

Letter to the Editor

IL-33 impacts on the skin barrier by down-regulating the expression of filaggrin

To the Editor:

IL-33 is a member of the IL-1 family of cytokines that is constitutively expressed in healthy skin and was found to be increased in the skin of patients with atopic dermatitis (AD). Because it can be released after tissue damage or physical stress including scratching of the skin,¹ it has been classified as an alarmin concerned with alerting the immune system.² It enhances T_H2 responses by inducing IL-5 and IL-13 as well as T_H1 responses via upregulation of IFN- γ . Keratinocytes are known producer cells of IL-33 and also express the receptor complex consisting of ST2 and IL-1RAcP on their surface. The aim of this study was to investigate the effect of IL-33 on keratinocytes, skin biopsies, and living skin equivalents with regard to the regulation of the skin barrier molecule filaggrin (FLG). FLG is a structural protein promoting the outer epidermal barrier by aggregation of intermediate filaments³ and influencing cell differentiation, which is considered to be a key step in establishing the structure and function of the stratum corneum.^{E1} Furthermore, processed FLG contributes to natural moisturizing factors important for skin hydration.^{E2} There is evidence that a lack of FLG breakdown products favors transepidermal water loss,^{E3} allergen penetration,⁴ and skin colonization with *Staphylococcus aureus*.^{E4} FLG loss of function may also play a role in the development of AD^{5,6} because it has been shown that up to 50% of the patients with AD carry such a mutation. Several cytokines such as IL-4,⁷ IL-17,^{E5} IL-22,^{E5} IL-25,^{E6,E7} and IL-31^{E8} have been described to negatively affect the expression of FLG in keratinocytes. In this study, we analyzed the effect of IL-33 on FLG expression in subconfluent and differentiated monolayer keratinocytes, skin biopsies, and skin equivalents.

In the first approach, monolayer keratinocytes and skin samples were stimulated with IL-33 or IL-4 to analyze FLG mRNA expression (see additional information in the [Methods](#) section in this article's Online Repository at [www.jacionline.org](#)). We confirmed the effect of IL-4 on FLG in Ca²⁺-differentiated human primary keratinocytes (Fig 1, A). On investigating the impact of IL-33 on FLG transcription, we observed a tendency toward downregulation using the same experimental setup, whereas no effect of IL-33 on FLG expression was observable in undifferentiated keratinocytes cultured in the absence of calcium (Fig 1, B). When we analyzed keratinocytes from patients with AD that were kept in cell culture in a semi-confluent manner with or without Ca²⁺, we observed significant decreases in FLG mRNA on stimulation with IL-33 (Fig 1, B). The median effect on mRNA reduction was more pronounced compared with keratinocytes from healthy donors (median 0.54 vs 0.73, respectively). Subsequently, we investigated the expression of FLG mRNA in skin biopsies after incubation for 24 hours with IL-33 (Fig 1, C). Here, we could also confirm the downregulatory effect of IL-4. Although the effect of IL-33 was not significant, which may be explained by the time point of measurement after 24-hour incubation, a clear tendency could be observed ($P = .08$). An even shorter stimulation (4 hours) of skin samples with IL-33 or IL-4 did not show a clear

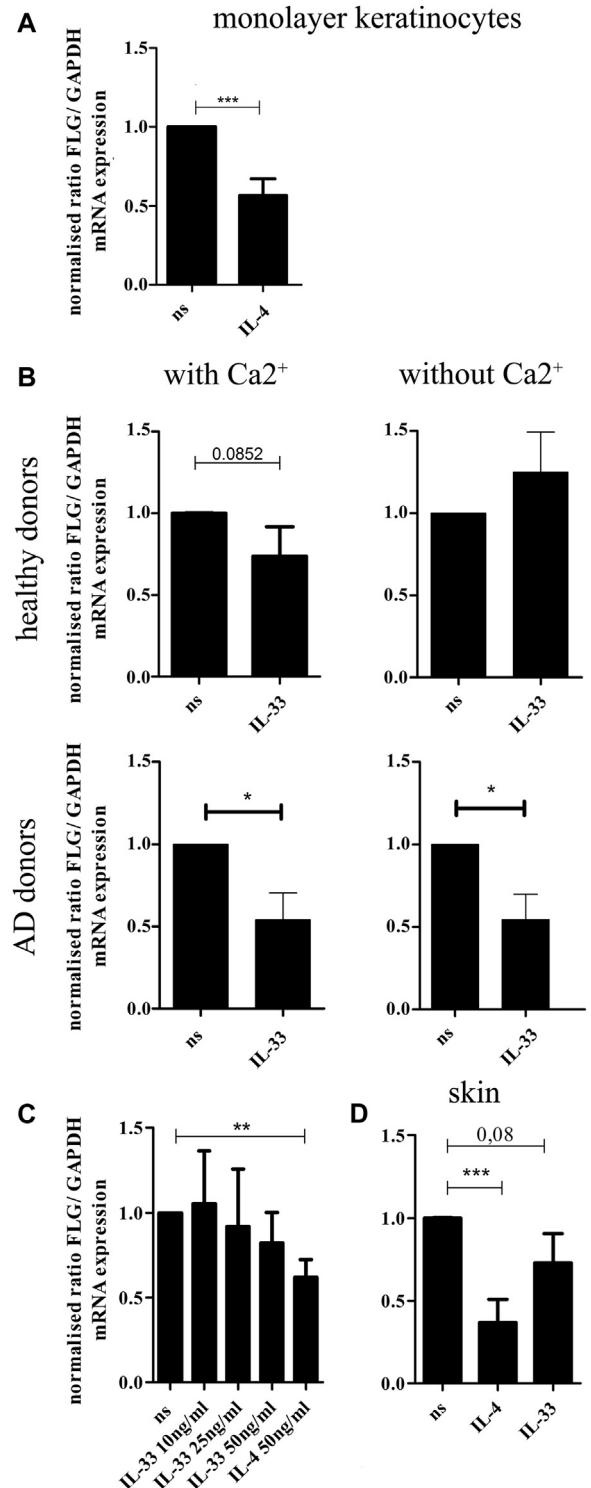


FIG 1. mRNA expression of FLG in monolayer keratinocytes and skin samples measured by quantitative RT-PCR. **A**, Ca²⁺-differentiated keratinocytes from healthy donors stimulated for 4 hours with 50 ng/mL of IL-4 ($n = 7$). **B**, Ca²⁺-differentiated and undifferentiated keratinocytes from healthy donors and patients with AD stimulated for 4 hours with 50 ng/mL of IL-33 as indicated ($n = 3-7$). **C**, Whole-skin samples stimulated with 50 ng/mL of IL-4 or IL-33 for 24 hours ($n = 6$). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ns, nonstimulated. Means and SEM are depicted. t test: * $P < .05$, ** $P < .005$, *** $P < .001$.

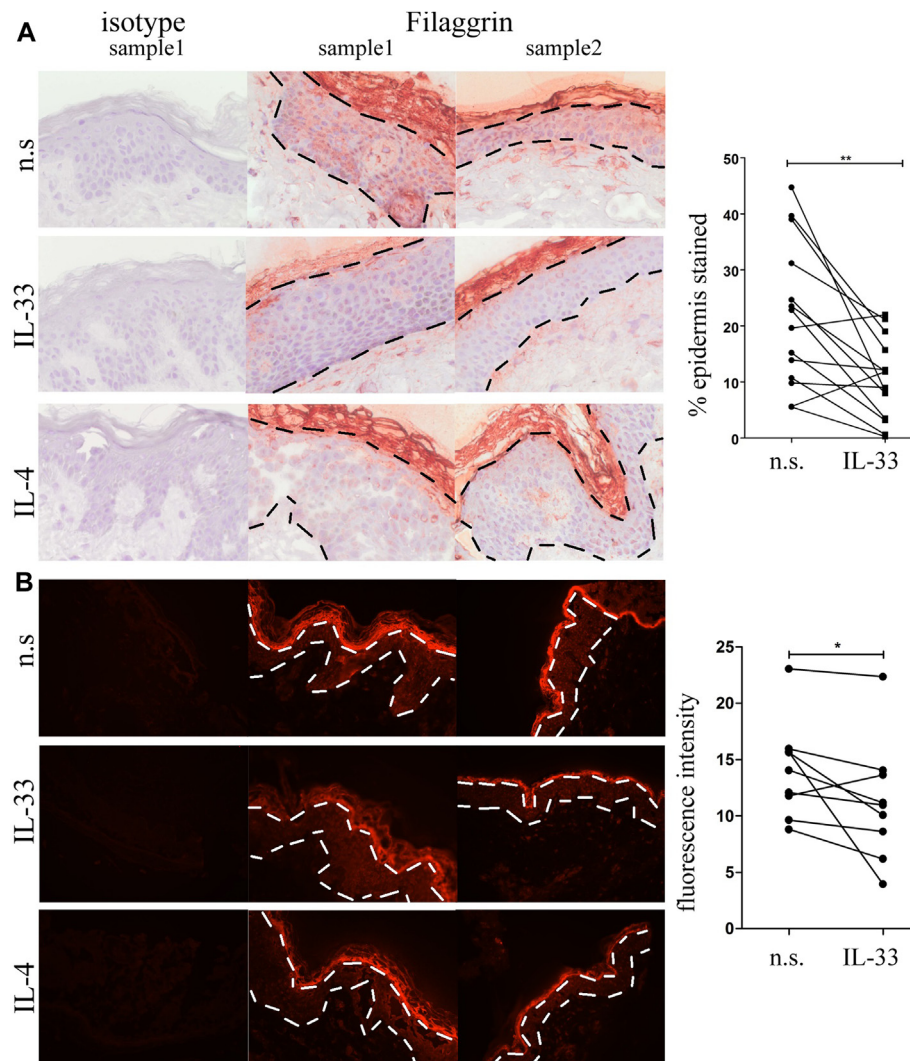


FIG 2. Detection of FLG protein expression in the skin. Samples were stimulated with 50 ng/mL of IL-33 or IL-4 for 24 hours. **A**, Immunohistochemistry staining. Stained area within the epidermis except the stratum corneum (dotted line) was measured using cell Sens Dimension software (Olympus). **B**, Immunofluorescence staining. Fluorescence intensity within the epidermis except the stratum corneum (dotted line) was measured using ImageJ (NIH). n.s., Nonstimulated. Paired *t* test: **P* < .05, ***P* < .005.

effect in the regulation of FLG mRNA expression (data not shown).

Histologic staining of FLG expression in the skin confirmed our results on the protein level. When compared with the nonstimulated skin, addition of IL-4 or IL-33 led to a decrease in FLG protein expression in the epidermis (Fig 2). We analyzed the staining regarding intensity and area. Because existing FLG within the stratum corneum is not expected to be affected by 24-hour stimulation, evaluation was restricted to keratinocytes with detectable nuclear counterstaining. By immunohistochemical staining and software-assisted analysis, we measured the stained area within the epidermis and observed a significant difference between nonstimulated and IL-33-stimulated samples (Fig 2, A). Stimulation with IL-4 also led to FLG loss as expected (nonstimulated, $17.72\% \pm 11.92$; IL-4, $8.97\% \pm 7.06\%$; % epidermis stained, *n* = 6). In the next step, we studied the expression of FLG with fluorescence antibodies, in which the signal strength is not dependent on an enzymatic reaction. Here,

the intensity of staining with anti-FLG was reduced in skin samples on IL-33 incubation (Fig 2, B). Under these conditions, IL-4 stimulation did not lead to a distinct decrease in FLG expression (nonstimulated, 14.07 ± 4.3 ; IL-4, 13.08 ± 5.29 ; fluorescence intensity, *n* = 9).

Finally, *in vitro* skin equivalents were continuously stimulated with IL-33 to investigate a putative effect on keratinocyte differentiation and we observed a disturbed barrier formation by trend. After 8 days of culture in the air-liquid interphase, FLG was reduced by IL-33 stimulation (see Fig E1 in this article's Online Repository at www.jacionline.org). This effect was still observable after 14 days. Software-assisted measurement of FLG staining depicts a tendency toward reduced protein expression under the influence of IL-33. To investigate the effect on the penetration of allergens, we applied biotinylated natural peanut allergen Ara h 2 onto the skin equivalent. After 2 hours of incubation, the allergen was detected in fixed paraffin sections by streptavidin-phycoerythrin. In control skin equivalents, we

were not able to detect allergen penetrance, while IL-33 treatment led to a modest but visible effect on facilitated allergen penetrance into the epidermal part of the skin equivalent (see Fig E2 in this article's Online Repository at www.jacionline.org).

On the basis of these results, we hypothesize that IL-33 contributes to eczematous inflammation not only by acting as an alarmin but also by virtue of its barrier-weakening function. We here observed a direct action of IL-33 on downregulation of FLG expression independent of cytokines from mast cells or T_H2 cells. The observed stronger reduction in cells derived from patients with AD than in cells derived from healthy donors fits our findings of a higher expression of ST2 on the surface of AD keratinocytes in a previous study.⁸

Filaggrin expression was shown to be controlled by the activator protein 1 (AP1) family of transcriptional regulators,^{E11} POU-domain transcription factors,^{E12} and p63.^{E13} Signaling of IL-33 via ST-2 may affect these pathways because it has been shown to result in the recruitment of MyD88, IRAK, IRAK4, and TRAF6, followed by phosphorylation of the downstream targets extracellular-signal-regulated kinase1/2, p38, IκBα, and JUN amino-terminal kinase, finally resulting in nuclear factor-κB activation.^{E14} The mitogen-activated protein kinases Erk1/2, p38, and JNK stimulate AP1 activity and transcription,^{E15} whereas nuclear factor-κB has been shown to counteract AP1 by downregulation of cFos via Elk-1.^{E16}

Under most experimental conditions, the effect of IL-33 on FLG reduction was weaker than that observed for IL-4. We recently described another IL-4-mimetic action of IL-33 regarding the regulation of human beta defensin-2,⁹ supporting the idea of IL-33 being a dichotomous cytokine having IL-1 family-like and T_H2-associated properties.

In conclusion, we propose that IL-33 has skin barrier-modulating effects apart from its well-described function as an innate alarmin mediating the activation of the adaptive immune system by promoting T_H2 as well as T_H1 responses. IL-33 bears the potential to directly or indirectly promote epidermal barrier impairment and thereby plays a crucial role in sustaining and enhancing the local inflammatory responses in diseases such as AD.

We thank Ute Staar, Gabriele Begemann, and Petra Kienlin for excellent technical assistance.

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This work was supported by the Deutsche Forschungsgemeinschaft (DFG) (grant no. GRK144/1) and The Royal Society.

Disclosure of potential conflict of interest: J. Seltmann has received research support from the German Research Foundation (grant no. GRK144/1). M. Wittmann has received research support from The Royal Society; and unrelated to this work she has received consultancy fees from Novartis and research support from the 2014 Inflammation Competition Research Programme (I-CRP, Pfizer). The rest of the authors declare that they have no relevant conflicts of interest.

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<http://dx.doi.org/10.1016/j.jaci.2015.01.048>

METHODS

Cell culture and skin samples

Foreskin for keratinocyte culture was obtained from anonymized donors. Keratinocytes from patients with AD were isolated from plucked hairs as described before.^{E9} Cells were cultured in serum-free medium (KGM-2, Promocell, Heidelberg, Germany). Human dermal fibroblasts were isolated from foreskin samples by incubation of 1 g of tissue for 2 hours in 5 mg/mL of Collagenase (Worthington, Freehold, NJ) and 2.5 mg/mL of hyaluronidase (Sigma-Aldrich, St Louis, Mo). Released fibroblasts were cultured in RPMI (Lonza, Basel, Switzerland) supplemented with 10% FCS. Full-thickness skin from otherwise discarded tissue in the context of routine surgery was obtained from anonymized healthy donors. Punch biopsies (4 mm) were taken from full-thickness skin, placed in serum-free medium (KGM-2, Promocell), and cultured at 37°C 5% CO₂. The study was approved by the medical ethics committee of Hannover Medical School and conducted according to the principles of Declaration of Helsinki. Informed written consent was given by the patients or their parents. For differentiation experiments, keratinocytes were expanded until they reached confluency. The CaCl₂ concentration in the medium was increased to 1.5 mM for a total of 5 days before the cells were used for experiments. Hydrocortisone and epidermal growth factor were omitted from the culture medium before stimulation.

In vitro skin equivalents

In vitro skin equivalents were prepared as described before.^{E10} Briefly, 1×10^6 dermal fibroblasts in 1 volume FCS per gel were mixed with 8 volumes of collagen (Pure Col, Advanced BioMatrix, San Diego, Calif) and 1 volume $10 \times$ HBSS (Gibco/Invitrogen, Darmstadt, Germany) neutralized with 1 mol/L NaOH and poured into 6-well inserts (BD Falcon, Franklin Lakes, NJ) in a deep 6-well tray. After gelation in a humidified atmosphere at 37°C (without CO₂) for 2 hours, the gels were equilibrated with KGM (Lonza) for another 2 hours at 37°C/5% CO₂. Afterwards, 1×10^6 keratinocytes in 2 mL per gel were added to the inserts. The skin equivalents were incubated overnight. The next day the medium was removed from the inserts and the external well. The medium of the external well was replaced by a modified serum-free medium without bovine pituitary extract, but supplemented with 1.3 mM of calcium (Promocell) and 10 µg/mL of transferrin and 50 µg/mL of ascorbic acid (both Sigma-Aldrich), for the air-liquid interphase culture. The medium was changed every second day up to 14 days.

To investigate allergen penetrance into the skin equivalent, we applied 5 µL of biotinylated natural peanut allergen Ara h 2 (1.1 mg/mL, Indoor Biotech, Charlottesville, Va) onto IL-33-stimulated or unstimulated skin equivalents. After 2 hours of incubation followed by 4% formaldehyde/PBS fixation, the allergen was detected in paraffin sections by immunofluorescence as described below.

Stimulation procedure

Keratinocytes and skin biopsies were stimulated with 50 ng/mL of IL-33, IL-4 (both R&D Systems, Wiesbaden, Germany), or their combination for 4 hours or 24 hours for either mRNA isolation or immunohistochemistry. Skin equivalents were either not stimulated or treated with 50 ng/mL of IL-33 every 2 days once the air-liquid interphase culture was started for a total of 8 or 14 days. A punch biopsy was taken and immediately embedded in TissueTek O.C.T Compound (Sakura Finetek, Torrance, Calif) to prepare frozen sections for immunohistochemistry. The remaining epidermis was detached, and RNA was isolated from the keratinocytes for PCR analysis.

RNA isolation, cDNA synthesis, and PCR

RNA from keratinocytes was isolated with the Mini RNA Isolation II Kit (Zymo Research, Freiburg, Germany). RNA from skin biopsies was isolated with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using the Quantitect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was run on a Light Cycler using SYBR

Green with Quantitect primer assays for filaggrin FLG and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (both Qiagen).

Immunostaining and quantification

Immunohistochemistry. Embedded tissues were cut into 6-µm cryosections and fixed for 10 minutes at 4°C in acetone. Staining was performed according to the DAKO EnVision+ System-HRP protocol for mouse or rabbit antibody (DAKO, Glostrup, Denmark). Briefly, sections were blocked for 5 minutes with Peroxidase Block before primary anti-human FLG antibody (clone SPM181, Abcam, Cambridge, United Kingdom) (0.5 µg/mL) or the isotype control IgG was applied for 1 hour. Afterwards, the slides were incubated for 30 minutes each, first with peroxidase-labeled polymer and substrate chromogen. Slides were counterstained with hematoxylin and mounted with DAKO Ultramount medium. The percentage of area stained within the epidermis was measured using cell Sens Dimension software (version 1.8.1, Olympus, Hamburg, Germany). The area stained with the specific color spectrum was measured within the epidermis except the stratum corneum (skin biopsies, see Fig 2) or within the epidermis and the stratum corneum (skin equivalents, see Fig E1).

Immunofluorescence. Cryosections (6 µm) of skin biopsies were fixed for 10 minutes at 4°C in acetone. Initial blocking was performed by incubation with 2% BSA and 10% normal goat serum for 30 minutes. Anti-human FLG antibody (see above) or isotype control was applied (4 µg/mL) in antibody diluent (DAKO) overnight at 4°C. AlexaFluor546-labeled goat-anti-mouse IgG (Life Technologies, Carlsbad, Calif) was applied subsequently for 1 hour. Slides were mounted with VectaShield (VectorLabs, Burlingame, Calif). Intensity of fluorescence staining was measured using ImageJ version 1.44o (NIH, Bethesda, Md). Staining of the epidermis except the stratum corneum was analyzed. Background staining was subtracted.

Sections (5 µm) of paraffin-embedded skin equivalents were subjected to deparaffinization and a prior blocking step by applying antibody diluent (Dako, Glostrup, Denmark). Staining of biotinylated Ara h 2 was achieved by incubation with streptavidin-phycoerythrin (Jackson Laboratories, Bar Harbor, Me).

Statistical analysis

Statistical analysis was performed with PRISM 5.0 (GraphPad Prism, La Jolla, Calif). Data that passed the normality test were analyzed with unpaired *t* test or paired *t* test when indicated. *P* values of less than .05 were considered significant (**P* < .05, ***P* < .005, ****P* < .001).

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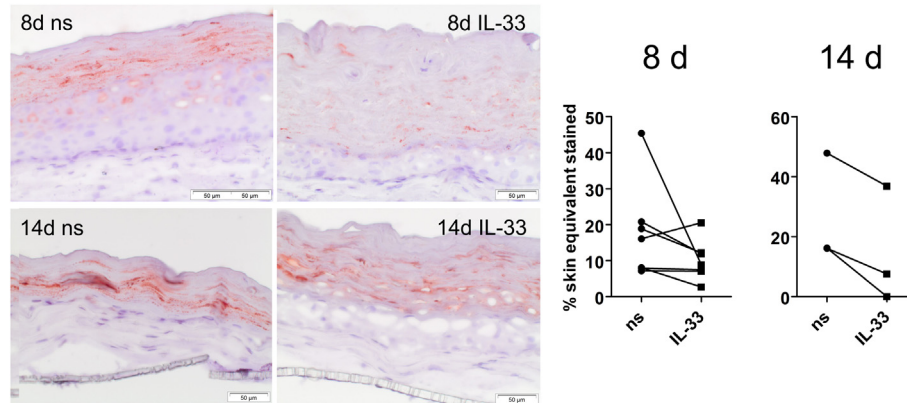


FIG E1. FLG expression in 3D skin equivalents. Skin equivalents were stimulated with IL-33 at the beginning of the air-liquid interphase for a total of 8 or 14 days. FLG staining in skin equivalents: 1 representative out of 7 independent experiments is shown. Time points of measurements as indicated. Stained area was quantified using cell Sens Dimension software (Olympus). *ns*, Not stimulated.

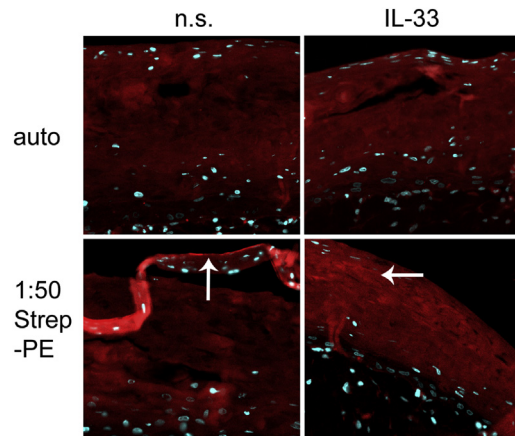


FIG E2. Penetration of Ara h 2 into the skin equivalent. Skin equivalents were either stimulated with IL-33 at the beginning of the air-liquid interphase for a total of 14 days or left unstimulated. Biotin-Ara h 2 that entered the skin equivalent was detected by streptavidin-PE. Area of positive staining is indicated by *white arrows*. *auto*, Autofluorescence; *n.s.*, not stimulated; *PE*, phycoerythrin.