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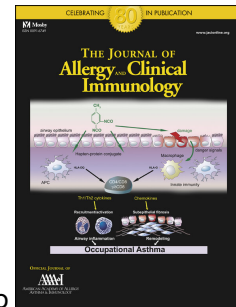
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Keratin-dependent TSLP expression suggests a link between skin blistering and atopic disease

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Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

To the editor:

Atopic dermatitis (AD) is a pruritic and inflammatory skin disorder characterized by barrier disruption. The cytokine TSLP (thymic stromal lymphopoietin), a common denominator of atopic diseases, promotes allergic skin inflammation and is predominantly produced by barrier epithelial cells, triggering Th2-mediated inflammatory responses. The mechanisms governing upregulation of TSLP in keratinocytes remain incompletely understood, although barrier disruption represents one major trigger of TSLP expression (1, 2). Keratin (K) proteins form the intermediate filament cytoskeleton of epithelia and are crucial barrier constituents. Dominant-negative mutations in the *KRT5* or *KRT14* genes cause epidermolysis bullosa simplex (EBS), a disorder characterized by skin fragility, inflammation and barrier defects. In addition to protecting epithelia against mechanical stress, keratins regulate immune responses by controlling alarmins, IL-18 and additional cytokines. Remarkably, gene expression and cytokine profiling of keratin-deficient mice and disorders such as atopic dermatitis show considerable overlap (3–5). The notion that itch of unknown cause is the most common and bothersome complication in EBS (6) raises the question whether keratin mutations are involved in itch through upregulation of TSLP. Here, we report keratinocyte-intrinsic and keratin-dependent upregulation of TSLP independent of barrier defects in keratinocytes isolated from keratin-deficient mice and identify a link between *KRT5* and *KRT14* mutations and increased TSLP serum levels in EBS patients.

To investigate the role of keratins in epidermal differentiation and pathogenesis, we generated mice lacking all type II (KtyII^{-/-}) or all type I (KtyI^{-/-}) keratin genes and corresponding keratinocyte cell lines (7). Both strains lack the entire epidermal keratin cytoskeleton, with highly similar phenotypes. Global gene expression and qPCR analysis of prenatal KtyII^{-/-} mice revealed a strong upregulation of *Tslp* gene expression (Fig 1A; Table E1A). A few cytokines and chemokines were additionally upregulated *in vivo* but not in cultured keratinocytes, correlating with barrier defects triggering their induction (Table E1). *Tslp* protein levels in sera of postnatal day 5 mosaic KtyII^{-/-} mice (8) reached up to 3.000 pg/ml whereas controls had levels below 300 pg/ml (Fig 1A). Perinatal mortality in global KtyII^{-/-} and KtyI^{-/-} mice precluded the analysis of serum levels and the assessment of a potential scratching behavior of keratin-deficient mice due to elevated *Tslp*.

Tslp induction may result from barrier defects or from novel, keratinocyte-intrinsic mechanisms due to the absence of keratins. To address this, immunofluorescence of KtyI^{-/-} and WT mouse skin was performed. Whereas WT epidermis hardly expressed *Tslp* (Fig 1B and E1A), in agreement with ELISA data (Fig 1A), the entire KtyI^{-/-} epidermis showed strong *Tslp* reactivity (Fig E1A). To substantiate the inverse relationship between *Tslp* and keratin protein expression, the unique setting of mosaic KtyII_m^{-/-} mice with keratin-deficient and positive keratinocytes in the same specimen was probed (8). This confirmed *Tslp* presence in keratin-deficient and absence in WT keratinocytes (Fig 1B). Thus, high *Tslp* levels in keratinocytes and release therefrom strongly correlate with absence of keratins in mouse epidermis.

To support cell-intrinsic and barrier-independent *Tslp* upregulation, we analyzed its expression by qPCR and ELISA in isolated KtyI^{-/-} keratinocytes. Here, very strong upregulation of *Tslp* RNA and protein (Fig 1C) levels occurred, even in low Ca²⁺-medium. Most other cytokine RNAs remained unaffected by the cells' keratin status (Table E1B). To examine keratin dependence, mouse Krt14 was re-expressed in KtyI^{-/-} keratinocytes (Kty^{-/-} K14), restoring keratin filaments together with endogenous Krt5 (Fig E1B) (9). This significantly reduced both *Tslp* RNA and protein levels (Fig 1C), strongly supporting keratinocyte-intrinsic and keratin-dependent *Tslp* expression. Given that dominant KRT14 mutations cause EBS by formation of cytoplasmic keratin aggregates, murine keratinocytes stably expressing the dominant K14p.Arg₁₃₁Pro mutation (KtyI^{-/-} K14R131P) were generated, equivalent to the human mutation K14p.Arg₁₂₅Cys which results in severe EBS with keratin aggregates in keratinocytes (10). The mutation led to a ~four-fold *Tslp* increase relative to WT controls (Fig 1C), accompanied by extensive cytoplasmic keratin aggregation (Fig E1B), a hallmark of severe generalized EBS (10). Collectively, keratin aggregates comprising the dominant K14 EBS-mutant or lack of keratins were sufficient to trigger increased *Tslp* in a keratinocyte-intrinsic way. Since keratin-dependent *Tslp* regulation could be of clinical interest, we addressed molecular mechanisms controlling *Tslp* expression.

To examine the role of calcium, cells were treated for 3h with the intracellular calcium chelator 1,2-Bis(2-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM). This decreased *Tslp* RNA levels by ~50 %, whereas chelation of residual extracellular calcium by 3 mM EGTA had negligible effects on *Tslp* (Fig E2A, B), suggesting increased signaling of a calcium-dependent pathway. MAPK signaling is a possible downstream effector of Ca^{2+} . Given elevated ERK1/2 activity upstream of AP-1-dependent transcription in keratin-deficient keratinocytes (11), ERK1/2 activity and *Tslp* expression were analyzed upon MEK1/2 inhibition. Reduction of *Tslp* by 45 % was accompanied by decreased P-ERK1/2 (Fig 1D and E2D). Combined inhibition of Ca^{2+} and MEK1/2, followed by the analysis of P-ERK1/2, further supported a Ca^{2+} -ERK1/2-axis in *Tslp* regulation (Fig E2C). Activation of MAPK with the PKC-activator PMA (Phorbol-12-myristate-13-acetate) strongly induced *Tslp* via ERK1/2 in *KtyI^{-/-}* cells, emphasizing the role of MAPK signaling in *Tslp* regulation in keratinocytes (Fig 1D and E2E). To examine whether keratin-deficient keratinocytes secrete soluble factors triggering *Tslp*, WT cells were incubated with *KtyI^{-/-}* keratinocyte-conditioned media. Modest *Tslp* upregulation compared to that in unstimulated *KtyI^{-/-}* cells further substantiated cell-intrinsic mechanisms (Fig 1D).

Given the prevalence of itch in EBS (6), we considered elevated TSLP in EBS. Analysis of TSLP serum levels from 17 EBS patients, presenting with diverse *KRT5* and *KRT14* mutations including K14p.Arg₁₂₅Cys, identified one group of eight patients with enriched TSLP levels, well correlating with a high disease score for EB (++, +++; Fig 2A, inset). A second group of nine patients displayed low TSLP levels (<50pg/ml) including milder affected patients (controls with variable TSLP levels are shown in Fig E3). Elevated TSLP levels in controls could correspond to smoking and allergic sensitization, known to increase TSLP (12). We found no correlation with markers for allergic sensitization (sx1, fx5) and CCL17 suggested to correlate with atopic conditions (Fig 2A) (13).

The good correlation between TSLP levels and EBS disease severity is further supported by lower TSLP levels in an EBS patient with minimal skin defects (patient 7 in Fig E4A). However, patient 8, carrying the same mutation as patient 7 but displaying severe skin lesions, showed high TSLP serum levels (Fig E4B). Finally, TSLP immunohistochemistry of EBS patient skin samples corresponded well with the expression of mutant keratins and TSLP and confirmed the data from the analysis of sera (Fig E4C-F).

Collectively, we propose a model (Fig 2B) in which TSLP upregulation occurs downstream of barrier defects (left, marked in black) and of mutant or absent keratins (right, marked in red). It is well known that epidermal-specific barrier defects (caused by filaggrin or kallikrein protease mutations) can trigger TSLP expression downstream of PAR2 (14), inducing TSLP expression via NF κ B or NFAT. We identified a novel cell-intrinsic mechanism for *Tslp* induction in epidermal mouse keratinocytes where mutant keratins or their absence activate MAPK components MEK1/2 and ERK1/2 through yet unknown mechanisms. Lack of keratins (red lines with black X) or presence of the severe EBS mutant K14p.Arg₁₃₁Pro (red aggregates) cause cell-intrinsic upregulation and secretion of *Tslp* (marked in

light green), possibly through AP-1 and NFκB. Our data suggest that keratin mutations contribute to inflammatory and barrier disorders of epithelial tissues.

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Bulleted statement: We identified a keratinocyte-intrinsic and keratin-dependent mechanism mediating strong upregulation of TSLP. High TSLP serum levels in keratin-deficient mice and in EBS patients suggest that TSLP contributes to itch in EBS.

Key words: atopic dermatitis, allergy, epidermolysis bullosa simplex, keratins, itch, thymic stromal lymphopoietin (TSLP)

Abbreviations

AD	- Atopic dermatitis
AP-1	- Activating protein 1
BAPTA-AM	- 1,2-Bis(2-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CCL17	- thymus and activation-regulated chemokine
EBS	- Epidermolysis bullosa simplex
ERK1/2	- Extracellular signal-regulated kinase 1/2
Fx1	- food allergens
K5	- Keratin 5
K14	- Keratin 14
Krt5	- Keratin 5
Krt14	- Keratin 14
KLK5	- Kallikrein-related peptidase 5
KLK7	- Kallikrein-related peptidase 7
KtyI ^{-/-}	- Keratin type 1 gene cluster knockout
KtyII ^{-/-}	- Keratin type 2 gene cluster knockout

139	KtyII _m ^{-/-}	- Keratin type 2 gene cluster knockout mosaic
140	LEKTI	- Lympho-epithelial Kazal-type-related inhibitor
141	MAPK	- Mitogen-activated protein kinases
142	MEK1/2	- Mitogen/extracellular signal regulated kinase
143	NFAT	- Nuclear factor of activated T-cells
144	NFκB	- Nuclear factor kappa-light-chain-enhancer of activated B cells
145	PAR2	- Protease-activated receptor 2
146	PMA	- Phorbol-12-myristate-13-acetate
147	SPINK5	- Serine protease inhibitor Kazal-type 5
148	Sx1	- aeroallergens
149	Th1	- T helper type 1
150	Th2	- T helper type 2
151	Th17	- T helper type 17
152	TSLP	- Thymic stromal lymphopoietin

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Figure 1.

Loss of keratins or keratin mutation triggers TSLP upregulation via ERK1/2

- A** Tslp RNA in WT, *KtyI*^{-/-} and *KtyII*^{-/-} mice skin (n=2) and protein levels in WT (n=4) and *KtyII*_m^{-/-} (n=6) sera determined by qPCR and/or ELISA.
- B** Immunofluorescence staining of K5 (green) and Tslp (red) in *KtyII*_m^{-/-} mice (n≥3).
- C** Tslp RNA and protein levels in WT, *KtyI*^{-/-}, *KtyI*^{-/-} K14 and *KtyI*^{-/-} K14R131P examined by qPCR and/or ELISA. (n=3).
- D** qPCR and Western blot analysis of *KtyI*^{-/-} cells treated with MEK1/2 inhibitor U0126 and MAPK signaling activator PMA. WT cells treated with conditioned medium of *KtyI*^{-/-} cells. (n=3).

Error bars represent standard error. p-values = *P< 0.05; ** P< 0.01; ***P< 0.001. ns = non-significant. *KtyI*^{-/-} = keratin type 1 cluster knockout. *KtyII*_m^{-/-} = keratin type 2 mosaic knockout.

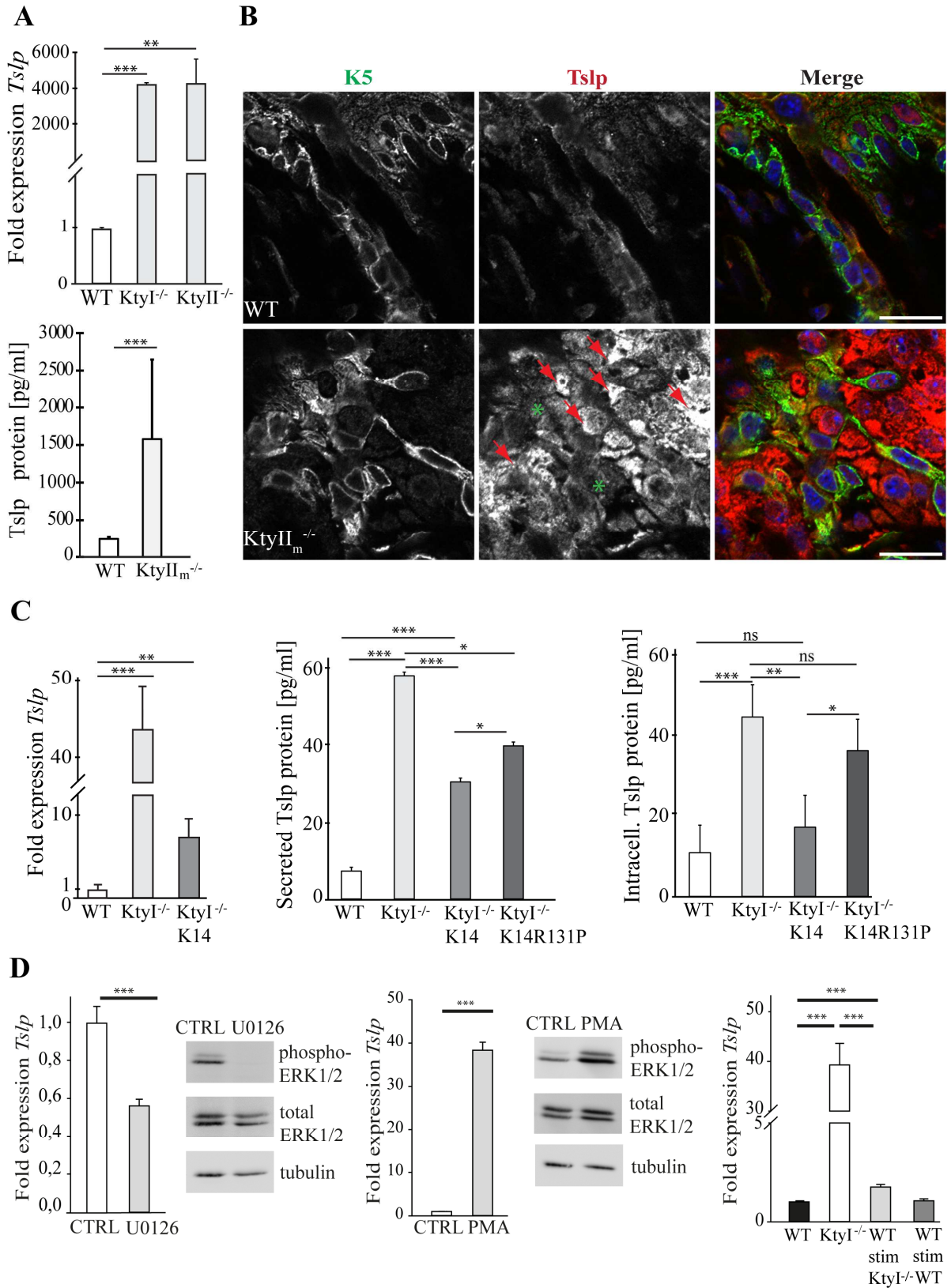
Figure 2.

8 out of 17 EBS patients show elevated TSLP serum level

- A** Skin defects (disease severity score EB) are indicated by + (few blisters on hands and feet), ++ (severe blistering on extremities) or +++ (severe, generalized blistering). Elevated TSLP levels (> 50 pg/ml) correlate with a high disease score (++, +++). Error bars represent standard deviations. Correlation and statistical analysis between TSLP level and this severity score are indicated in the top right. AD/As/Allergy = atopic dermatitis, asthma or allergy,

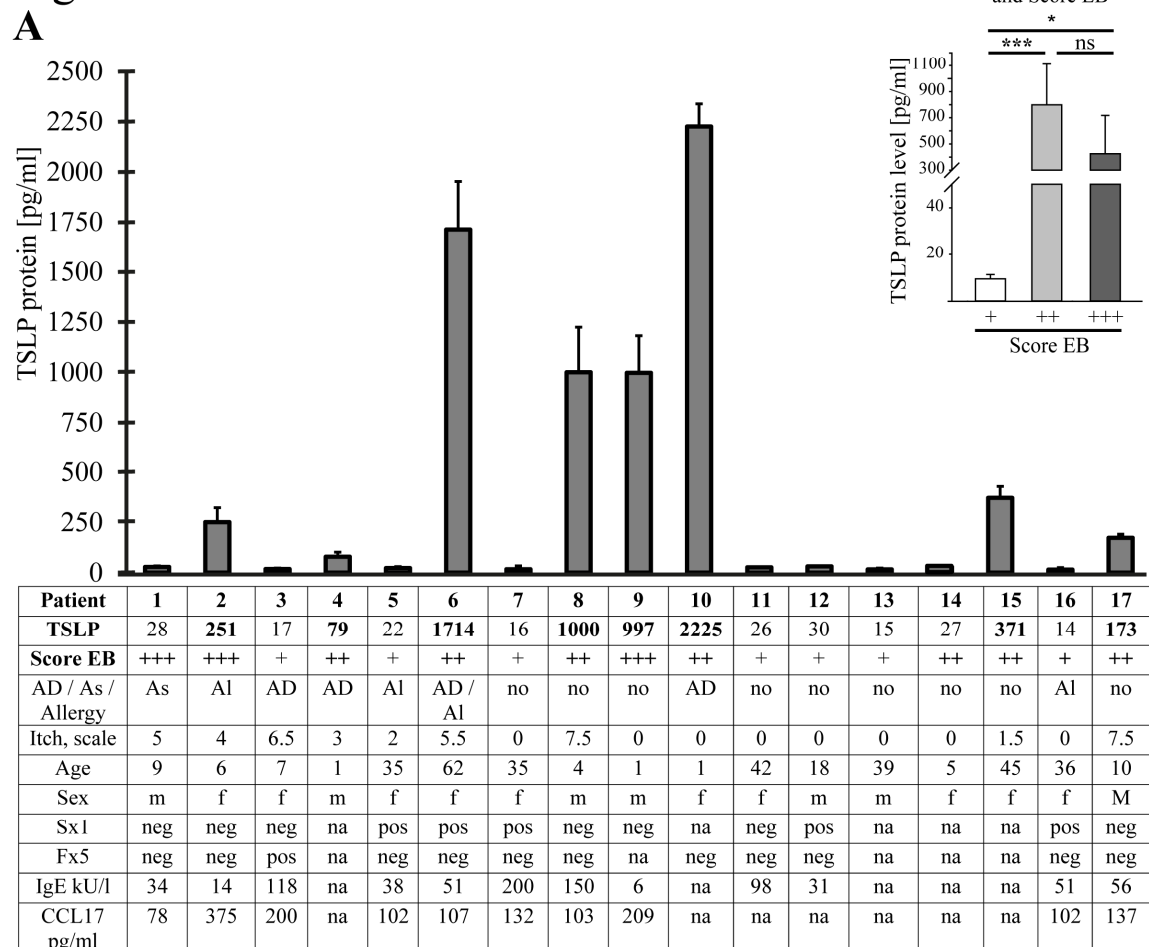
respectively. Itch evaluation = scale from 0-10 (0 = no itch; 10 = worst imaginable itch).
Sx1=aeroallergens, Fx1= food allergens, CCL17 = thymus and activation-regulated
chemokine, na = not available, no = absence.

B Model of barrier- and keratin-dependent TSLP upregulation induced via calcineurin and IKK or
MEK1/2 –ERK1/2, respectively. Upon barrier defects induced by filaggrin (FLG) or LEKTI
mutations, protease-activated receptor PAR2 is activated by different kallikreins (KLK5/7/14) and
acts via Ca^{2+} - calcineurin or IKK to trigger *Tslp* expression by the transcription factors NFAT or
NF κ B (indicated with black arrows). Loss of keratins or keratin mutations (keratin aggregates)
induce *Tslp* expression via MEK1/2 – ERK1/2 of a so far unknown activation mechanism
(indicated with red arrows).

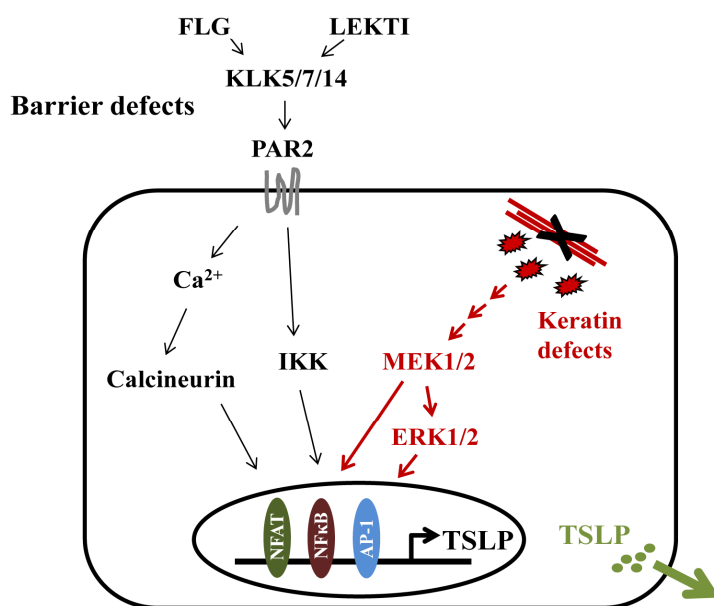


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Figure 2



B



Supplementary Data

Keratin-dependent TSLP expression suggests a link between skin blistering and atopic disease

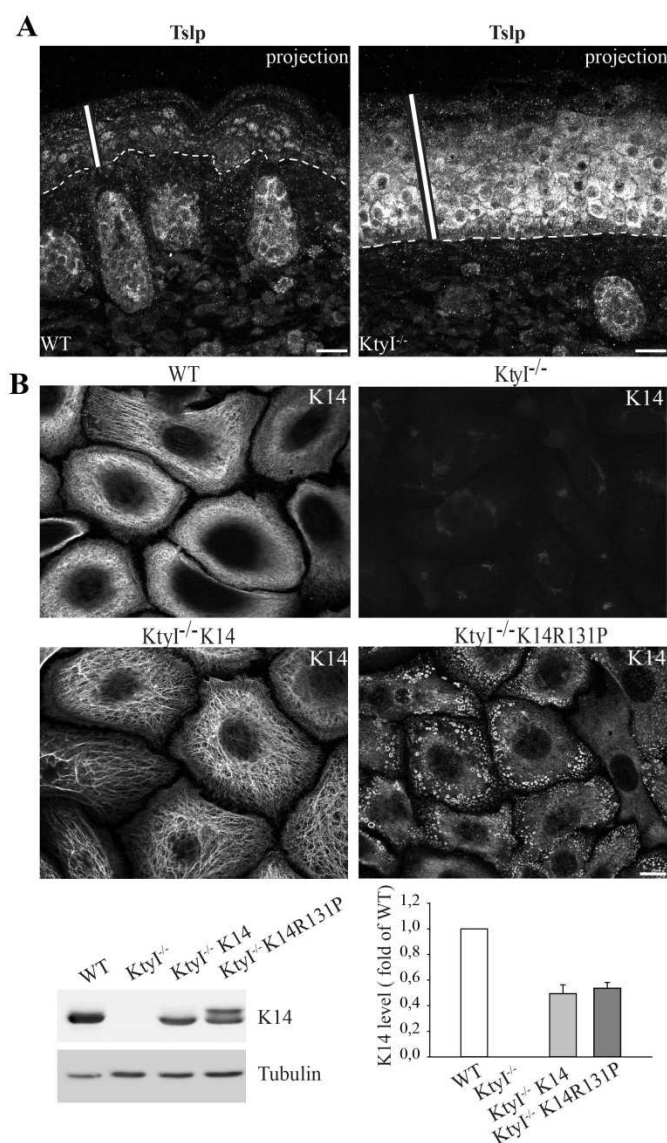
Vinod Kumar¹⁺, Matthias Behr¹⁺, Dimitra Kiritsi², Andrea Scheffschick¹, Anja Grahner¹, Melanie Homberg¹, Agnes Schwieger-Briel², Thilo Jakob^{2,3}, Leena Bruckner-Tuderman² and Thomas M. Magin^{1*}

Supplementary Abbreviations

<i>Ccl2</i>	- Chemokine (C-C motif) ligand 2
<i>Ccl4</i>	- Chemokine (C-C motif) ligand 4
<i>Ccl11</i>	- Chemokine (C-C motif) ligand 11
<i>Cxcl11</i>	- Chemokine (C-X-C motif) ligand 1
<i>Cxcl2</i>	- Chemokine (C-X-C motif) ligand 2
<i>Cxcr4</i>	- C-X-C chemokine receptor type 4
<i>Il1α</i>	- Interleukin 1 alpha
<i>Il1β</i>	- Interleukin 1beta
<i>Il4</i>	- Interleukin 4
<i>Il6</i>	- Interleukin 6
<i>Il16</i>	- Interleukin 16
<i>Il33</i>	- Interleukin 33
<i>Mmp9</i>	- Matrix metalloproteinase 9
<i>Mmp12</i>	- Matrix metalloproteinase 12
<i>Mmp13</i>	- Matrix metalloproteinase 13
<i>Mmp23</i>	- Matrix metalloproteinase 23
<i>Spp1</i>	- Secreted phosphoprotein 1
<i>Tnfα</i>	- Tumour necrosis factor α

Supplementary Data

Fig E1

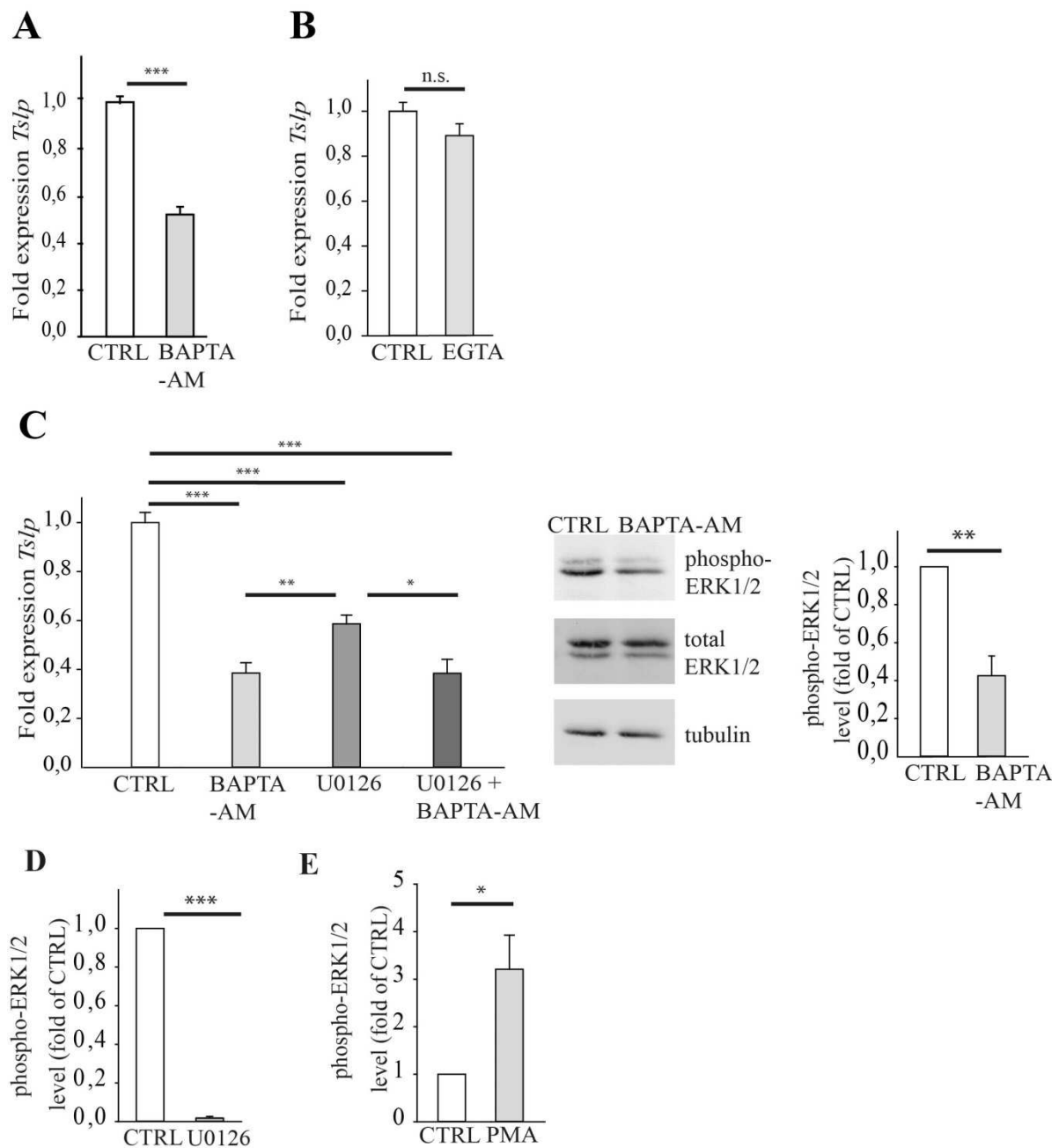


Elevated epidermal Tslp protein levels in the absence of keratins and characterization of keratinocyte cell model

A Immunofluorescence staining of Tslp in WT and KtyI^{-/-} skin. White dashes underline basement membrane. White bar illustrates epidermal thickness elevated in KtyI^{-/-} skin. Scale bar = 20 μm.

B Mouse keratinocytes stained for K14. Note presence of keratin aggregates in KtyI^{-/-} K14R131P transfectants, presence of normal keratin filaments in KtyI^{-/-} K14 and KtyI WT keratinocytes and absence in KtyI^{-/-} cells. Quantification of K14 level is indicated. Scale bar = 10 μm.

41 **Fig E2**



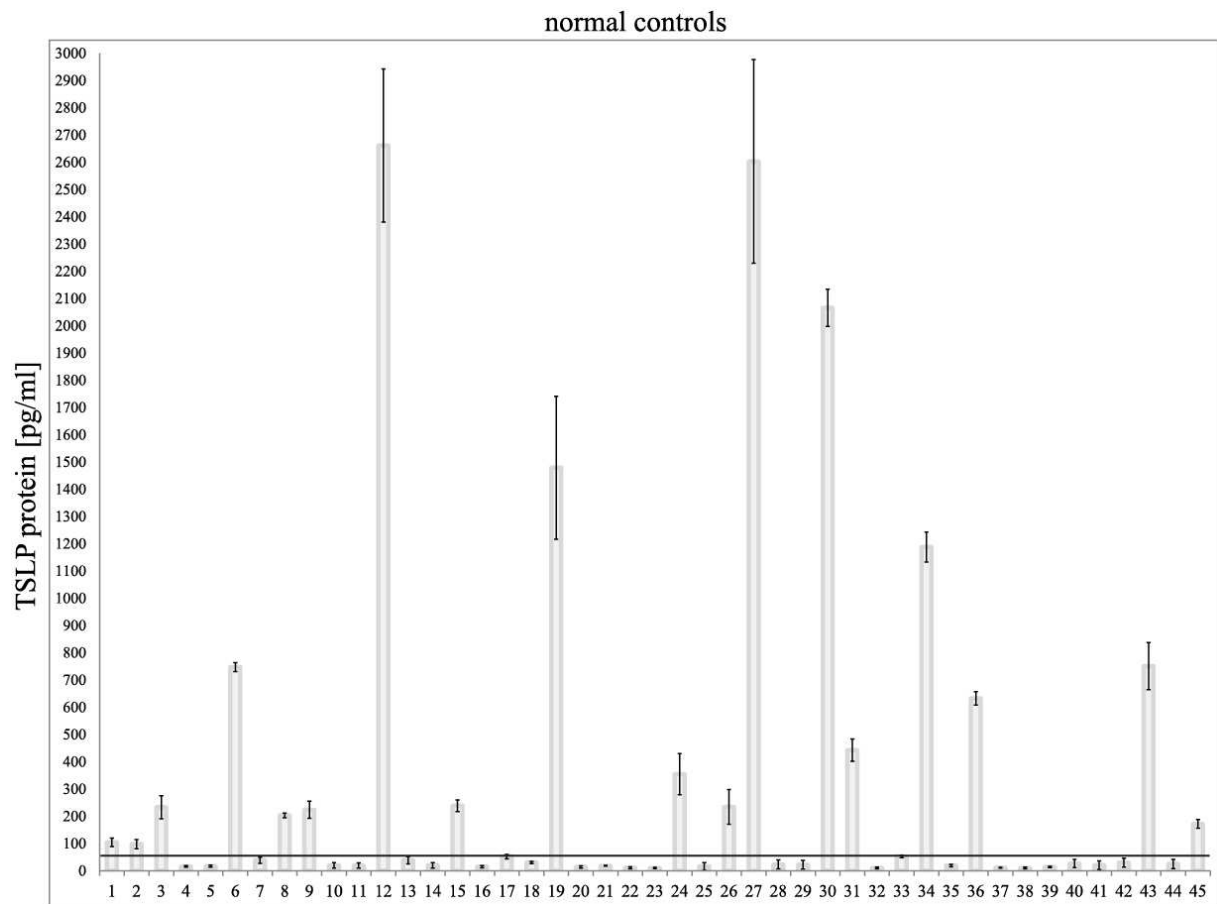
Intracellular Calcium signaling contributes to *Tslp* levels via MAPK signaling in keratinocytes

A, B *Tslp* RNA expression in KtyI^{-/-} cells treated with intracellular calcium chelator BAPTA-AM (A) or with extracellular calcium chelator EGTA (B), respectively.

C *Tslp* RNA expression in KtyI^{-/-} cells treated with BAPTA-AM, MEK1/2 inhibitor U0126 and Cotreatment of BAPTA-AM and U0126. Western blot analysis and quantification of ERK1/2 phosphorylation of KtyI^{-/-} cells following BAPTA-AM treatment is indicated.

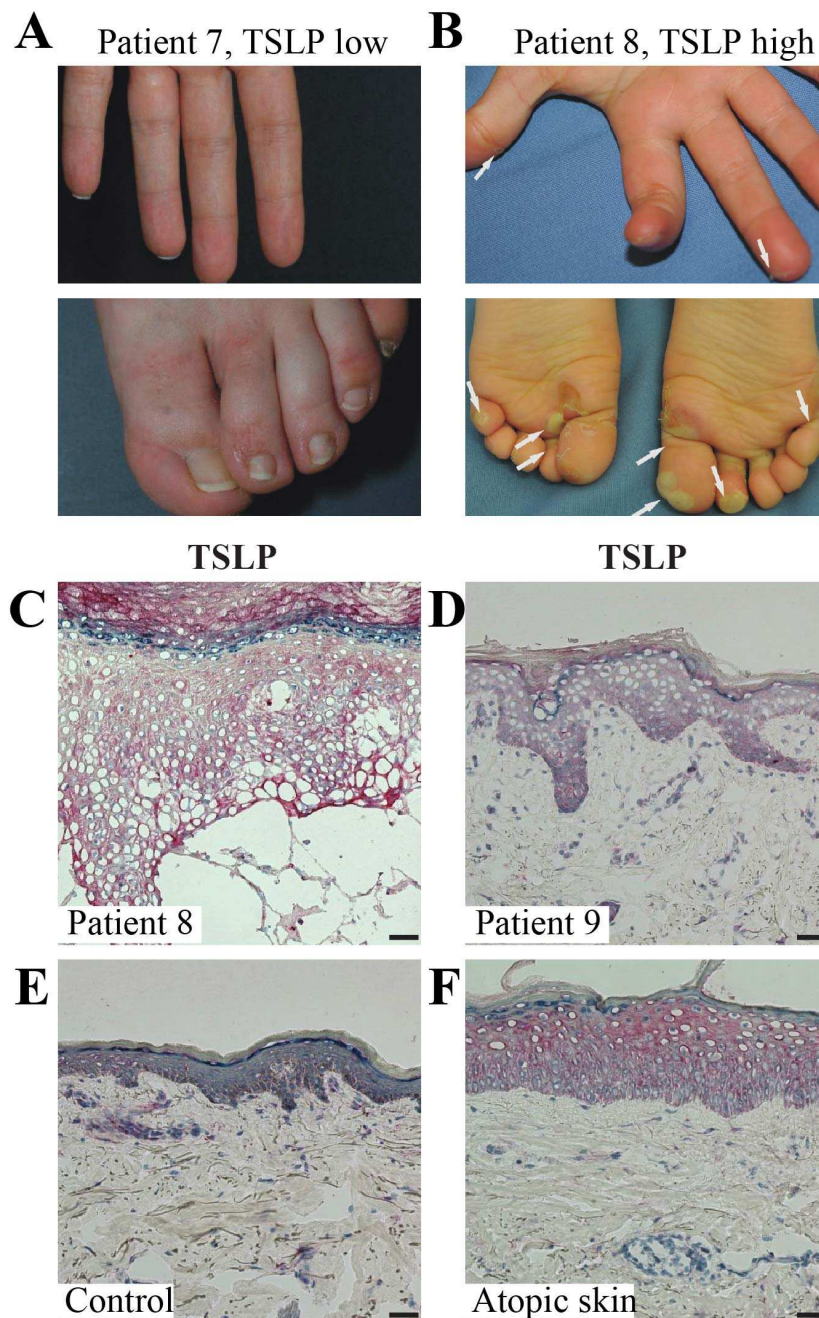
D, E Quantification of ERK1/2 phosphorylation of KtyI^{-/-} cells treated with U0126 and PMA is indicated (Western blots of Fig 1D).

Asterisks represent p-values *P< 0.05; ** P< 0.01; ***P< 0.001; ns= not significant. Error bars indicate standard error of the mean (SEM).

53 **Fig E3**

TSLP protein levels from normal controls. Representative TSLP protein levels from normal controls (n=45, obtained by the Universities of Freiburg and Leipzig (blood bank) were typically below 50 pg/ml (black line)). Note, that TSLP protein levels can be enriched in normal controls, due to smoking behaviour, unspecified infections and skin lesions.

Fig E4



Elevated TSLP levels in EBS patients correlate with skin lesions

- A** EBS patient 7 with basal TSLP levels (see Fig 2 A) has minimal EB-related skin manifestations.
- B** EBS patient 8 with high TSLP levels (see Fig 2 A) has several blisters on hands and feet (arrows).
- C-F** Representative immunohistochemical images support ELISA data showing TSLP accumulation (red staining) within the epidermis from EBS patients when compared with atopic skin and control skin. Note local cytolysis and hyperkeratosis. Scale bar = 50 μ m.

72 **Table E1**

A Genes differentially expressed in skin encoding cytokines/chemokines (Th1, Th2, Th17)

Th1	KtyII ^{-/-}	Th2	KtyII ^{-/-}	Th17	KtyII ^{-/-}
<i>Ccl4</i>	15.2	<i>Tslp</i>	146.8	<i>Mmp13</i>	60.6
<i>Tnfa</i>	8.6	<i>Il33</i>	70.8	<i>Cxcl1</i>	53.1
<i>Spp1</i>	8.4	<i>Ccl2</i>	13.9	<i>Mmp9</i>	4.2
<i>Il1β</i>	7.7	<i>Il4</i>	4.0	<i>Mmp12</i>	3.9
<i>Il1α</i>	3.1	<i>Ccl11</i>	3.5	<i>Cxcl2</i>	3.6
		<i>Il16</i>	-2.1		
		<i>Cxcr4</i>	-2.4		

B Genes expressed in keratinocytes encoding cytokines/chemokines (Th1, Th2, Th17)

Th1	KtyII ^{-/-}	Th2	KtyII ^{-/-}	Th17	KtyII ^{-/-}
<i>Tnfa</i>	2.0	<i>Tslp</i>	3.8	<i>Mmp12</i>	2.2
<i>Ccl4</i>	1.0	<i>Il4</i>	2.0	<i>Mmp9</i>	2.2
<i>Spp1</i>	1.0	<i>Il33</i>	1.0	<i>Mmp13</i>	1.0
<i>Ilβ</i>	1.0	<i>Ccl2</i>	1.0	<i>Cxcl1</i>	1.0
<i>Il1α</i>	1.0	<i>Ccl11</i>	1.0	<i>Cxcl2</i>	-2.3
		<i>Il16</i>	1.0		
		<i>Cxcr4</i>	1.0		

Chemokine/cytokine RNA levels in mice skin and keratinocytes.

A Transcriptome profile-based expression (KtyII^{-/-} skin) of genes encoding cytokines/chemokines for Th1, Th2 and Th17. The fold-change values of differentially expressed genes are indicated.

B As **A** but for KtyII^{-/-} keratinocytes.

Supplementary Material

Transcriptome profiling

This was performed as described in Roth et al. (4). The MouseWG-6v2.0 Expression BeadChip kit (Illumina, Inc., San Diego, CA, USA) was used to probe triplicate WT, KtyI^{-/-}, KtyII_{res}^{-/-} skin and keratinocytes samples from E18.5 embryos. Data analysis was based on the R Statistical language (R Development Core Team 2007, 2.8.0) and Beadstudio 3.1.1.0 software. Data were quantile-normalized. A fold-change/P-value filter was used to select differentially expressed genes; P-values smaller than 0.1, expression changes higher than 2-fold and a difference between mean intensity signals greater than background were considered statistically significant. The false discovery rate (FDR) of P-values was adjusted by the Benjamini–Hochberg method. The gene expression data sets for WT, KtyI^{-/-}, KtyII_{res}^{-/-} skin and keratinocytes were deposited in the GEO database (GSE79590 and GSE79596).