Overall, higher levels of human β-defensin 2 (hBD-2) and human β-defensin 3 (hBD-3) have been detected in AD skin relative to healthy controls. Compared with psoriasis, however, β-defensin levels are diminished in AD, likely due to down-regulatory effects of the Th2 cytokines IL-4 and IL-13 (Albanesi et al., 2007; de Jongh et al., 2005; Howell et al., 2006; Nomura et al., 2003). Although dysregulated immune processes clearly disturb cutaneous antimicrobial defenses, the effects of filaggrin deficiency on the innate immune response are not yet fully understood.

The impact of inflammatory cascades and filaggrin deficiency in AD pathogenesis is well established, but little is known about the direct interplay between proinflammatory processes and filaggrin deficiency. In this study, we report the effects and interdependencies of the Th2-derived cytokines IL-4 and IL-13, both alone and in combination, in normal and filaggrin knockdown skin equivalents. Focus is placed on the cornified envelope proteins involucrin, filaggrin, and loricrin, the skin surface pH, the regulation of the TJ proteins occludin, claudin-1, and claudin-23, and hBD-1, hBD-2, and hBD-3 expression.

RESULTS
Supplementation with Th2 cytokines increased epidermal thickness and disturbed skin acidification

As expected, skin equivalents supplemented with IL-4, IL-13, or IL-4/IL-13, as well as those possessing filaggrin knockdown (FLG−) alone, were characterized by epidermal thickening, spongiosis, and parakeratosis (Figure 1a and b). FLG− skin equivalents showed higher proliferation rates, as indicated by increased Ki-67 expression in the basal skin layer (see Supplementary Figure S1 online). However, no further histologic differences between untreated and cytokine-stimulated FLG+ and FLG− equivalents were observed. Furthermore, skin surface pH measurements revealed no increases in skin surface pH in untreated FLG− equivalents, in line with previous work (Vávrová et al., 2014). Interestingly, although exposure to IL-4 or IL-13 alone resulted in a slight increase in skin surface pH, incubation with IL-4/IL-13 in combination produced a major shift toward higher pH values (Figure 1c).

Th2 cytokines diminished skin barrier protein expression and disturbed counterregulation in FLG− skin equivalents

As expected, filaggrin expression was significantly reduced in untreated FLG− skin equivalents (Figure 2a and d). A compensatory 3-fold up-regulation of involucrin (P = 0.075) and a 1.5-fold increase of loricrin was detected by densitometry of western blots (Figure 2b and c) and immunostaining (Figure 2d and e). After addition of either IL-4 or IL-13, the compensatory up-regulation of involucrin was significantly disturbed and was abolished altogether when IL-4 and IL-13 were applied in combination. The drastic effect of this combination was less pronounced in FLG+ skin equivalents. A similar trend, although less marked, was also observed for loricrin (Figure 2c and e). Concordant regulation at the mRNA level was detected (see Supplementary Figure S2a–c online).

Th2 cytokines disturbed the up-regulation of occludin but not of claudin-1 and claudin-23 in FLG− skin equivalents

In concordance with involucrin (Figure 2), occludin expression was markedly up-regulated (3.3-fold) in FLG− skin equivalents, something that again was significantly hampered by cytokine supplementation (Figure 3a and Supplementary Figure S3.1 online). For claudin-1, a clear
trend toward increased expression ($P = 0.0137$; Figure 3 and Supplementary Figure S3.1) was also detected in FLG skin equivalents; however, unlike involucrin, this was not affected by cytokine supplementation. Both TJ proteins proved to be membrane bound (see Supplementary Videos S1 and S2 online). The impact of the Th2 cytokines on claudin-23 expression was comparable to claudin-1, although a trend for reduced claudin-23 expression was observed in FLG skin equivalents after Th2 supplementation (Figure 3c). Similar regulation, although not statistically significant, was detected at gene expression levels (see Supplementary Figure S3.2).

Thymic stromal lymphopoietin expression was significantly enhanced in FLG skin equivalents

In FLG skin equivalents, thymic stromal lymphopoietin (TSLP) expression was significantly increased by 2.3-fold, even in the absence of any cytokine stimulation (Figure 4). After IL-4 or IL-13 supplementation, TSLP secretion was respectively 3-fold and 2-fold higher in FLG skin equivalents compared to Th2-stimulated FLG skin equivalents. The IL-4/IL-13 combination did not further enhance TSLP levels (Figure 4). Concordant mRNA levels were detected (see Supplementary Figure S4 online).

Filaggrin deficiency triggered hBD-2 production

Interestingly, filaggrin deficiency alone induced significant up-regulation of hBD-2 expression (~5-fold) compared to FLG skin equivalents (Figure 5). Supplementation with IL-4, IL-13, or most markedly with IL-4/IL-13 in combination significantly reduced hBD-2 levels (Figure 5). For hBD-3 expression, no significant differences in FLG+ and FLG− (untreated and IL-treated) skin equivalents were observed (see Supplementary Figure S5.1 online). The constitutively expressed hBD-1 was unaffected by cytokine supplementation or filaggrin

Figure 2. Impact of IL-4 and IL-13 on protein expression of filaggrin, involucrin, and loricrin. (a–c) Western blots and relative protein expression semiquantified via densitometry of untreated and IL-4, IL-13, and IL-4/IL-13 supplemented normal (FLG+) and filaggrin knockdown (FLG−) skin equivalents. Values are given as mean ± SEM. n = 3. Asterisk indicates statistical significance from FLG+ untreated: *$P \leq 0.05$, **$P \leq 0.01$. Plus sign indicates statistical significance from FLG− untreated: +$P \leq 0.05$, ++$P \leq 0.01$. (d, e) Representative immunostaining against filaggrin (FLG, red), involucrin (IVL, green), and loricrin (LOR, green) in untreated and IL-4, IL-13, and IL-4/IL-13 supplemented normal (FLG+) and filaggrin knockdown (FLG−) skin equivalents. Bar = 100 μm. Exposure times: red channel 1/10 second; green channel 1/13 second; blue channel 1/55 second. Counterstaining was performed with 4',6-diamidin-2-phenylindol (DAPI; blue).
knockdown (see Supplementary Figure S5.2); siRNA negative control also had no effect (see Supplementary Figure S5.3 online).

As a positive control, the effects of tumor necrosis factor-α (TNF-α) on hBD-2/hBD-3 expression were tested. TNF-α is a key player in the pathogenesis of psoriasis, which itself is characterized by significantly increased hBD levels. As expected, TNF-α stimulation resulted in a ~6.5-fold increase in hBD-2 expression in FLG+ skin equivalents and ~11-fold increase in FLG− skin equivalents (Figure 5a). These data were confirmed by immunostaining (Figure 5b).

At the mRNA level, filaggrin deficiency induced significant up-regulation of hBD-2 (7.4-fold) compared to normal keratinocytes (see Supplementary Figure S5.4 online). TNF-α stimulation resulted in a ~22-fold increase in hBD-2 expression in normal keratinocytes and ~65-fold increase in filaggrin-deficient keratinocytes (see Supplementary Figure S5.4). Expression of hBD-3 was also significantly increased in filaggrin-deficient keratinocytes, although this could not be verified at the protein level (see Supplementary Figure S5.4b).

To identify the mechanism of hBD regulation, filaggrin-deficient monolayer keratinocytes were preincubated with an IL-1R antagonist and neutralizing antibodies against IL-6, toll-like receptor-2, or TSLP. Interestingly, up-regulation of the β-defensins was not antagonized (see Supplementary Figure S5.5).

**Th2 cytokines slightly diminished the barrier function of skin equivalents**

The skin barrier function of untreated or Th2-supplemented FLG+ and FLG− skin equivalents was assessed by skin permeation studies. No distinct differences in skin permeability between untreated FLG+ and FLG− skin equivalents were observed. Supplementation with IL-4, IL-13, or IL-4/IL-13 did not significantly weaken the skin barriers of FLG+ or FLG− equivalents (see Supplementary Figure S6 online), although a clear tendency toward higher permeability was observed compared to untreated skin equivalents (see Supplementary Figure S6).

**DISCUSSION**

In this study, we investigated the effects of the Th2 cytokines IL-4 and IL-13 on the expression of important structural and TJ proteins, skin surface pH, cutaneous innate immune response, and skin barrier function in normal (FLG+) and filaggrin-deficient (FLG−) skin equivalents. The impact of mutations in the filaggrin gene (Brown and McLean, 2012; McAleer and Irvine, 2013; Palmer et al., 2006) as well as the contribution of a Th2-polarized immune response (Homey et al., 2006) in the pathogenesis of AD are well established. Nevertheless, the direct interplay between filaggrin deficiency and inflammatory conditions has not yet been investigated. Whether immune dysregulation results from skin barrier abnormalities, such as a lack of filaggrin, or is the initial
trigger leading to an acquired barrier deficiency by down-regulation of, for example, filaggrin expression, is still debated (Howell et al., 2009).

Recent publications, although not focused on filaggrin-deficient skin, have described the effects of TNF-α and Th2 cytokines (IL-4, IL-13, and IL-31) on normal
keratinocytes and skin equivalents, revealing impaired skin lipid composition and organization, hyperproliferation, and attenuated skin protein expression, leading to a disruption of keratinocyte integrity and skin barrier function (Danso et al., 2014; Omori-Miyake et al., 2014). These findings are well in line with our own results (Figures 1 and 2, and Supplementary Figure S1).

In this study, a distinct compensatory increase in proteins of the cornified envelope, particularly involucrin, was observed in the FLG—skin equivalents. This was abolished after IL-4/IL-13 supplementation, demonstrating the detrimental synergistic effects of these cytokines (Figure 2). The effects of IL-4/IL-13 on involucrin, loricrin, and filaggrin expression were most pronounced in FLG—skin equivalents. Although involucrin and, to a lesser extent, loricrin initially are up-regulated to compensate for a lack of filaggrin, this feedback mechanism is disturbed by the actions of these cytokines likely by activation of the STAT-6 signaling pathway (Kim et al., 2008) or the S100 calcium-binding protein A11 (Howell et al., 2008). Similar compensatory mechanisms involving cornified envelope components have previously been reported only in loricrin-deficient mice (Koch et al., 2000) and in flaky tail mice (Presland et al., 2000). Comparable compensatory mechanisms were also observed for desmosome and TJ proteins in mice (Furuse et al., 2002). Consistently, we also detected significant up-regulation of occludin and a statistically nonsignificant increase of claudin-1 and claudin-23 in the FLG—skin equivalents (Figure 3), likely a mechanism to prevent aggravation of the disease phenotype. As with involucrin, expression of occludin was greatly reduced after the addition of Th2 cytokines. The same trend was also observed for claudin-23, although this was less pronounced. By contrast, no detrimental effects on claudin-1 expression were observed after incubation with the interleukins. This is consistent with the findings of De Benedetto et al. (2011), who found no negative effects of IL-4 and IL-13 on claudin-1 expression in human keratinocytes. Nonetheless, these results support the hypothesis that TJ dysfunction in AD patients is secondary to inflammatory processes and is not directly linked to a lack of filaggrin, in keeping with recent findings in filaggrin-null mice and in mice with hapten-induced dermatitis (Yokouchi et al., 2015). Moreover, the compensatory up-regulation of involucrin and occludin in FLG—skin equivalents might explain the lack of skin permeability differences between untreated FLG+ and FLG—equivalents (see Supplementary Figure S6). This mechanism was disturbed after cytokine supplementation, resulting in slightly increased skin permeability (see Supplementary Figure S6c). However, these data must be interpreted cautiously because skin equivalents do not exhibit a fully developed skin barrier and thus are characterized by a significantly weaker barrier function compared to native human skin.

Consistent with studies in humans (Sano et al., 2013), flaky tail mice (Moniaga et al., 2013), and epidermal layers (Lee et al., 2011), basal TSLP levels were significantly enhanced in filaggrin-deficient skin equivalents (Figure 4). TSLP is considered a key player in AD pathogenesis (Ziegler, 2010). It directly activates dendritic cells that, in turn, induce naïve T cell proliferation and primes differentiation into Th2 cells, followed by secretion of high levels of IL-4, IL-5, IL-13, or TNF-α (Soumelis et al., 2002). Moreover, a significant influence of TSLP on innate lymphoid cells was recently recognized. In particular, group 2 innate lymphoid cells seem to play a prominent role. They are abundantly expressed in lesional AD skin and are activated by TSLP, IL-25, and IL-33 (Kim et al., 2013; Salimi et al., 2013). Besides TSLP, the involvement of epithelium-derived cytokines IL-25 and IL-33 in the pathophysiology of AD is apparent and will be an important point of focus in future studies.

AD patients are typically more prone to recurrent microbial infections than are patients suffering from other skin diseases, possibly because of lower AMP levels and increased skin surface pH (Eberlein-König et al., 2000; Kuo et al., 2013; Schmid-Wendtner and Korting, 2006). Overall, changes in the balance of the microbiome and the cutaneous immune response aggravate AD (Kong et al., 2012), and disease severity is strongly associated with lower skin microbiome diversity (Salava and Lauerma, 2014). However, whether these changes are due to an imbalance of the immune system or occur secondarily to permeability changes remains unclear (Cogen et al., 2008).

To evaluate the effects of IL-4, IL-13, and filaggrin deficiency on the cutaneous innate immune response, the expression of hBD-1, hBD-2, and hBD-3 was assessed. Although it is well established that AMP secretion is higher in psoriatic skin than in AD skin (de Jongh et al., 2005; Kopnagel et al., 2013), AD patients still show increased expression relative to healthy skin (Harder et al., 2010), something also noted in this study.

We observed greatly increased hBD-2 expression after TNF-α stimulation, which is a key mediator of psoriasis. Interestingly, significantly higher hBD-2 levels were also detected in FLG—skin equivalents, even without stimulation (Figure 5). To the best of our knowledge, this effect has not been described before and suggests that there seems to be no general defect, at least in the induction of hBD-2, in filaggrin-associated skin diseases. Clausen et al. (2013) previously studied hBD-2 expression in AD patients with and without filaggrin mutations. Here, a clear trend toward increased hBD-2 expression was detected in filaggrin mutation carriers, although without statistical significance, perhaps because of the low patient number and distinct intersubject differences. In this study, stimulation with Th2 cytokines greatly reduced hBD-2 levels (Figure 5), which might result from a Th2-mediated activation of the STAT-6 signaling pathway as previously demonstrated (Albanesi et al., 2007), and may be a potential reason for the lower AMP levels seen in AD patients compared to other skin diseases. Interestingly, other T cell-derived cytokines, such as IL-17 and IL-22, have been found to synergistically increase hBD-2 expression and thus also seem to play an important role in the regulation of skin innate immunity (Liang et al., 2006). However, IL-17 and IL-22 are also known to exacerbate AD disease severity (Koga et al., 2008; Mirshafiey et al., 2015), thus illustrating the complex interdependencies and effects of cytokines in the pathogenesis of AD.

In contrast to this study, van Drongelen et al. (2014) observed no effect of filaggrin knockdown on hBD expression. This possibly results from the use of a N/TERC cell line for skin equivalent construction, which in all probability responds differently than the primary skin cells used here.
Expression of hBD-2/hBD-3 is physiologically up-regulated by mechanical or metabolic injury to the skin barrier (Ahrens et al., 2011), whereas IL-1, IL-6, or toll-like receptor-2 all were identified as endogenous trigger factors (Liu et al., 2002; Park et al., 2013). However, antagonizing IL-1, IL-6, TSLP, or toll-like receptor-2 did not diminish hBD-2/hBD-3 expression in untreated FLG− keratinocytes (see Supplementary Figure S5.5), indicating the existence of a currently unknown pathway of hBD induction that requires clarification in future studies.

Previous investigations from our group have shown that filaggrin deficiency alone does not cause the frequently described increase in skin surface pH often seen in AD patients. A compensatory feedback mechanism for the lack of filaggrin in the FLG− skin equivalents was identified, wherein the proton pump NHE-1 and the secretory phospholipase A2 are up-regulated (Vávrová et al., 2014). In this study, a considerable increase in skin equivalent surface pH was detected, particularly after suplementation with IL-4 and IL-13 in combination. The impact of IL-4 or IL-13 alone was less pronounced (Figure 1c). It is tempting to speculate that the proinflammatory cytokines IL-4 and IL-13 disturb the aforementioned feedback mechanism, ultimately increasing skin surface pH. This hypothesis will be clarified in future investigations.

In summary, our results show a distinct impact of FLG deficiency on hBD-2 expression and demonstrate that a compensatory up-regulation of skin barrier and TJ proteins such as involucrin or occludin can initially balance a lack of filaggrin. However, this counterregulation is disturbed by the Th2-derived cytokines IL-4 and IL-13, ultimately resulting in reduced hBD-2 levels, increased skin equivalent surface pH, and a weakened skin barrier. Overall, filaggrin-deficient skin equivalents were more sensitive to the detrimental effects of Th2-derived cytokines IL-4 and IL-13, ultimately resulting in reduced skin surface pH, and a weakened skin barrier. Overall, filaggrin-deficient skin equivalents were more sensitive to the detrimental effects of IL-4 and IL-13 in combination with 30 ng/ml IL-4, 30 ng/ml IL-13, or 15 ng/ml IL-4 and IL-13 for 5 days. Subsequent real-time PCR was performed as described earlier.

The same experimental setup was used to identify potential mediators responsible for increased hBD expression. Here, FLG− keratinocytes were incubated with 5 μg/ml IL-6 antibody, 5 μg/ml toll-like-receptor-2 antibody, 20 μg/ml TSLP antibody, or 10 μg/ml IL-1R receptor antagonist for 5 days, and subsequent real-time PCR was performed.

For histologic analysis, skin equivalents were punched, immediately frozen, and cut into 8 μm vertical slices using a Leica CM 1510 S cryotome (Leica Biosystems, Nussloch, Germany). Sections were then stained with hematoxylin and eosin according to standard procedures. Epidermal thickness was measured from the stratum corneum to the beginning of the dermal equivalent in three hematoxylin and eosin-stained sections (per skin equivalent). In total, 10 measuring points were evaluated in each section using BZ image analysis software (Keyence, Neu-Isenburg, Germany).

**MEDIAcHNIcS**

**Skin equivalent construction and cytokine supplementation**

Normal (FLG+) and filaggrin-deficient (FLG−) skin equivalents were generated according to previously published procedures (Eckl et al., 2011; Kuchler et al., 2011) (see Supplementary Materials). Skin equivalents were cultivated for 14 days with media changes every second day. Starting at day 10, the culture media was supplemented with 30 ng/ml IL-4, 30 ng/ml IL-13, 15 ng/ml IL-4 and IL-13, or 20 ng/ml TNF-α.

**Real-time PCR and histologic analysis**

To determine knockdown efficiency, skin equivalents were punched into 10 mm discs, and epidermis was gently removed, frozen, and then milled for 30 seconds at 25 Hz using a TissueLyzer (Qiagen, Hilden, Germany). Subsequently, RNA was isolated using Nucleo-Spin RNA II (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. For cDNA synthesis, a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) was used. Real-time PCR was performed using SYBR Green 1 Masterplus (Roche, Penzberg, Germany). Primer sequences are listed in Supplementary Table S1. Glyceraldehyde-3-phosphate dehydrogenase served as a housekeeping gene.

Gene expression levels of filaggrin, involucrin, loricrin, occludin, claudin-1, claudin-23, and β-defensin 1-3 were determined in 2D cell cultures. Primary, human keratinocytes (untreated and IL-treated; FLG+ and FLG−) were cultivated in high calcium (1.3 μM calcium chloride) keratinocyte growth medium supplemented with 30 ng/ml IL-4, 30 ng/ml IL-13, or 15 ng/ml IL-4 and IL-13 for 5 days. Subsequent real-time PCR was performed as described earlier.

ELISA

The skin equivalents were lysed in radioimmunoprecipitation assay buffer, and the proteins were quantified via BCA Protein Assay (Thermo Scientific, Waltham, MA). Subsequently, hBD-2 and hBD-3 amounts in skin equivalents were quantified using the BD-2 and BD-3 OmnkineTM ELISA Kits (AssayBioTech, Sunnyvale, CA) according to the manufacturer’s instructions.

**Skin surface pH measurements**

Optical sensor foils for pH imaging containing pH indicator microcrystals (fluorescein isothiocyanate) and reference microcrystals (ruthenium(II)-tris(4,7-diphenyl-1,10-phenanthroline) were applied to skin equivalents (Vávrová et al., 2014). After equilibration, an RGB image was recorded using a VisiSens system for 2D pH imaging (Presens, Regentsburg, Germany) and calculations performed with corresponding VisiSens AnalytiCal 2 software (Presens, Regentsburg, Germany).

**Skin absorption testing**

To assess skin barrier function in the skin equivalents, skin permeation tests were performed according to validated test procedures (Schäfer-Korting et al., 2008). Radioactively labeled testosterone served as a lipophilic test compound (for details, see Supplementary Materials and Supplementary Figure S6).

**Statistical analysis**

One-way analysis of variance, followed by either Dunnett post hoc testing or Bonferroni correction for multiple testing, was performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA).
Asterisk indicates statistical significance over FLG+ untreated, and plus sign indicates statistical significance over FLG− untreated. \( P \leq 0.05 \) indicates statistical significance. Data from at least three independent experiments are presented as mean ± standard error of the mean.

ORCID
Sarah Hedrich http://orcid.org/0000-0001-6770-3657

CONFLICT OF INTEREST
The authors state no conflict of interest.

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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.2015.11.007.

REFERENCES


