Role of fibroblasts in the pathogenesis of atopic dermatitis

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Background: Atopic dermatitis (AD) is a common dermatosis that highly impairs a patient's quality of life. The recent discovery that epidermal barrier defects caused by an aberrant differentiation process of keratinocytes are comparably important to the well-characterized changes in immune response patterns attributed a crucial role to the keratinocytes. Fibroblasts are able to alter proliferation and differentiation of keratinocytes, but their role in AD is not yet fully understood.
Objective: We sought to determine the role of fibroblasts in skin proliferation and differentiation in patients with AD.
Methods: We used human 3-dimensional organotypic skin cultures consisting of atopic fibroblasts and healthy keratinocytes, as well as healthy fibroblasts and atopic keratinocytes, and compared them with their controls. The expression of differentiation markers in these organotypic cultures were analyzed by using immunohistology and quantitative RT-PCR. Furthermore, the fundamental role of fibroblast-secreted leukemia inhibitory factor was assessed by using small interfering RNA–mediated knockdown cultures.
Results: We observed that atopic fibroblasts influence the proliferation of keratinocytes and the terminal differentiation process, resulting in an *in vivo*–like morphology of AD. Subsequently, healthy fibroblasts were able to restore the structural deficits of the epidermis consisting of atopic keratinocytes. Partially, these effects were due to a reduced expression of the differentiation-associated cytokine leukemia inhibitory factor by atopic fibroblasts.
Conclusion: These data demonstrate that fibroblasts and the modulation of fibroblast-derived factors might be new therapeutic targets for the alleviation of AD. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: *Atopic dermatitis, leukemia inhibitory factor, fibroblast, keratinocyte, organotypic culture, differentiation, stratification*

Abbreviations used
AD: Atopic dermatitis
FK-OTC: Organotypic culture with healthy fibroblasts and healthy keratinocytes
Fk-OTC: Organotypic culture with healthy fibroblasts and atopic keratinocytes
fK-OTC: Organotypic culture with atopic fibroblasts and healthy keratinocytes
fk-OTC: Organotypic culture with atopic fibroblasts and atopic keratinocytes
LIF: Leukemia inhibitory factor
OTC: Organotypic culture
siRNA: Small interfering RNA
SOCS3: Suppressor of cytokine signaling 3
STAT: Signal transducer and activator of transcription

Atopic dermatitis (AD) is one of the most common skin disorders in industrial countries; its prevalence has increased in recent decades. The key symptoms of this chronic, inflammatory, highly pruritic skin disease include xerosis, erythema, lichenification, pruritic papules, and plaques, leading to a significant impairment in quality of life. AD is the result of a multifactorial and complex interaction of epidermal barrier dysfunctions and immunologic aberrations, which are influenced by genetic factors.

For quite a long time, AD was regarded as a primarily immune-driven disease. One of the major hurdles for specific immune interventions is the change in immune phenotype from a T_H2-dominated acute exudative phase to a T_H1-dominated chronic phase. Hence effective immune interventions are based on general immunosuppression, as best demonstrated for corticosteroids or the calcineurin inhibitors.

The view of the pathogenesis of AD underwent a complete change with the landmark discovery of filaggrin mutations in atopic subjects.[1-3] Filaggrin is a keratin-aggregating protein that is crucial for keratinocyte differentiation, formation of natural moisturizing factors, and, ultimately, constitution of a functional epidermal barrier. Therefore loss-of-function mutations within the filaggrin gene predispose to skin barrier dysfunction, which enables allergens to penetrate into the skin and induce an inflammatory immune reaction. Another cause for disturbed epidermal barriers might be the altered regulation and localization of tight junction proteins, which has been reported for chronic inflammatory skin conditions, such as AD and psoriasis.[4-6] Therefore keratinocytes and the skin barrier moved into focus as targets for future therapeutic interventions.

A continuous steady state of proliferation, differentiation, and shedding of keratinocytes results in the constant renewal of the epidermis, thereby maintaining and repairing skin barrier function. However, the steady state of keratinocyte proliferation and differentiation can be modulated by exogenous triggers, such as wounding or burning,[7] and has been reported to be deregulated in patients with skin diseases, such as psoriasis.[8] Therefore the intrinsic program of keratinocyte proliferation and differentiation can be altered by exogenous and endogenous factors. Fibroblasts

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and keratinocytes mutually interact through soluble factors, such as interleukins (eg, IL-1 and IL-6), growth factors (eg, GM-CSF and keratinocyte growth factor/fibroblast growth factor), and other secreted factors (eg, stromal cell–derived factor 1 and pleiotrophin).[9-11] Hence we were interested in whether fibroblasts are involved in the pathogenesis of AD by influencing the structure of the epidermal skin barrier. To address this issue, we developed and analyzed organotypic culture (OTC) skin models derived from fibroblasts and keratinocytes obtained from either healthy or atopic donors. Using comparative analyses of OTCs derived from different combinations of healthy and atopic cells, we provide strong evidence for a significant influence of atopic fibroblasts on the proliferation and differentiation of keratinocytes and, consequently, on the corresponding formation and stratification of the epidermis. We show that a reduced concentration of leukemia inhibitory factor (LIF) in atopic fibroblasts is an important factor for the phenotypic aberrations of the OTC epidermis.

Moreover, our data indicate that expression of filaggrin by keratinocytes is regulated by dermal fibroblasts. Cocultivation with atopic fibroblasts strongly reduces the filaggrin expression of healthy keratinocytes. Conversely, healthy fibroblasts were able to stimulate the expression of filaggrin in atopic keratinocytes. We conclude that fibroblasts are an important factor for the manifestation of epidermal characteristics of AD.

METHODS

Clinical studies

Clinical studies were carried out according to the recommendations of the current version of the Declaration of Helsinki and the guidelines of the International Conference on Harmonization Good Clinical Practice as applicable to a nondrug study. All participants (healthy volunteers and atopic patients) provided written informed consent. Punch biopsy specimens were obtained within a clinical study approved by the ethics committees of the Medical Association of Hamburg and Kiel, Germany. Biopsy specimens (6 mm) were obtained after achievement of local anesthesia from the dorsal forearm (perilesional areas in atopic patients). Total SCORAD scores, local SCORAD scores, and IgE levels were assessed in atopic patients (see Table E1 in this article's Online Repository at www.jacionline.org). Suction blisters were prepared by applying a negative pressure of 180 mbar, followed by 320 mbar after 30 minutes, to the perilesional areas. Suction blister fluids and roofs were isolated and immediately stored at −80°C until further use.

Isolation of primary human keratinocytes and dermal fibroblasts

Primary human dermal fibroblasts were isolated from punch biopsy specimens by using the dispase II digestion technique (Roche, Penzberg, Germany) for 3 hours. All incubations, unless otherwise stated, were performed at 37°C in a humidified atmosphere containing 7% CO_2. Fibroblasts were cultivated in Dulbecco modified Eagle medium supplemented with 1% penicillin/streptomycin, 1% glutamine (all from Invitrogen, Darmstadt, Germany), and 10% FCS (PAA, Linz, Austria). Keratinocytes were obtained by incubating the detached epidermis in trypsin-EDTA solution (PAA) for 5 to 10 minutes. Keratinocytes were cultured in KBM-2 supplemented with KGM-2 SingleQuots (Lonza, Cologne, Germany) and 0.1 mmol/L Ca^{++} (Sigma-Aldrich, Munich, Germany). In our experiments only single-cell populations from individual donors were used. Single-cell populations from different donors were never pooled.

LIF small interfering RNA knockdown in fibroblasts

Primary human dermal fibroblasts were transfected with Stealth RNAi small interfering RNA (siRNA) specific for LIF using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions with modifications. For the siRNAs used and the transfection protocol, see Table E2 in this article's Online Repository at www.jacionline.org.

OTCs

Type I collagen (Sigma-Aldrich) was dissolved in 0.1% acetic acid overnight at 4°C. The collagen solution was buffered with 10× Hanks solution (Invitrogen) and neutralized with 1 mol/L NaOH. Primary fibroblasts were incubated with 2 μg/mL mitomycin (Sigma-Aldrich) for 4 hours. These proliferation-arrested fibroblasts were trypsinized, resuspended in the neutralized collagen solution (1×10^5 cells/mL), and seeded into Cell Culture Inserts (BD Biosciences, Heidelberg, Germany). After incubation for 1 hour, the gelatinized dermal equivalents were cultured overnight in rFAD medium consisting of Dulbecco modified Eagle medium (Invitrogen) and Ham F12 (Sigma-Aldrich) with 1% penicillin/streptomycin (Invitrogen), 10% FCS (PAA), 0.4 μg/mL hydrocortisone, and 50 μg/mL ascorbic acid (Sigma-Aldrich). Subsequently, human keratinocytes (5×10^5 cells/cm^2) were seeded onto the fibroblast-containing collagen matrix. The resulting OTCs were cultured in rFAD medium under submerged conditions. After 2 days, the OTCs were air exposed and incubated for an additional 6 or 12 days.

Real-time quantitative RT-PCR

Isolation of total RNA from suction blister epidermis, cultured fibroblasts, or keratinocytes was performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was processed with the Qiagen AllPrep DNA/RNA/Protein Mini Kit (Qiagen). The concentration and purity of isolated RNA were assessed spectrophotometrically by using a NanoDrop ND-1000 (Peqlab, Erlangen, Germany). For cDNA synthesis, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) was used. Expression levels were detected by using real-time RT-PCR with gene expression assays mixed with TaqMan Master Mix Reagents Kit performed in a 7900HT Fast Real-Time PCR System (all from Applied Biosystems). All assays were run in duplicates. Gene expression was quantified by using the comparative ΔΔCT method, normalizing each target gene expression to endogenous control 18s rRNA levels. Normalized gene expression levels were compared and indicated as relative target mRNA concentrations. See Table E2 for further details on gene expression assays.

Histologic analysis

For immunohistochemistry, cryosections (6 μm) were fixed with ice-cold acetone for 10 minutes. Subsequently, they were incubated with 1% (wt/vol) BSA in PBS for 1 hour at room temperature to prevent nonspecific binding. The sections were incubated with antibodies directed against human keratin 1 (1:1000, PRB-149P-100; Covance, Munich, Germany), human keratin 5/14 (1:1000, MAB1984; Abnova, Heidelberg, Germany), Ki67 (1:100, ab16667; Abcam, Cambridge, United Kingdom), filaggrin (1:100, 15C10; Leica, Newcastle upon Tyne, United Kingdom), signal transducer and activator of transcription (STAT) 3 (1:1000, 124H6; Cell Signaling Technology, Danvers, Mass), and phospho-STAT3 (1:100, Tyr705, Cell Signaling Technology) overnight at 4°C. The sections were stained with fluorophore-coupled secondary antibodies (anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594, Invitrogen) for 1 hour at room temperature. The slides were mounted with fluorescence mounting medium (Dako, Hamburg, Germany). Immunofluorescence microscopy was performed with the Zeiss Axio Observer Z1 (Carl Zeiss, Oberkochen, Germany). Quantification of nuclei-containing cells was done by counting after 4′-6-diamidino-2-phenylindole dihydrochloride staining (1:2000 [stock: 5 mg/mL], Sigma-Aldrich). Epidermal layer thickness was quantified with ImageJ Software (see Fig E1 in this article's Online Repository at www.jacionline.org).

Detection of LIF protein

LIF protein levels were detected with a cytokine assay from Bio-Rad (Munich, Germany). Recombinant human LIF was obtained from Millipore (Billerica, Mass).

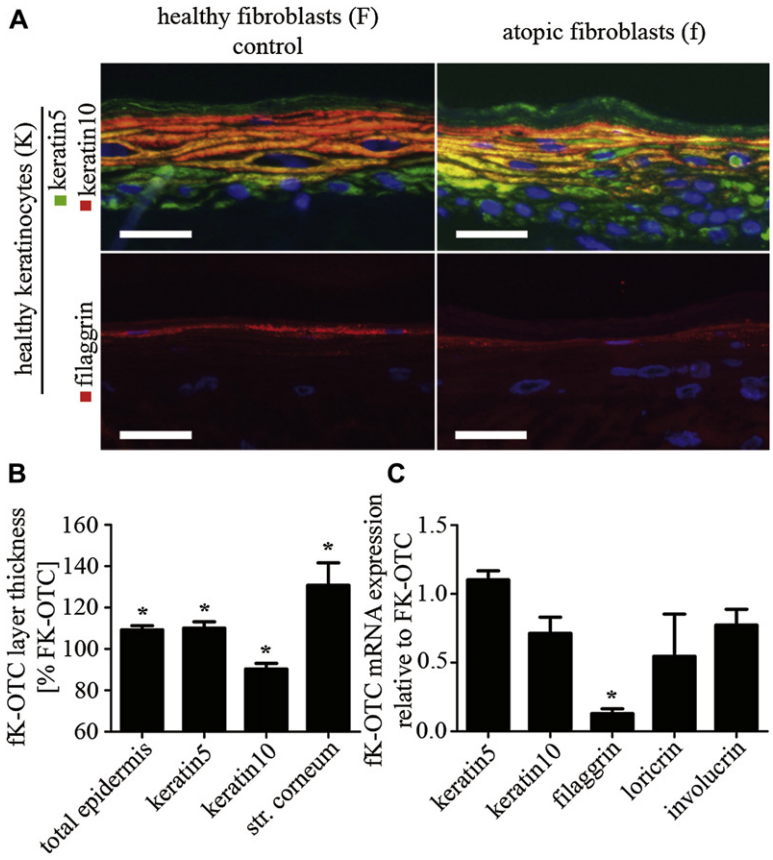

FIG 1. Atopic fibroblasts influence the proliferation and differentiation of healthy keratinocytes in OTCs. **A,** Histologic sections of OTCs consisting of healthy *(F)* or atopic *(f)* fibroblasts and healthy keratinocytes *(K)* were stained for keratin 5 *(green)*/keratin 10 *(red)* and filaggrin *(red)*. **B,** The layer thickness of OTCs cultured with atopic fibroblasts and healthy keratinocytes (fK-OTCs) were compared with that of OTCs cultured with healthy fibroblasts and healthy keratinocytes (FK-OTCs; set at 100%, n = 6). **C,** RNA derived from the newly formed epidermis of the OTCs was reverse transcribed into cDNA. The indicated genes were analyzed with real-time RT-PCR (n = 10). *Scale bar* = 50 μm. **P* < .05, unpaired *t* test.

RESULTS

Atopic fibroblasts impair the proliferation and differentiation of keratinocytes in OTCs

OTCs consisting of fibroblasts and keratinocytes derived from the skin of patients with atopic eczema (perilesional) and healthy skin from nonatopic volunteers were used to analyze the role of dermal fibroblasts in modulating epidermal morphology in patients with AD. Four types of OTCs were compared, comprising different combinations of atopic (f/k, atopic fibroblasts/keratinocytes) and healthy skin cells (F/K, fibroblasts/keratinocytes): OTC with healthy fibroblasts and healthy keratinocytes (FK-OTC), OTC with atopic fibroblasts and healthy keratinocytes (fK-OTC), OTC with healthy fibroblasts and atopic keratinocytes (Fk-OTC), and OTC with atopic fibroblasts and atopic keratinocytes (fk-OTC).

Immunohistologic analyses of the epidermal structures in the OTCs (Fig 1, *A*) revealed a significant increase in mean epidermal thickness in OTCs comprising atopic fibroblasts and healthy keratinocytes (fK-OTCs) in comparison with healthy controls (FK-OTCs). This effect was due to a thickening of the keratin 5–positive basal layer, as well as an increase in the stratum corneum of 30% ± 10.8%. However, the thickness of keratin 10–expressing suprabasal layers was reduced by 10% ± 2.7% (Fig 1, *B*). Immunohistochemical analysis of filaggrin localization and expression levels displayed strongly reduced filaggrin expression in the fK-OTCs (Fig 1, *A*). These results were confirmed by using quantitative real-time PCR analyses of the epidermal differentiation markers keratin 5, keratin 10, involucrin, and filaggrin (Fig 1, *C*). Additionally, the expression of loricrin mRNA was reduced in healthy keratinocytes cocultivated in OTCs with atopic fibroblasts (Fig 1, *C*).

The hyperplastic phenotype induced by atopic fibroblasts in the fK-OTCs was mirrored by an increase of total cell count, as well as an increased number of Ki67-positive proliferating keratinocytes after 6 days of culture (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). These results were confirmed by the proliferative effect of the supernatants of atopic fibroblasts compared with those of healthy fibroblasts on healthy keratinocytes, as shown by an increase of 33% in cell counts (see Fig E2, *B*). Immunohistologically, this effect was also visible in the multilayered structure of the keratin 5–positive stratum basal of the fK-OTCs (Fig 1, *A*).

To confirm the effect of fibroblasts in patients with AD, we reversed the OTC composition by comparing atopic OTCs (fk-OTCs) with OTCs consisting of healthy fibroblasts and atopic keratinocytes (Fk-OTCs). The morphology of the epidermis was highly disturbed in atopic OTCs. Immunohistologic analyses of keratin 5 and keratin 10 expression showed no clear stratification patterning of the seeded keratinocytes at up to 12 days of culture

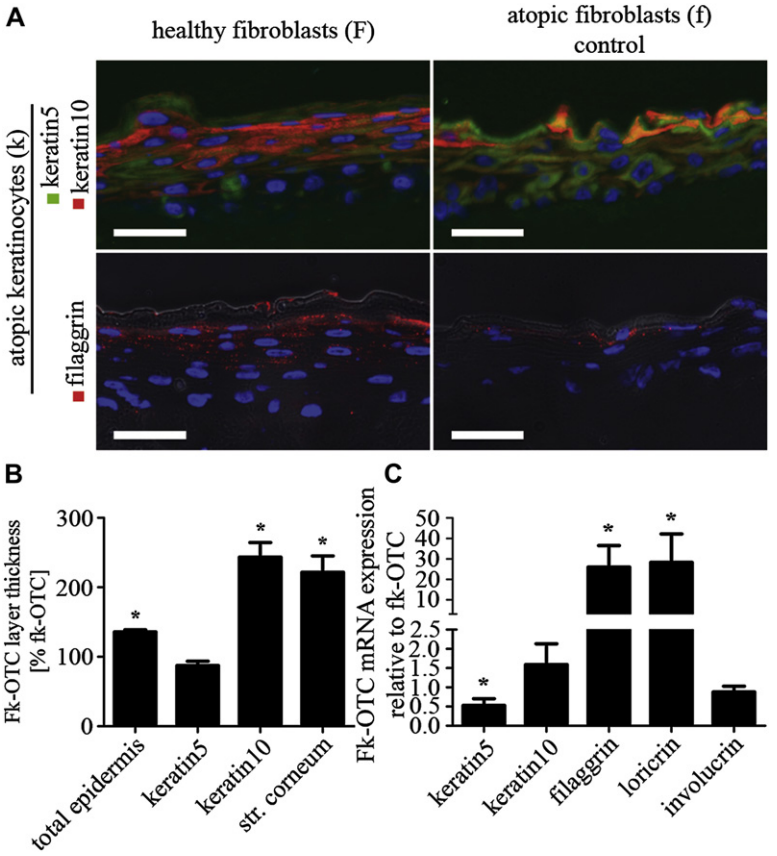

FIG 2. Healthy fibroblasts rescue the morphology of the epidermis in OTCs cultured with atopic keratinocytes. **A,** Histologic sections of OTCs consisting of healthy *(F)* or atopic *(f)* fibroblasts and atopic keratinocytes *(k)* were stained for keratin 5 *(green)*/keratin 10 *(red)* and filaggrin *(red)*. **B,** The layer thickness of OTCs cultured with healthy fibroblasts and atopic keratinocytes (Fk-OTCs) were compared with that of fully atopic OTCs (fk-OTCs; set at 100%, n = 4). **C,** RNA derived from the newly formed epidermis of the OTCs was reverse transcribed into cDNA. The indicated genes were analyzed by using real-time RT-PCR (n = 5). *Scale bar* = 50 μm. *$P < .05$, unpaired *t* test.

(Fig 2, *A*). Also, basal and suprabasal layers were not morphologically differentiable. Additionally, almost no stratum corneum was formed. These structural epidermal defects were partially rescued by healthy fibroblasts (Fig 2, *A*). Total epidermal thickness, especially regarding the suprabasal layers (+143% ± 21%) and the stratum corneum (+122 ± 23%), was increased. Furthermore, the thickness of the keratin 5–positive basal layer was reduced by 13% ± 4% (Fig 2, *B*). Subsequently, the expression level of keratin 5 was reduced, and the expression level of the differentiation marker keratin 10 was increased by healthy fibroblasts compared with the level seen in full atopic control OTCs. Filaggrin mRNA expression and loricrin expression were strongly induced in the Fk-OTCs (Fig 2, *C*). Histologically, the stratification pattern was also recovered with distinguishable basal and differentiated keratin 10–positive suprabasal layers.

Reduced expression of LIF in atopic skin cells influences epidermal structure

Fibroblasts cultivated from atopic and healthy skin were subjected to comparative analysis regarding their expression profiles of inflammatory cytokines to identify fibroblast-secreted factors that are differentially regulated under atopic conditions. From more than 50 analyzed cytokines, most prominently, the expression of LIF mRNA was significantly downregulated to 0.15 ± 0.03 of the relative expression of healthy fibroblasts (Fig 3, *A*). This result correlated with a decrease in secreted LIF protein levels in the supernatant of atopic fibroblasts by 79% ± 3.3% (Fig 3, *B*). However, expression of the LIF receptor, as well as suppressor of cytokine signaling 3 (SOCS3), an important suppressor of LIF signaling, was not altered (Fig 3, *A*). Suction blister fluids and epidermis obtained from perilesional skin of atopic volunteers or healthy control subjects were analyzed to examine whether LIF expression is also impaired in atopic skin *in vivo*. LIF mRNA derived from suction blister epidermis, predominantly consisting of keratinocytes, was reduced significantly by 39% ± 7.6% (Fig 3, *C*), indicating an altered expression of LIF in keratinocytes. Consistently, the level of secreted LIF protein was reduced in atopic skin by 55% ± 11% (Fig 3, *D*). We were not able to detect LIF protein in supernatants of cultured normal and atopic keratinocytes. The expression of LIF receptor and SOCS3 was quantified in suction blister epidermis to address the question of whether the atopic epidermis is able to respond to LIF protein signaling (Fig 3, *C*). Similar to fibroblasts, keratinocytes derived from atopic skin showed no differences in LIF receptor and SOCS3 expression. To assess whether the reduced concentrations of LIF in atopic skin cells affect the activation level of the LIF downstream effector STAT3, we used

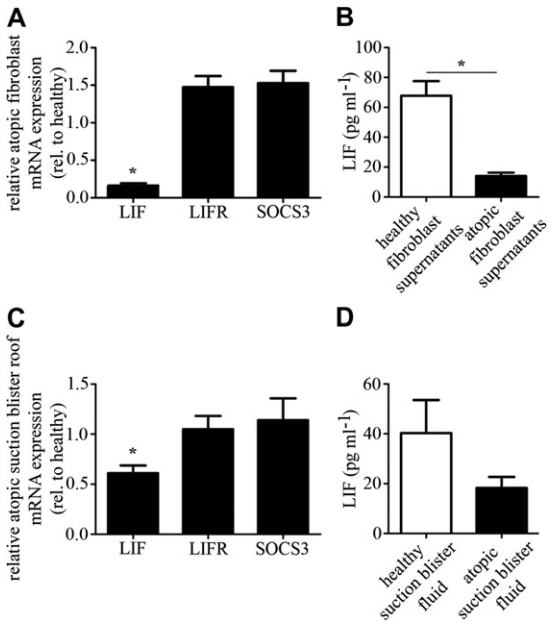

FIG 3. LIF gene expression is reduced in atopic fibroblasts and suction blisters. RNA derived from human dermal fibroblasts and suction blister roofs was reverse transcribed into cDNA, which was analyzed by using real-time RT-PCR. **A** and **C,** Gene expression in atopic fibroblasts (n = 9; Fig 3, *A*) and atopic suction blister roofs (n = 12; Fig 3, *C*) were compared with that in healthy controls (set at 1). **B** and **D,** By using the ELISA technique, the LIF protein concentrations in cell-culture supernatants of healthy and atopic fibroblasts (n = 10; Fig 3, *B*) and suction blister fluid (n = 8; Fig 3, *D*) were determined. Protein concentrations were normalized to protein content of corresponding cell lysates. **P* < .05, unpaired *t* test.

immunofluorescence microscopy to determine the STAT3 phosphorylation level in OTCs. In OTCs consisting of healthy fibroblasts and healthy keratinocytes, 55% of total nuclei were phospho-STAT positive, whereas only 33% were phospho-STAT positive in OTCs cultured with atopic fibroblasts. Addition of recombinant LIF increased the phospho-STAT–positive number of nuclei to 45% in these OTCs. Recombinant LIF or cocultivation with LIF-releasing healthy fibroblasts induced phosphorylation of STAT3 in atopic keratinocytes similar to that seen in healthy keratinocytes. In OTCs consisting of atopic fibroblasts and atopic keratinocytes, only 30% of total nuclei were phospho-STAT positive. Addition of recombinant LIF increased the phospho-STAT–positive number of nuclei to 58%. Taken together, in the absence of LIF, either by LIF-specific siRNA or by cultivation with atopic fibroblasts, the phospho-STAT3 signal was reduced in OTC epidermis (see Fig E3 in this article's Online Repository at www.jacionline.org).

By using siRNA to knockdown LIF expression in healthy fibroblasts, we investigated whether LIF was critical for the observed rescue effect of healthy fibroblasts on atopic keratinocytes (Fig 2, *A*). LIF siRNA strongly reduced the expression of LIF mRNA by 85%, even 120 hours after siRNA application (data not shown). Analyses of the corresponding OTCs revealed a reduced expression of terminal differentiation markers, including filaggrin, loricrin, and involucrin (Fig 4, *A* and *C*) induced by LIF deficiency. Phenotypically, LIF knockdown reduced the thickness of the OTC epidermis, mainly because of a thinning of the keratin 10–positive layer comparable with the effect of atopic fibroblasts (Fig 4, *B*).

To confirm the functional relevance of fibroblast-derived LIF for the stratification and differentiation of keratinocytes, we supplied recombinant human LIF protein to OTCs comprising atopic fibroblasts and healthy keratinocytes (the fK-OTCs; see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). Over a cultivation period of 12 days, the application of LIF resulted in a significant decrease in the mean epidermal thickness of the fK-OTCs by 16% ± 1.8% (see Fig E4, *B*). Furthermore, LIF almost completely abrogated the increase in keratinocyte cell numbers and the thickening of the keratin 5/14–positive basal layer (see Fig E4, *B* and *C*), as well as the increased number of Ki67-positive proliferating keratinocytes (see Fig E2, *C*), which are characteristic of the fK-OTCs. However, gene expression analyses of differentiation markers using real-time quantitative PCR showed no significant effect of LIF on filaggrin or loricrin expression (see Fig E4, *D*). In the fk-OTCs group supplementation of LIF only slightly improved the stratification of the epidermis (see Fig E4, *A*).

DISCUSSION

A functional skin barrier prevents epidermal water loss and also impedes the entry of allergens, chemicals, and microorganisms that cause cutaneous inflammation.[12] Recently, the importance of epidermal skin barrier function has gained attention regarding atopic diseases.[13-15] The discovery that filaggrin mutations predispose to AD has focused research on keratinocyte-dependent defects in skin barrier function. Clinical characteristics, such as hyperplasia and parakeratosis, were attributed to inherent proliferation and differentiation defects of atopic keratinocytes or aberrant immune responses.[16,17]

It is known that fibroblasts contribute to the microenvironment of other cell types by releasing paracrine mediators.[11] Skin pigmentation and thickness are influenced by dermal fibroblast–secreted factors.[18] Additionally, secreted factors are of importance during cutaneous wound healing, stimulating keratinocyte proliferation and re-epithelialization.[7,19,20] In patients with psoriasis, fibroblasts were shown to induce hyperproliferation of keratinocytes and to enhance the formation of the cornified envelope.[21-23] However, although some dermal changes, such as pruritus-related papules and nodes, are diagnosed, little is known about the effect of fibroblasts in patients with AD.[24]

Using atopic-like OTCs, we provide evidence that dermal fibroblasts contribute to the atopic epidermal phenotype by increasing proliferation and impairing the stratification of keratinocytes. Fibroblasts derived from perilesional atopic skin not only maintained their atopic disposition *in vitro* but also induced an *in vivo*–like atopic epidermal phenotype in healthy keratinocytes characterized by hyperproliferation and reduced expression of differentiation markers, such as filaggrin. Moreover, impaired terminal differentiation was also indicated by higher numbers of nuclei in suprabasal layers in the fK-OTCs, pointing to an incomplete degradation of nuclei during terminal differentiation of keratinocytes. This phenotype might be related to parakeratosis of atopic skin[25] and should be investigated in future experiments. We hypothesized that the characteristics of atopic skin would be even more pronounced in the fk-OTCs, which contains keratinocytes and fibroblasts from atopic donors. However, our results showed that cocultures of these 2 atopic cell types did not form a stratified epidermis. Strong proliferation and differentiation defects in these OTCs were indicated by the indistinct distribution of

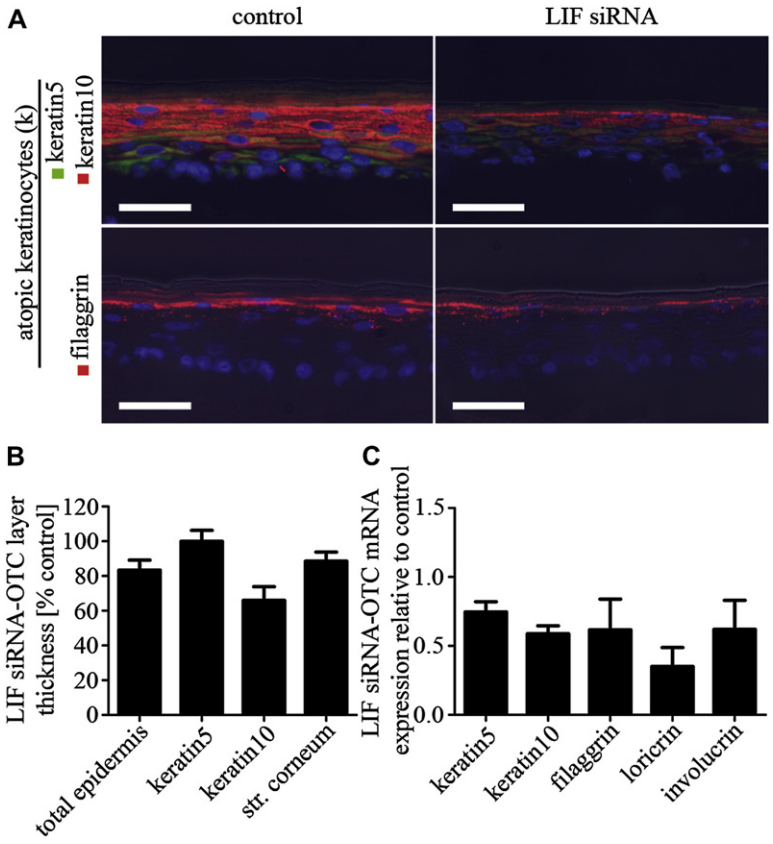

FIG 4. LIF siRNA knockdown in healthy fibroblasts reduces the ability to rescue epidermal morphology in atopic keratinocyte OTCs. **A,** Histologic sections of OTCs consisting of healthy fibroblasts treated with either scrambled or LIF siRNA were stained for keratin 5 *(green)*/keratin 10 *(red)* and filaggrin *(red)*. **B,** The layer thickness of LIF siRNA OTCs was compared with that of the controls (scrambled siRNA OTCs; set at 100%, n = 3). **C,** RNA derived from the newly formed epidermis of the OTCs was reverse transcribed into cDNA. The indicated genes were analyzed by using real-time RT-PCR (n = 3). *Scale bar* = 50 μm.

differentiation markers. *In vivo*, the structure of the epidermis is also modulated by paracrine factors released by noncutaneous cells. We speculate that in atopic skin peripheral nerve endings and nonresident immune cells produce mediators, such as calcitonin gene-related peptide and substance P, influencing proliferation of keratinocytes and altering tissue homeostasis and structure.[26,27]

Nevertheless, healthy fibroblasts were able to rescue the stratification and differentiation of the epidermis in OTCs containing atopic keratinocytes. Consequently, an increase of the expression of terminal differentiation markers, such as filaggrin and loricrin, was observed, indicating an important role for fibroblasts on epidermal structure. Filaggrin is an important molecule for the formation of the cornified envelope, and mutations in the filaggrin gene predispose to AD.[3,28] However, in some atopic patients reduced filaggrin expression is not due to a mutation in the filaggrin gene[29] but to the cutaneous micromilieu. Indeed, several proinflammatory cytokines, such as IL-4, IL-13, and IL-31, were reported to reduce filaggrin expression in keratinocytes *in vitro*.[29,30] Our results provide the first evidence that atopic fibroblasts negatively modulate the basal expression of filaggrin.

Instead of increased concentrations of mitogenic and inflammatory factors, such as keratinocyte growth factor, GM-CSF, and IL-6,[9,11,31] a lack of proliferation- and/or differentiation-restricting factors could explain the atopic fibroblast–dependent epidermal phenotype. Here, our *in vitro*, *ex vivo*, and *in vivo* data consistently point to LIF as an important factor. LIF is a member of the IL-6 family of hematopoietic growth factors.[32] LIF signaling through the Janus kinase–STAT3 pathway is associated with proliferation and differentiation processes.[33-37] The important role of LIF-STAT3 signaling for the atopic fibroblast–induced phenotype is supported by the rescue effect of healthy fibroblasts on Fk-OTCs and enhanced STAT3 phosphorylation in Fk-OTC keratinocytes. Both effects were dependent on LIF expression by fibroblasts, as confirmed by LIF siRNA knockdown in healthy fibroblasts. These results might be explained by a threshold concentration of LIF that is granted by healthy but not atopic fibroblasts. Consistently, although fK-OTCs and FK-OTCs contained healthy keratinocytes, recombinant LIF-dependent effects were more distinct in fK-OTCs. LIF-induced reduction of keratinocyte numbers and improvement of the epidermal structure might be explained by inhibition of proliferation or induction of terminal differentiation. Supporting the latter, activation of the LIF receptor induces differentiation processes in keratinocytes[38] and mucosal epithelia,[39] whereas STAT3-deficient keratinocytes do not undergo differentiation.[38] However, the effect could also be based on a general inhibition of keratinocyte proliferation because reduced numbers of Ki67-positive keratinocytes and a thinning of all differentiation layers were observed. Of note, other glycoprotein 130 activators, IL-6,

oncostatin M, and GM-CSF, were reported to promote human epidermal keratinocyte proliferation,[40-42] indicating a nonredundant role of LIF and other IL-6 family members. According to our results, LIF is an important but not the sole factor involved in aberrant dermal-epidermal communication in patients with AD. The supplementation of recombinant LIF to fK-OTCs was not able to completely mimic the effects of healthy fibroblasts. For example, in contrast to healthy fibroblasts, recombinant LIF did not induce filaggrin expression in healthy keratinocytes. Studies with LIF knockout mice revealed a key anti-inflammatory role for LIF *in vivo*, hypothetically because of its regulatory function on the expression of IL-6, IL-7, IL-2Ra, and IFN-γ.[43] Application of low doses of LIF suppressed both IL-1β and nerve growth factor upregulation during complete Freund adjuvant–induced cutaneous inflammation in LIF knockout mice.[44] Hence a reduced LIF concentration in atopic skin might result in a proinflammatory condition. Interestingly, a recent report showed a synergistic potentiating of LIF function by glucocorticoids in AtT-20 cells, revealing a gene cluster that requires both stimuli for activation.[45] Most of these clustered genes were involved in different cell defense mechanisms. It is tempting to speculate that topical application of glucocorticoids might enhance the effect of LIF in atopic skin, a factor that could be considered in the new approach of proactive treatment of AD.

Taken together, our data indicate that atopic fibroblasts contribute to the pathogenesis of AD. Moreover, organotypic skin models containing skin cells derived from atopic donors appear to be a relevant tool to investigate new approaches for atopic skin.

Key messages

- **Atopic fibroblasts modulate the proliferation, differentiation, and stratification of healthy keratinocytes in an OTC, resembling an *in vivo*–like atopic phenotype.**
- **Atopic skin is characterized by low concentrations of LIF.**
- **Addition of recombinant LIF ameliorates the effects of atopic fibroblasts in OTCs.**

REFERENCES

1. Korge B. [Epidermal barrier function abnormalities in atopic dermatitis caused by filaggrin mutations]. Hautarzt 2009;60:522.
2. McGrath JA. Filaggrin and the great epidermal barrier grief. Australas J Dermatol 2008;49:67-74.
3. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet 2006;38:441-6.
4. De Benedetto A, Rafaels NM, McGirt LY, Ivanov AI, Georas SN, Cheadle C, et al. Tight junction defects in patients with atopic dermatitis. J Allergy Clin Immunol 2010;127:773-86.e1-7.
5. Kirschner N, Houdek P, Fromm M, Moll I, Brandner JM. Tight junctions form a barrier in human epidermis. Eur J Cell Biol 2010;89:839-42.
6. Suarez-Farinas M, Tintle SJ, Shemer A, Chiricozzi A, Nograles K, Cardinale I, et al. Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities. J Allergy Clin Immunol 2011;127:954-64, e1-4.
7. El Ghalbzouri A, Hensbergen P, Gibbs S, Kempenaar J, van der Schors R, Ponec M. Fibroblasts facilitate re-epithelialization in wounded human skin equivalents. Lab Invest 2004;84:102-12.
8. Grossman RM, Krueger J, Yourish D, Granelli-Piperno A, Murphy DP, May LT, et al. Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. Proc Natl Acad Sci U S A 1989;86:6367-71.
9. Maas-Szabowski N, Shimotoyodome A, Fusenig NE. Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism. J Cell Sci 1999;112(suppl):1843-53.
10. Smola H, Thiekotter G, Baur M, Stark HJ, Breitkreutz D, Fusenig NE. Organotypic and epidermal-dermal co-cultures of normal human keratinocytes and dermal cells: Regulation of transforming growth factor alpha, beta1 and beta2 mRNA levels. Toxicol In Vitro 1994;8:641-50.
11. Smola H, Thiekotter G, Fusenig NE. Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. J Cell Biol 1993;122:417-29.
12. Tupker RA, Pinnagoda J, Coenraads PJ, Nater JP. Susceptibility to irritants: role of barrier function, skin dryness and history of atopic dermatitis. Br J Dermatol 1990;123:199-205.
13. Lee SH, Jeong SK, Ahn SK. An update of the defensive barrier function of skin. Yonsei Med J 2006;47:293-306.
14. Morar N, Willis-Owen SA, Moffatt MF, Cookson WO. The genetics of atopic dermatitis. J Allergy Clin Immunol 2006;118:24-36.
15. Cork MJ, Danby SG, Vasilopoulos Y, Hadgraft J, Lane ME, Moustafa M, et al. Epidermal barrier dysfunction in atopic dermatitis. J Invest Dermatol 2009;129:1892-908.
16. Proksch E, Folster-Holst R, Jensen JM. Skin barrier function, epidermal proliferation and differentiation in eczema. J Dermatol Sci 2006;43:159-69.
17. De Benedetto A, Agnihothri R, McGirt LY, Bankova LG, Beck LA. Atopic dermatitis: a disease caused by innate immune defects? J Invest Dermatol 2009;129:14-30.
18. Yamaguchi Y, Passeron T, Hoashi T, Watabe H, Rouzaud F, Yasumoto K, et al. Dickkopf 1 (DKK1) regulates skin pigmentation and thickness by affecting Wnt/beta-catenin signaling in keratinocytes. FASEB J 2008;22:1009-20.
19. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999;341:738-46.
20. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev 2003;83:835-70.
21. Saiag P, Coulomb B, Lebreton C, Bell E, Dubertret L. Psoriatic fibroblasts induce hyperproliferation of normal keratinocytes in a skin equivalent model in vitro. Science 1985;230:669-72.
22. Krueger GG, Jorgensen CM. Experimental models for psoriasis. J Invest Dermatol 1990;95:56S-8S.
23. Ishikawa H, Mitsuhashi Y, Takagi Y, Hashimoto I. Psoriatic fibroblasts enhance cornified envelope formation in normal keratinocytes in vitro. Arch Dermatol Res 1997;289:551-3.
24. Lee JH, Chen SY, Yu CH, Chu SW, Wang LF, Sun CK, et al. Noninvasive in vitro and in vivo assessment of epidermal hyperkeratosis and dermal fibrosis in atopic dermatitis. J Biomed Opt 2009;14:014008.
25. Hirao T, Terui T, Takeuchi I, Kobayashi H, Okada M, Takahashi M, et al. Ratio of immature cornified envelopes does not correlate with parakeratosis in inflammatory skin disorders. Exp Dermatol 2003;12:591-601.
26. Peters EM, Ericson ME, Hosoi J, Seiffert K, Hordinsky MK, Ansel JC, et al. Neuropeptide control mechanisms in cutaneous biology: physiological and clinical significance. J Invest Dermatol 2006;126:1937-47.
27. Roggenkamp D, Köpnick S, Stäb F, Wenck H, Schmelz M, Neufang G. Epidermal nerve fibers modulate keratinocyte growth and differentiation via neuropeptide signaling in an innervated skin model. J Invest Dermatol 2013 [Epub ahead of print].
28. Esparza-Gordillo J, Weidinger S, Folster-Holst R, Bauerfeind A, Ruschendorf F, Patone G, et al. A common variant on chromosome 11q13 is associated with atopic dermatitis. Nat Genet 2009;41:596-601.
29. Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, De Benedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. J Allergy Clin Immunol 2007;120:150-5.
30. Cornelissen C, Marquardt Y, Czaja K, Wenzel J, Frank J, Luscher-Firzlaff J, et al. IL-31 regulates differentiation and filaggrin expression in human organotypic skin models. J Allergy Clin Immunol 2012;129:426-33, e1-8.
31. Szabowski A, Maas-Szabowski N, Andrecht S, Kolbus A, Schorpp-Kistner M, Fusenig NE, et al. c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. Cell 2000;103:745-55.
32. Gearing DP. The leukemia inhibitory factor and its receptor. Adv Immunol 1993;53:31-58.
33. Brown GS, Brown MA, Hilton D, Gough NM, Sleigh MJ. Inhibition of differentiation in a murine F9 embryonal carcinoma cell subline by leukemia inhibitory factor (LIF). Growth Factors 1992;7:41-52.
34. Adachi T, Takanaga H, Kunimoto M, Asou H. Influence of LIF and BMP-2 on differentiation and development of glial cells in primary cultures of embryonic rat cerebral hemisphere. J Neurosci Res 2005;79:608-15.
35. Elliott J, Cayouette M, Gravel C. The CNTF/LIF signaling pathway regulates developmental programmed cell death and differentiation of rod precursor cells in the mouse retina in vivo. Dev Biol 2006;300:583-98.
36. Murray P, Edgar D. The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor (LIF). Differentiation 2001;68:227-34.

37. Xie P, Chan FS, Ip NY, Leung MF. Induction of gp130 and LIF by differentiation inducers in human myeloid leukemia K562 cells. Leuk Res 1999;23:1113-9.
38. Hauser PJ, Agrawal D, Hackney J, Pledger WJ. STAT3 activation accompanies keratinocyte differentiation. Cell Growth Differ 1998;9:847-55.
39. Wu R, Sun S, Steinberg BM. Requirement of STAT3 activation for differentiation of mucosal stratified squamous epithelium. Mol Med 2003;9:77-84.
40. Boniface K, Diveu C, Morel F, Pedretti N, Froger J, Ravon E, et al. Oncostatin M secreted by skin infiltrating T lymphocytes is a potent keratinocyte activator involved in skin inflammation. J Immunol 2007;178:4615-22.
41. Braunstein S, Kaplan G, Gottlieb AB, Schwartz M, Walsh G, Abalos RM, et al. GM-CSF activates regenerative epidermal growth and stimulates keratinocyte proliferation in human skin in vivo. J Invest Dermatol 1994;103:601-4.
42. Hernandez-Quintero M, Kuri-Harcuch W. Gonzalez Robles A, Castro-Munozledo F. Interleukin-6 promotes human epidermal keratinocyte proliferation and keratin cytoskeleton reorganization in culture. Cell Tissue Res 2006;325:77-90.
43. Zhu M, Oishi K, Lee SC, Patterson PH. Studies using leukemia inhibitory factor (LIF) knockout mice and a LIF adenoviral vector demonstrate a key anti-inflammatory role for this cytokine in cutaneous inflammation. J Immunol 2001; 166:2049-54.
44. Banner LR, Patterson PH, Allchorne A, Poole S, Woolf CJ. Leukemia inhibitory factor is an anti-inflammatory and analgesic cytokine. J Neurosci 1998;18:5456-62.
45. Langlais D, Couture C, Balsalobre A, Drouin J. Regulatory network analyses reveal genome-wide potentiation of LIF signaling by glucocorticoids and define an innate cell defense response. PLoS Genet 2008;4:e1000224.

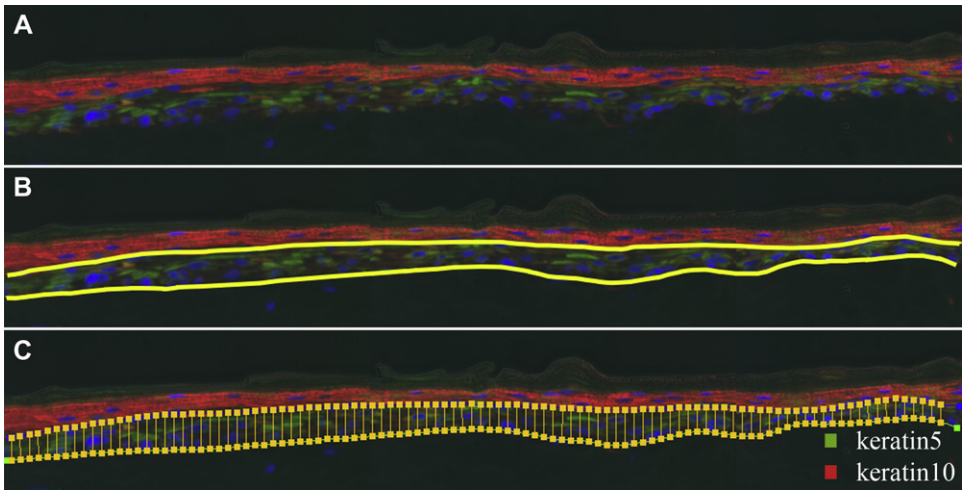

FIG E1. Image analysis of an OTC. **A,** The OTCs were stained for keratin 5 *(green)* and keratin 10 *(red)* to determine the layer thickness of the different epidermal layers. **B,** Lines were drawn at the borders of the different epidermal layers. **C,** By using ImageJ Software, the layer thickness was quantified as the median distance between the drawn lines. Five images of each OTC, approximately 4.5 to 5 mm in width, of different positions within the OTCs were analyzed.

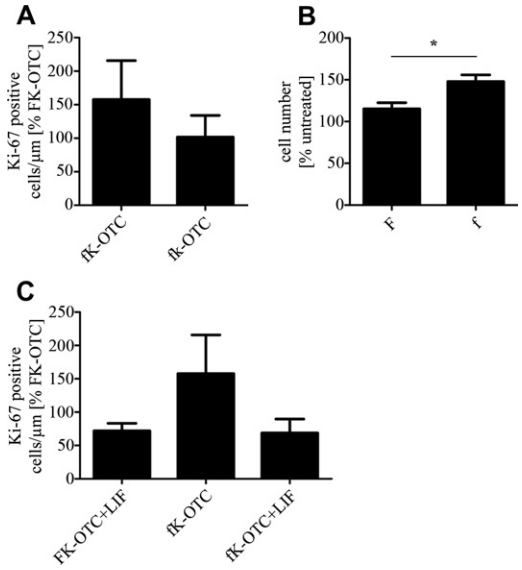

FIG E2. Addition of LIF alters the amount of Ki67-positive keratinocytes in OTCs. **A,** Ki67-positive cells were counted in OTCs cultured with atopic fibroblasts and healthy *(fK)* or atopic *(fk)* keratinocytes and compared with numbers in healthy controls (set at 100%, n = 4). **B,** Healthy keratinocytes were serum starved for 24 hours before being treated with supernatants of either healthy *(F)* or atopic *(f)* fibroblasts. The total cell count was determined after 96 hours and compared with that in untreated controls (set at 100%, n = 5). **C,** Ki67-positive keratinocytes (healthy) were counted at day 6 in OTCs cultured with healthy fibroblasts with additional LIF *(FK-OTC+LIF)*, atopic fibroblasts without *(fK-OTC)* and with additional LIF *(fK-OTC+LIF)* and were compared with numbers in healthy untreated controls (set at 100%, n = 6).

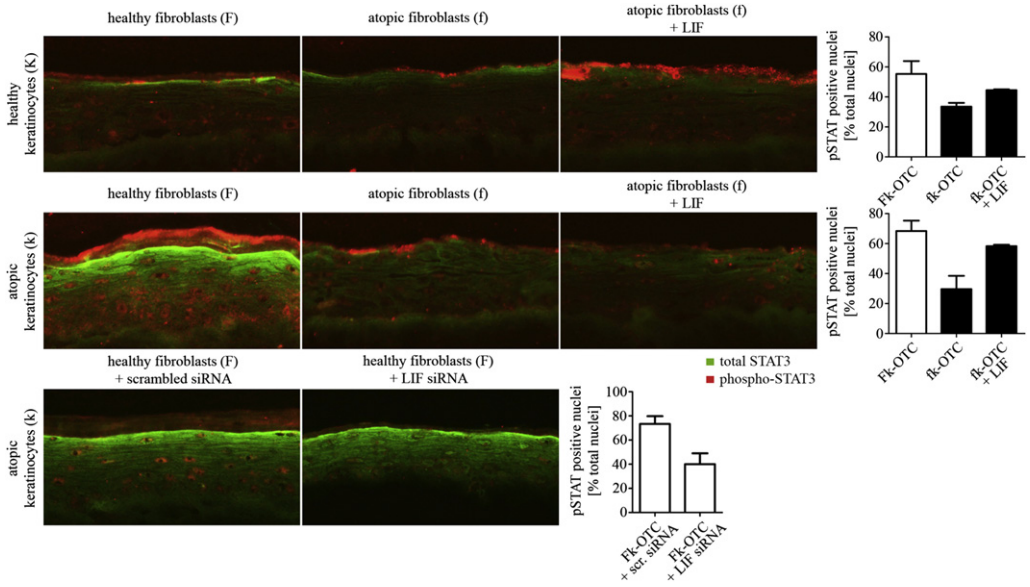

FIG E3. Phosphorylation of STAT3 in OTCs. OTCs were stained for total STAT3 *(green)* and phospho-STAT3 *(red)* as indicated. The number of phospho-STAT–positive nuclei of 3 donors of each OTC were counted and compared with total nuclei numbers.

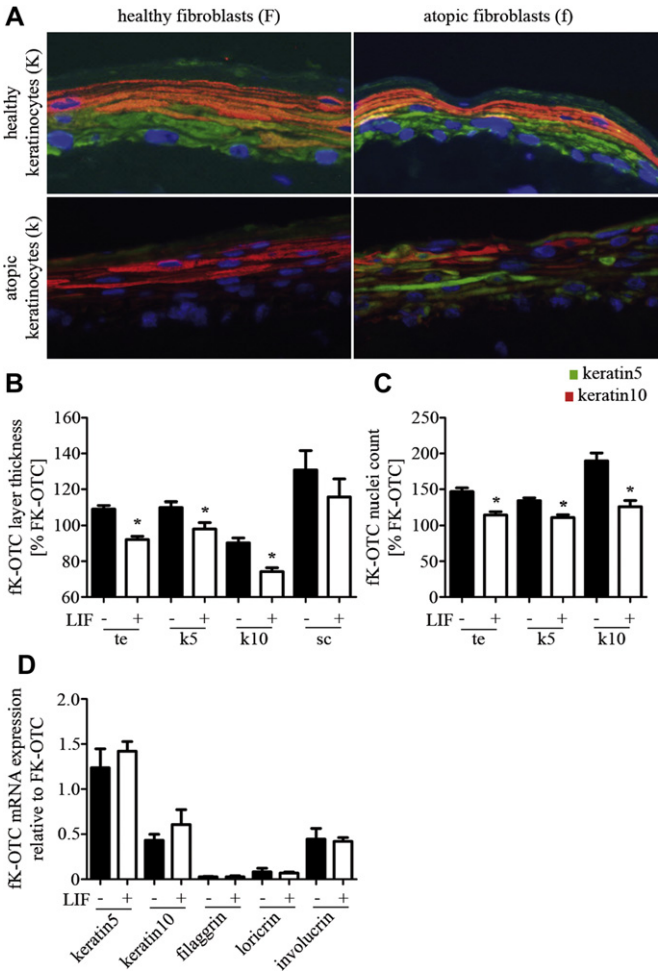

FIG E4. Addition of LIF leads to a reduction in layer thickness and nuclei counts in OTCs cultured with atopic fibroblasts and healthy keratinocytes. **A,** Histologic sections of OTCs consisting of either healthy *(F)* or atopic *(f)* fibroblasts and healthy *(K)* or atopic *(k)* keratinocytes were stained for keratin 5 (green) and keratin 10 (red). **B,** The layer thickness of untreated fK-OTCs *(black bars)* and fK-OTCs treated daily with 25 ng/mL human recombinant LIF *(white bars)* were compared with OTCs cultured with healthy fibroblasts and healthy keratinocytes (FK-OTC; set at 100%, n = 6). **C,** The nuclei count of untreated fK-OTCs *(black bars)* and fK-OTCs treated daily with 25 ng/mL human recombinant LIF *(white bars)* were compared with OTCs cultured with healthy fibroblasts and healthy keratinocytes (FK-OTC; set at 100%). **D,** RNA derived from the newly formed epidermis of the OTCs was reverse transcribed into cDNA. The indicated genes were analyzed by using real-time RT-PCR. *k5*, Keratin 5–positive layer; *k10*, keratin 10–positive layer; *sc*, stratum corneum; *te*, total epidermis. **P* < .05, unpaired *t* test.

TABLE E1. Clinical data of patients with AD

Age (y)	Sex	SCORAD (total)	SCORAD (local)	IgE (kU/L)	Filaggrin mutation
35	F	30.3	23.7	48.1	No
44	M	35.2	19	4,057	No
35	M	25.6	33	2,301	R2447X
48	M	38	30.8	11,645	No
41	F	28.3	9.5	2,640	R501X
25	M	27.5	19.5	–	No
26	M	No data	17.5	–	No data
36	F	No data	24.5	–	No data
44	F	No data	12.3	–	No data
49	M	No data	19.3	–	No data
Average 38	4 F, 6 M	Average 30.8	Average 20.9	Average 4,138	–

F, Female; *M*, male.

TABLE E2. List of used gene expression assays and siRNAs

siRNAs	
LIF siRNA	HSS106045 (Invitrogen)
Scrambled control siRNA	AllStars Neg. Control siRNA (Qiagen)
Gene expression assays	
LIF	Hs00171455_m1
LIF-R	Hs01123581_m1
SOCS3	Hs02330328_s1
Keratin 5	Hs00361185_m1
Keratin 10	Hs00166289_m1
Filaggrin	Hs00856927_g1
Loricrin	Hs01894962_s1
Involucrin	Hs00846307_s1

Fibroblasts were transfected by using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions with modifications. One day before the transfection, 2×10^5 fibroblasts were seeded into 6-well plates with 1.25 mL of culture medium without antibiotics. siRNA (1.5 μL of a 20 μmol/L) solution was mixed with 123.5 μL of OPTI-MEM medium (Gibco). RNAiMAX (1.5 μL) was mixed with 123.5 μL of OPTI-MEM medium. The siRNA solution and the RNAiMAX solution were mixed and incubated at room temperature for 15 minutes before transfer to the fibroblasts. The transfected fibroblasts were cultured for 24 hours before they were used to create OTCs.