

Letter to the Editor

Keratin defects trigger the itch-inducing cytokine thymic stromal lymphopoietin through amphiregulin–epidermal growth factor receptor signaling

To the Editor:

Chronic inflammatory skin diseases, such as atopic dermatitis, result from an overactive immune response in combination with epidermal barrier defects. The subsequent intrusion of allergens or pathogens stimulates keratinocytes to produce antimicrobial peptides, cytokines, and chemokines to restore epidermal homeostasis. One of these cytokines is thymic stromal lymphopoietin (TSLP), which plays a crucial role in T_H2 immune responses by triggering dendritic cell activation and $CD4^+$ T-cell differentiation. Barrier disruption can trigger TSLP through the protease-activated receptor 2–calcineurin–nuclear factor of activated T cells pathway in keratinocytes, which causes itch in mice by stimulating skin-resident somatosensory neurons.¹ However, the mechanisms of TSLP upregulation remain incompletely understood.

Keratin proteins form the cytoskeleton in all epithelial cells and provide structural integrity and stress resilience to epithelia.² Keratin mutations cause the severe skin blistering disease epidermolysis bullosa simplex (EBS), which is characterized by skin inflammation and severe itch.³ Recently, we identified a keratinocyte-intrinsic and keratin-dependent upregulation of TSLP independent of epithelial barrier defects.⁴ In agreement with the upregulation of TSLP protein levels in the sera of 8 of 17 patients with EBS, we found increased TSLP levels in mouse keratinocytes expressing an EBS-associated keratin 14 mutation. How loss of keratins or keratin mutations trigger TSLP induction remained unknown. Here we report that increased autocrine amphiregulin (AREG)–epidermal growth factor receptor (EGFR) signaling stimulates TSLP expression in keratin-deficient keratinocytes.

Previously, we have found increased extracellular signal-regulated kinase (ERK) 1/2 signaling activity in mouse keratinocytes lacking the entire type I keratin gene cluster ($KtyI^{-/-}$) that was reduced by keratin re-expression.⁵ Furthermore, increased TSLP mRNA and protein levels were detected in single keratin deficiencies, namely $K16^{-/-}$ mice, and keratinocytes, resulting at least partially from increased ERK signaling.⁶ To unravel how ERK1/2 can mediate TSLP upregulation in keratin-free keratinocytes,⁴ we turned to the EGFR, a potential upstream regulator. Inhibition of EGFR signaling for 3 and 7 hours using 10 μ mol/L PD153035 reduced TSLP mRNA and protein expression around 50% (Fig 1, A), revealing EGFR signaling as a major pathway involved in TSLP upregulation in the absence of keratins. In this setting, EGFR signals through ERK1/2 kinases because coinhibition of EGFR and ERK1/2 using the EGFR and mitogen-activated protein kinase kinase 1/2 inhibitors PD153035 and U0126, respectively, reduced *Tslp* mRNA expression to the same extent (ie, around 50%; Fig 1 C). EGFR inhibition for 1 hour reduced phosphorylated ERK1/2 levels, further indicating that EGFR acts through ERK1/2 to induce TSLP (Fig 1, B). Immunoprecipitation (IP) of total EGFR followed by Western

blot analysis for tyrosine phosphorylation revealed a 2-fold stronger phosphorylation of EGFR in the absence of keratins, indicating a stronger activation of EGFR (Fig 1, D).

We performed quantitative PCR (qPCR) analysis of EGFR ligand expression in wild-type (WT) and $KtyI^{-/-}$ keratinocytes to identify the pathway underlying its increased activity. The epidermal EGFR ligand AREG displayed 4- and 15-fold increased mRNA levels in keratin-deficient keratinocytes and in the skin of mice (Fig 1, E and E'), respectively. Further, AREG precursor protein and secreted protein levels were significantly increased upon loss of keratins by 1.9- and 6-fold (Fig 1, E'' and E'''), respectively. Neutralization of AREG using 5 μ g/mL blocking antibody reduced secreted TSLP levels by approximately 35% in $KtyI^{-/-}$ cells (Fig 1, F), strongly supporting a role for AREG in TSLP upregulation. In this setting AREG signaled through ERK1/2 to induce TSLP because AREG blocking reduced ERK1/2 phosphorylation by 25% (Fig 1, F'). Increased *Areg* mRNA levels were completely reduced by 3 hours of EGFR inhibition with 10 μ mol/L PD153035 (Fig 1, G), suggesting that keratin-deficient keratinocytes upregulate AREG through EGFR signaling to induce TSLP.

To test the hypothesis that absence of keratins in $KtyI^{-/-}$ cells upregulates TSLP through stimulation of EGFR, growth factor-starved WT and $KtyI^{-/-}$ cells were treated for 3 and 7 hours with 200 ng/mL recombinant AREG, followed by TSLP mRNA and protein analysis. This revealed a strong induction of *Tslp* mRNA in WT and $KtyI^{-/-}$ cells caused by the AREG stimulus (Fig 2, A), with greater *Tslp* mRNA levels in $KtyI^{-/-}$ cells. Interestingly, AREG-EGFR stimulation of TSLP protein by 4-fold was restricted to $KtyI^{-/-}$ cells, indicating increased sensitization of keratin-deficient cells toward TSLP induction through EGFR signaling (Fig 2, B). Furthermore, we show that stable re-expression of keratin 14 using lentiviral transduction in keratin-free keratinocytes prevents a strong induction of TSLP by AREG compared with green fluorescent protein (GFP)–expressing keratinocytes, suggesting that keratins repress an AREG-EGFR-TSLP signaling pathway (Fig 2, C).

To substantiate the finding that AREG-mediated TSLP induction depended on EGFR and ERK1/2 (Fig 2, A and B), Western blot analysis of Raf and ERK1/2 kinases was performed. This confirmed their involvement in AREG-mediated TSLP induction (Fig 2, D). Nuclear factor κ B signaling, as measured based on p65 phosphorylation, remained unaltered between WT and $KtyI^{-/-}$ cells and was not induced upon AREG-EGFR stimulation (Fig 2, D'). This suggests that nuclear factor κ B is not significantly involved in the keratin-mediated TSLP induction in keratinocytes. Intriguingly, mRNA levels of the EGFR-regulated cytokine CCL20, which are known to be increased in blister fluid from patients with EBS,⁷ were also upregulated strongly after AREG-EGFR stimulation in $KtyI^{-/-}$ keratinocytes (Fig 2, E). Other inflammatory mediators, such as CCL2, CXCL1, CXCL10, IL-1A, Defensin β 4, and S100A7 remained uninduced or were regulated equally on AREG-EGFR stimulation in WT and $KtyI^{-/-}$ cells (see Fig E1, B, in this article's Online Repository at www.jacionline.org). This indicates that lack of keratins triggers expression of a distinct set of cytokines, chemokines, and antimicrobial peptides at the level of AREG-EGFR to induce a distinct inflammatory response.

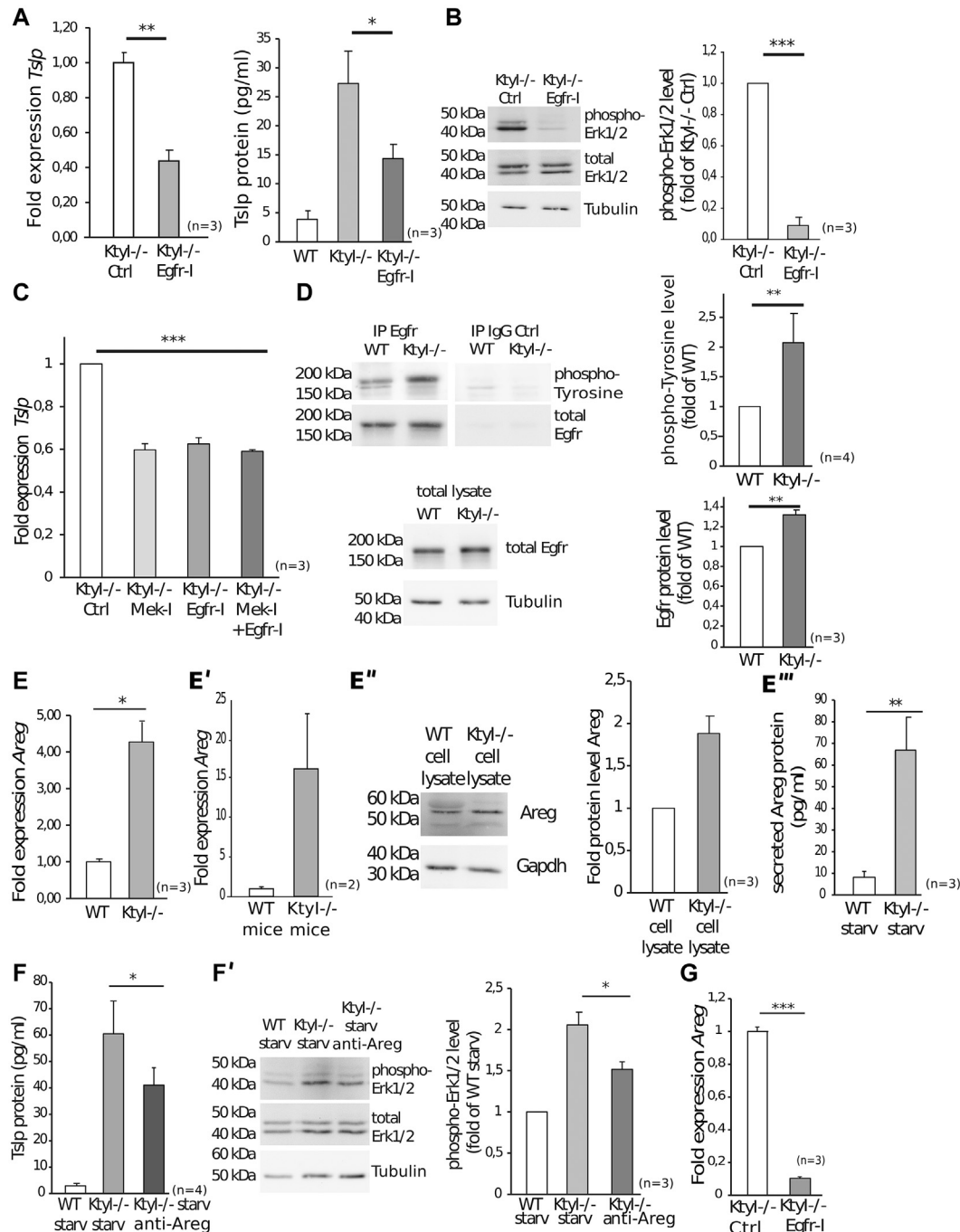


FIG 1. Loss of keratins results in increased AREG-EGFR-ERK1/2 signaling, leading to TSLP upregulation in keratinocytes. **A**, EGFR inhibition with 10 μ Mol/L PD153035 for 3 or 7 hours reduced increased TSLP mRNA and protein levels in Kt1l^{-/-} keratinocytes. **B**, Complete dephosphorylation of ERK1/2 upon EGFR inhibition for 1 hour. **C**, Single inhibition or co-inhibition of EGFR and ERK1/2 using PD153035 and U0126 reduced *Tslp* mRNA expression to the same extent in Kt1l^{-/-} cells. **D**, Stronger tyrosine phosphorylation of EGFR in the absence of keratins based on IP of total EGFR followed by Western blot analysis of phosphorylated tyrosine levels in WT and Kt1l^{-/-} cells. **E-E'''**, qPCR, Western blotting, and ELISA analysis revealed increased AREG mRNA and protein levels in keratin-deficient keratinocytes and increased *Areg* mRNA levels in Kt1l^{-/-} mice. **F** and **F'**, AREG blocking for 7 hours or 15 minutes with 5 μ g/mL AREG blocking antibody reduced TSLP protein levels and ERK1/2 phosphorylation in Kt1l^{-/-} cells. **G**, Full reduction of *Areg* mRNA expression on 3 hours of EGFR inhibition by using 10 μ Mol/L PD153035. * P < .05, ** P < .01, and *** P < .001.

To examine whether AREG is also involved in TSLP upregulation in patients with EBS, AREG and TSLP serum protein levels in sera from patients with EBS were analyzed (Fig 2, F). Six of 7

tested patients with EBS had increased TSLP serum protein levels (≥ 50 pg/mL), of whom 3 (50%) exhibited detectable AREG serum protein levels. Moreover, increased epidermal AREG levels

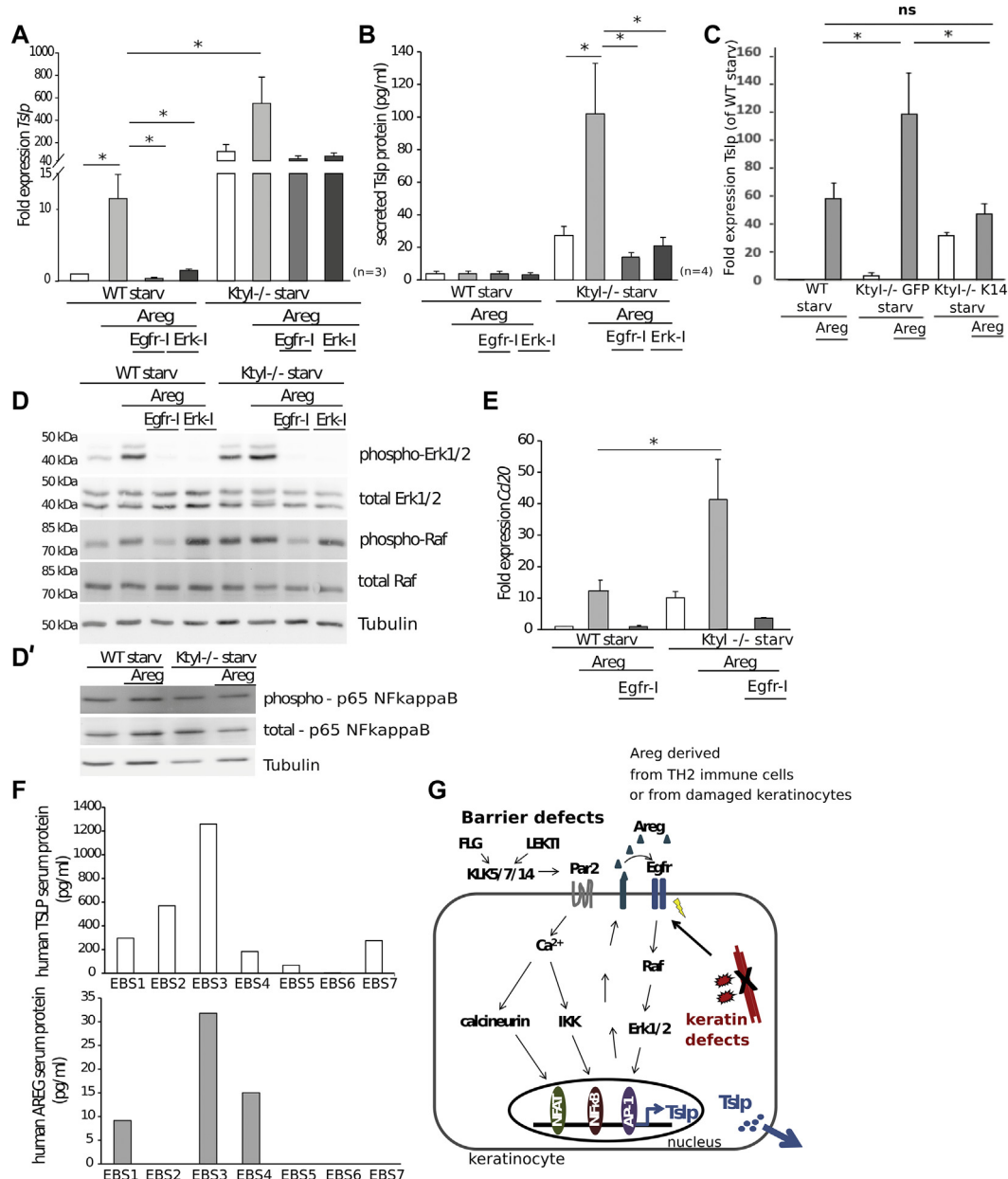


FIG 2. Loss of keratins renders keratinocytes more sensitive to EGFR activation by AREG, resulting in increased TSLP and CCL20 levels. **A-C**, Stimulation of serum-starved WT and Ktyl^{-/-} cells using recombinant AREG resulted in greater TSLP mRNA and protein levels in Ktyl^{-/-} cells. *Tslp* mRNA levels were greater in AREG-stimulated GFP-expressing Ktyl^{-/-} keratinocytes than in AREG-stimulated K14-reexpressing Ktyl^{-/-} keratinocytes. **D** and **D'**, Western blot analysis after AREG stimulation revealed involvement of Raf and ERK1/2 kinases in TSLP induction, whereas phosphorylation of p65 (a subunit of nuclear factor κ B transcription factor) was not altered between WT and Ktyl^{-/-} cells with and without AREG stimulus. **E**, *Ccl20* mRNA was induced more strongly in Ktyl^{-/-} cells compared with WT cells. **F** and **F'**, Increased AREG serum protein levels correlated with increased TSLP serum protein levels in patients with EBS. **G**, Model of TSLP upregulation, following barrier and/or keratin defects. Epidermal barrier disruption on filaggrin or LEKTI mutations induces TSLP through a protease-activated receptor 2–calcineurin–nuclear factor of activated T cells signaling axis, whereas loss of keratins renders keratinocytes more susceptible to EGFR activation, resulting in increased TSLP secretion. In the setting of EBS, TSLP is triggered by increased AREG levels arising from keratinocytes expressing mutant keratins and from wounded keratinocytes because of skin lesions or from T_H2-differentiated immune cells. AREG-EGFR signals through Raf and ERK1/2 to induce TSLP. **P* < .05. ns, Not significant.

were recently identified in a microarray analysis of 6 patients with EBS,⁸ which is in line with the known release of AREG from wound-activated keratinocytes.⁹ We hypothesize that increased

AREG levels in patients with EBS can trigger TSLP upregulation in keratinocytes with keratin defects to drive itch and T_H2 inflammation in patients with EBS.

Collectively, we present a new mechanism for TSLP induction in keratin-deficient keratinocytes mediated by an autocrine AREG-EGFR-ERK1/2 signaling axis (Fig 2, G). Increased AREG levels in EBS skin could result directly from fragile keratinocytes from patients with EBS or from infiltrating immune cells, which might potentiate TSLP production in mutant keratinocytes. The unaltered TSLP protein levels on EGFR stimulation in WT keratinocytes suggest that keratins synergize with AREG at the level of the EGFR to control TSLP expression through Raf and ERK1/2. How the absence of keratins activates EGFR to promote its proinflammatory activity in immune responses is currently unknown. Given that increased AREG-EGFR signaling in wound-activated keratinocytes is crucial for wound repair, we hypothesize that loss of keratins stimulates AREG-EGFR signaling in fragile keratinocytes to enhance tissue repair. Our data suggest AREG-EGFR neutralizing agents as potential targets to treat itch and inflammation in patients with EBS and possibly other keratinopathies.

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METHODS

Keratinocyte cell culture and drug treatments

Keratinocytes were isolated from *KtyI*^{-/-} mice and cultivated, as described previously.^{E1} Re-expression of keratin 14 (ie, *KtyI*^{-/-} K14 cells) and expression of GFP in *KtyI*^{-/-} keratinocytes (ie, *KtyI*^{-/-} GFP cells) was performed by using lentiviral transduction, as previously described.^{E1} For experiments, keratinocytes were seeded on collagen I (Invitrogen, Darmstadt, Germany)-coated wells and cultivated for 24 hours. Cells were treated for 3 or 7 hours with either 10 μ M/L PD153035 (EGFR inhibitor; Sigma, St Louis, Mo), 10 μ M/L U0126 (mitogen-activated protein kinase kinase 1/2 inhibitor; Sigma), or 1 μ g/mL actinomycin D (inhibitor of transcription; Sigma) or cotreated with 10 μ M/L PD153035 and 10 μ M/L U0126 in complete medium, followed by RNA isolation or ELISA analysis.

For AREG experiments, keratinocytes were washed twice with PBS and then starved overnight in medium without FCS, epidermal growth factor, and insulin-like growth factor to allow cell growth under autocrine conditions. The following day, cells were stimulated with 200 ng/mL AREG (989-AR-100, dissolved in sterile PBS; R&D Systems, Minneapolis, Minn) in the presence or absence of 10 μ M/L PD153035 (dissolved in dimethyl sulfoxide; Sigma) or 10 μ M/L U0126 (dissolved in dimethyl sulfoxide; Sigma) for 15 minutes, 3 hours, or 7 hours, followed by Western blot analysis, qPCR, or ELISA, respectively.

Starved keratinocytes were treated for 15 minutes or 7 hours with 5 μ g/mL AREG blocking antibody (Biotechne, #AF989), followed by Western blotting or ELISA analysis, respectively, to block AREG signaling. AREG blocking efficiency was checked by blocking AREG-mediated induction of phosphorylated ERK1/2 and TSLP protein. WT, *KtyI*^{-/-} GFP, and *KtyI*^{-/-} K14 cells were starved overnight and stimulated for 3 hours with recombinant AREG (200 ng/mL), followed by *Tslp* mRNA analysis, to investigate a keratin dependency of the AREG-EGFR-TSLP signaling pathway.

Cell lysates, SDS gel electrophoresis, and Western blotting

Cell lysates were generated with Laemmli sample buffer (50 mmol/L sodium phosphate pH 6.8, 5% SDS, 50 mmol/L dithiothreitol, 5 mmol/L EDTA, 5 mmol/L EGTA, 20% glycerol ultrapure, and 0.01% bromophenol blue). First, cells were washed once with ice-cold PBS, followed by cell lysis in 5 $\times$ Laemmli sample buffer preheated to 98°C. After 10 minutes of heating at 98°C, samples were vortexed, centrifuged, and either directly loaded onto SDS-PAGE or stored at -20°C. Protein separation on SDS-PAGE was performed on 10% polyacrylamide gels. Proteins were electrotransferred to nitrocellulose membranes (0.1- μ m pore width; GE Healthcare, Chicago, Ill) by using semidry or wet Western blotting at 300 mA for 45 minutes per gel or overnight at 10 V. Proteins were stained thereafter with 0.5% Ponceau-S, followed by blocking in either 5% BSA or 2.5% nonfat milk powder in TBS-Tween. Antibody incubation was carried out overnight at 4°C. The following antibodies were used: phosphorylated ERK1/2 (#4370; Cell Signaling Technology, Danvers, Mass), total ERK1/2 (#9102; Cell Signaling Technology), phosphorylated Raf (#94275; Cell Signaling Technology), total Raf (#94225; Cell Signaling Technology), AREG (sc-393433; Santa Cruz Biotechnology, Dallas, Tex), AREG blocking antibody (AF989; R&D Systems), EGFR (sc-03 [1005]; Santa Cruz Biotechnology), phosphorylated tyrosine (#9411; Cell Signaling Technology), IgG rabbit control (#2729; Cell Signaling Technology), and α -tubulin (#T9026; Sigma).

RNA isolation and quantitative real-time PCR

RNA isolation was performed with TRI Reagent (Sigma), followed by DNase digest with DNase I (Thermo Scientific, Waltham, Mass). cDNA synthesis was carried out with the RevertAid H Minus cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's instructions. qPCR was

performed with the Maxima SYBR green Kit with the primers depicted in Table E1. Normalization was carried out with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Statistics were generated with Excel software (Microsoft, Redmond, Wash). All experiments were performed at least in triplicates, except for AREG analysis in *KtyI*^{-/-} mice, which was only performed twice.

ELISA

AREG analysis in keratinocyte supernatants was carried out after overnight starvation (approximately 16 hours) of cells. TSLP analysis was performed in complete medium after overnight starvation and with AREG stimulation or AREG blocking at the indicated time points, as described. Human and mouse TSLP (DY555 and DY1298) and AREG (DY989 and DY262) DuoSet ELISAs were purchased from R&D Systems and performed according to the manufacturer's instructions. Four-parameter logistic curve fit on elisaanalysis.com was used for calculating protein concentrations.

IP

Keratinocytes were seeded on collagen I-coated 10-cm dishes and grown for 24 hours in complete medium. Cells were washed once in ice-cold PBS followed by cell lysis in IP buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100 [pH 7.4], 1 $\times$ protease inhibitor [#78438; Thermo Scientific], and 1 $\times$ phosphatase inhibitor [#78427; Thermo Scientific]) on ice by using a glass homogenizer (Dounce) and pestle (size S) (both Fisher Scientific, Germany) with 15 strokes. IP of total EGFR and the respective IgG control was carried out overnight at 4°C. After binding to protein G agarose (#20398; Thermo Scientific) at 4°C for 1 hour, IP samples were washed 3 times with IP buffer and a final PBS rinse, all performed on ice. Samples were processed for SDS-PAGE and Western blotting.

Patients' samples

TSLP serum and AREG serum protein levels were analyzed in sera from patients with EBS by using ELISA. Samples from patients with EBS were the same samples presented in Kumar et al.^{E1} Investigated patients were of white ancestry or European origin. After informed consent, EDTA blood and skin samples were obtained for molecular diagnostics of epidermolysis bullosa. The study was approved by the ethics committee of the University of Freiburg. Immunofluorescence staining of the skin was performed with antibodies directed to protein of the epidermal basement membrane zone to identify the EBS type. Subsequently, mutation analysis of the keratin 5 (*KRT5*) and *KRT14* genes was performed to confirm the diagnosis of EBS by means of DNA sequencing (data will be published elsewhere). Sera from healthy control subjects and patients with EBS were obtained by the University of Freiburg in addition to control sera from the University of Leipzig. Some control subjects occasionally had high TSLP levels, likely because of smoking behavior, unspecified infections, or skin wounding. Some control subjects showed also increased AREG serum levels, which could be related to skin wounding.

Statistics

Bars indicate SEs, if not otherwise indicated. Statistical significance ($P < .05$) was measured with Microsoft Excel (2010) by using Student *t* tests for paired comparison.

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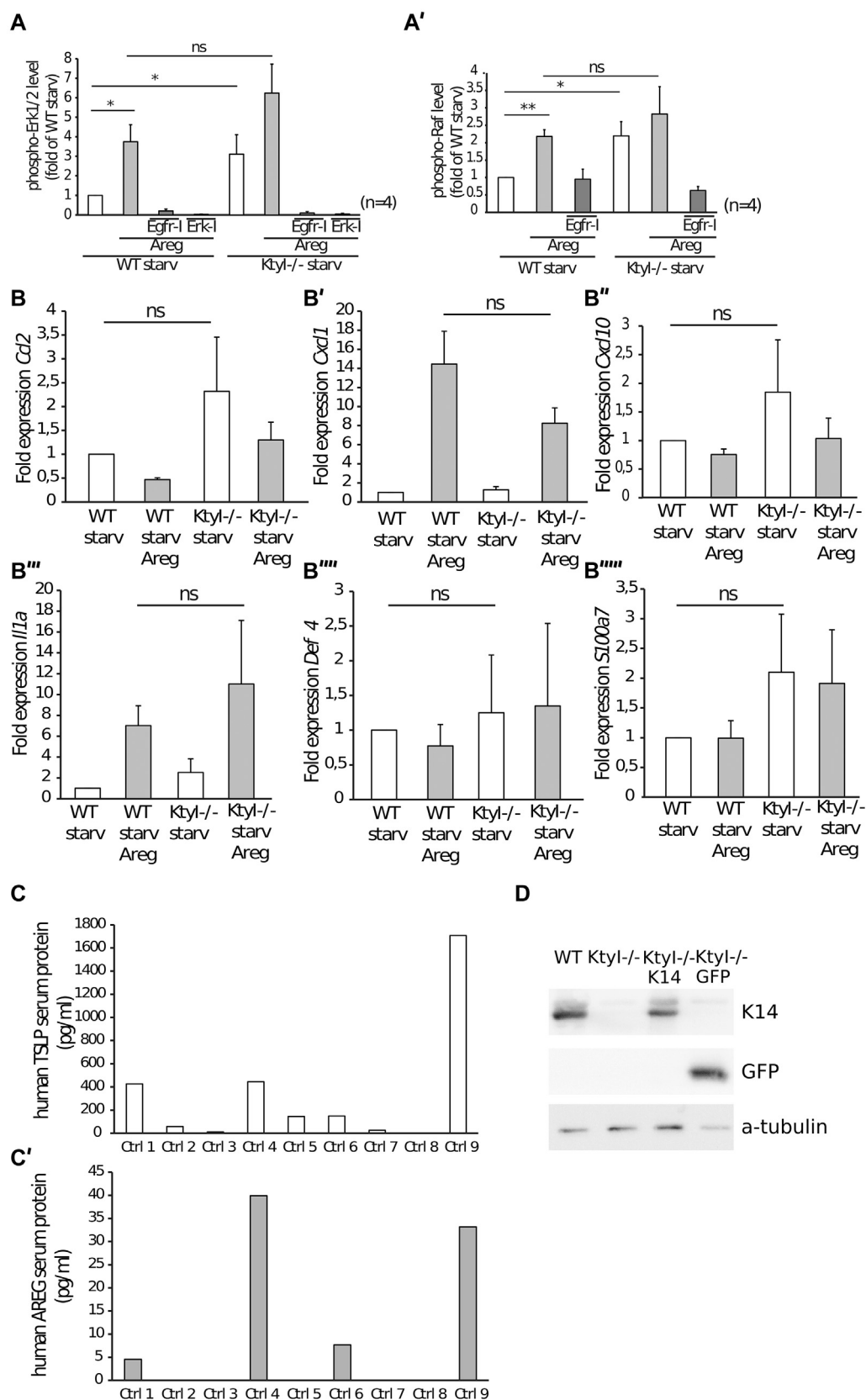


FIG E1. **A** and **A'**, Quantification of Western blots shown in Fig 2. **C**, Phosphorylated Raf and phosphorylated ERK1/2 levels were normalized to total Raf and ERK1/2 levels, respectively. Bars represent fold levels of WT starv. **B-B''''**, RNA expression analysis of CCL2, CXCL1, CXCL10, IL-1A, DEF4, and S100A7 of WT and Ktyl^{-/-} cells after AREG-EGFR stimulation. **C** and **C'**, Analysis of TSP and AREG serum protein levels in healthy subjects. **D**, Expression of keratin 14, GFP, and α-tubulin in WT, Ktyl^{-/-}, Ktyl^{-/-} K14, and Ktyl^{-/-} GFP cells. **P* < .05. ns, Not significant; WT starv, WT starved cells.

TABLE E1. qPCR primer

Gene	Sequence 5'- 3'
Mouse <i>Tslp</i>	Forward: CGACAGCATGGTTCTTCTCA Reverse: CGATTGCTCGAACTTAGCC
Mouse <i>Areg</i>	Forward: TTT GGT GAA CGG TGT GGA GAA Reverse: CGA GGA TGA TGG CAG AGA CAA
Mouse <i>Gapdh</i>	Forward: GTGTCCTACCCCAATGTG Reverse: AGGAGACAACCTGGTCCTCA