Journal of Dermatological Science xxx (2019) xxx–xxx

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

A proinflammatory role of KLK6 protease in Netherton syndrome

Eleni Zingkou[a], Georgios Pampalakis[a], Eleni Charla[a], Pauline Nauroy[b], Dimitra Kiritsi[b], Georgia Sotiropoulou[a,*]

[a] Department of Pharmacy, School of Health Sciences, University of Patras, Rion-Patras, Greece
[b] Department of Dermatology, Faculty of Medicine, Medical Center-University of Freiburg, Freiburg, Germany

ARTICLE INFO

Article history:
Received 26 February 2019
Received in revised form 6 June 2019
Accepted 11 June 2019

Keywords:
Kallikrein-related peptidase 6 (KLK6)
Netherton syndrome (NS)
Epidermal inflammation
Mouse models

ABSTRACT

Background: Netherton syndrome (NS) is a rare but severe type of ichthyosis characterized by atopy, allergies, and potentially lethal skin overdesquamation associated with highly elevated proteolytic activities in LEKTI-deficient epidermis. NS symptoms are recapitulated in *Spink5*$^{-/-}$ mouse where the gene encoding Lekti has been invalidated. *Spink5*$^{-/-}$ mice die within 5 h from birth due to their severe skin barrier defect leading to dehydration. *Spink5*$^{-/-}$ mice also serve as a model for atopic dermatitis. The KLK6 protease is expressed by epidermal keratinocytes and shown *in vitro* to cleave desmosomal components.
Objective: To investigate *in vivo* whether KLK6 is implicated in epidermal overdesquamation and/or inflammation associated with NS.
Methods: The role of KLK6 was evaluated by generating *Spink5*$^{-/-}$*Klk6*$^{-/-}$ double knockout mice. The phenotype was assessed by macroscopic observation, immunohistochemistry for differentiation markers, *in situ* zymography for proteolysis, and quantification of proinflammatory cytokines.
Results: Elimination of Klk6 in *Spink5*$^{-/-}$ remarkably suppresses the expression of Tslp, a major itching-inducing factor and driver of allergic reactions. Tnfα and the Th17 promoting cytokine Il-23 were also suppressed. *Spink5*$^{-/-}$*Klk6*$^{-/-}$ mice display normalized keratinocyte differentiation, nevertheless, epidermal proteolytic activities and the associated overdesquamation were not ameliorated, and *Spink5*$^{-/-}$*Klk6*$^{-/-}$ still died from a severe epidermal barrier defect as the *Spink5*$^{-/-}$.
Conclusions: Ablation of *Klk6* largely suppresses epidermal inflammation but cannot rescue overdesquamation leading to the lethal NS phenotype. Nonetheless, our findings demonstrate for the first time that KLK6 is implicated in skin inflammation and may represent a novel druggable target for NS and other inflammatory conditions *e.g.* atopic dermatitis.
© 2019 Japanese Society for Investigative Dermatology. Published by Elsevier B.V. All rights reserved.

1. Introduction

Netherton Syndrome (NS) is a severe type of ichthyosis characterized by extensive desquamation, hair shaft defect (bamboo hair) and constant atopic manifestations. NS is caused by inactivating mutations in the *SPINK5* gene encoding the serine protease inhibitor LEKTI [1] and is recapitulated in *Spink5*$^{-/-}$ mice [2]. Due to the atopic manifestations and the associated constitutive inflammation, *Spink5*$^{-/-}$ mice have served as an atopic dermatitis model [3] and as a model for rosacea [4]. In addition, variants in the *SPINK5* gene have been found in cases of atopic dermatitis [5–7]. Previously, it was reported that KLK5 drives the atopic manifestasions in NS by activating PAR2 signaling to induce NF-κB that in turn produces TSLP and other proinflammatory cytokines [3]. TSLP is a key molecule that is expressed by epidermal keratinocytes and binds to receptors in sensory neurons to promote itching [8]. TSLP is highly expressed in atopic dermatitis lesional skin [9] and its overexpression in mouse epidermis induces atopic dermatitis-like symptoms [10] and aggravates asthma [11].

From the kallikrein-related peptidase family (KLKs), KLK5, 7 and 14 have been implicated in skin desquamation [12]. Recently, we showed that elimination of *Klk5* in *Spink5*-null background rescues neonatal lethality and prevents inflammation [13]. KLK6 protease was originally cloned from a breast tumor and is also highly expressed by keratinocytes and neurons [14]. Human KLK6 (Klk6 for mouse) is strongly upregulated in lesional psoriatic skin [15]. Recently, it was shown that *Klk6*$^{-/}$-exponent skin is resistant to induction of psoriasiform inflammation with imiquimod and induction of resistance was independent on Par2 activation [16]. KLK6 has been shown to increase the motility of keratinocytes *in*

* Corresponding author at: Department of Pharmacy, School of Health Sciences, University of Patras, Rion-Patras, 265 04, Greece.
E-mail address: gdsotiro@upatras.gr (G. Sotiropoulou).

https://doi.org/10.1016/j.jdermsci.2019.06.004
0923-1811/© 2019 Japanese Society for Investigative Dermatology. Published by Elsevier B.V. All rights reserved.

vivo [17] and to cleave desmoglein 1 *in vitro* but the physiological significance of these findings have not been studied [18].

Here, we aimed to investigate the role of KLK6 in skin desquamation and inflammation *in vivo*. For this, we generated *Spink5*$^{-/-}$*Klk6*$^{-/-}$ double knockout mice to test whether elimination of *Klk6* will rescue the NS-like phenotype of these mice. *Spink5*$^{-/-}$*Klk6*$^{-/-}$ mice exhibited increased desquamation and a lethal barrier defect as the *Spink5*$^{-/-}$ mice. Interestingly, however, differentiation is normalized, and the constitutive inflammation observed in *Spink5*$^{-/-}$ is notably ameliorated in *Spink5*$^{-/-}$*Klk6*$^{-/-}$ mice manifested by the significantly normalized levels of pro-inflammatory cytokines, including Tslp, and immune infiltrates in their epidermis. This novel finding may open new ways for the development of new skin anti-inflammatory therapeutics based on KLK6 inhibitors for inflammatory skin conditions.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma-Aldrich or Merck. The following antibodies were used: involucrin (Santa Cruz, sc-15230), desmoglein 1 (Santa Cruz, sc-20114), keratin 5 (Abcam, ab24647), corneodesmosin (CusAb, CSBPA0051241A01HU), desmocolin 1 (Santa Cruz, sc-18115), loricrin (Abcam, ab24722), p65 (Cell signaling, 8242 P), Par2 (Santa Cruz, sc-13504), Erk1/2 (Cell Signaling, #4695) (for mouse), phosphorylated Erk1/2 (Cell Signaling, #4370) (for mouse), Erk1/2 (Sigma-Aldrich, M5670) (for human), phosphorylated Erk1/2 (Sigma-Aldrich, M8159) (for human), GAPDH (Santa Cruz, sc-47724), TSLP (Abcam, ab47943), β-actin (Santa Cruz, sc-47778), α-tubulin (Sigma-Aldrich, T5168). The chicken anti-KLK6 IgY antibody was developed in our laboratory [19].

2.2. Clinical specimens

Skin samples were obtained from three healthy donors and one Netherton Syndrome patient with written approval consents.

2.3. Animals

Spink5$^{-/-}$ mice were kindly provided by Prof Andrew McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK). *Klk6*$^{-/-}$ have been developed in collaboration with Prof Andras Nagy (University of Toronto, Toronto, Ontario, Canada). The design of *Klk6*$^{-/-}$ inactivation cassette has been published previously [20]. All experiments with animals were conducted in accordance with EU and national legislation.

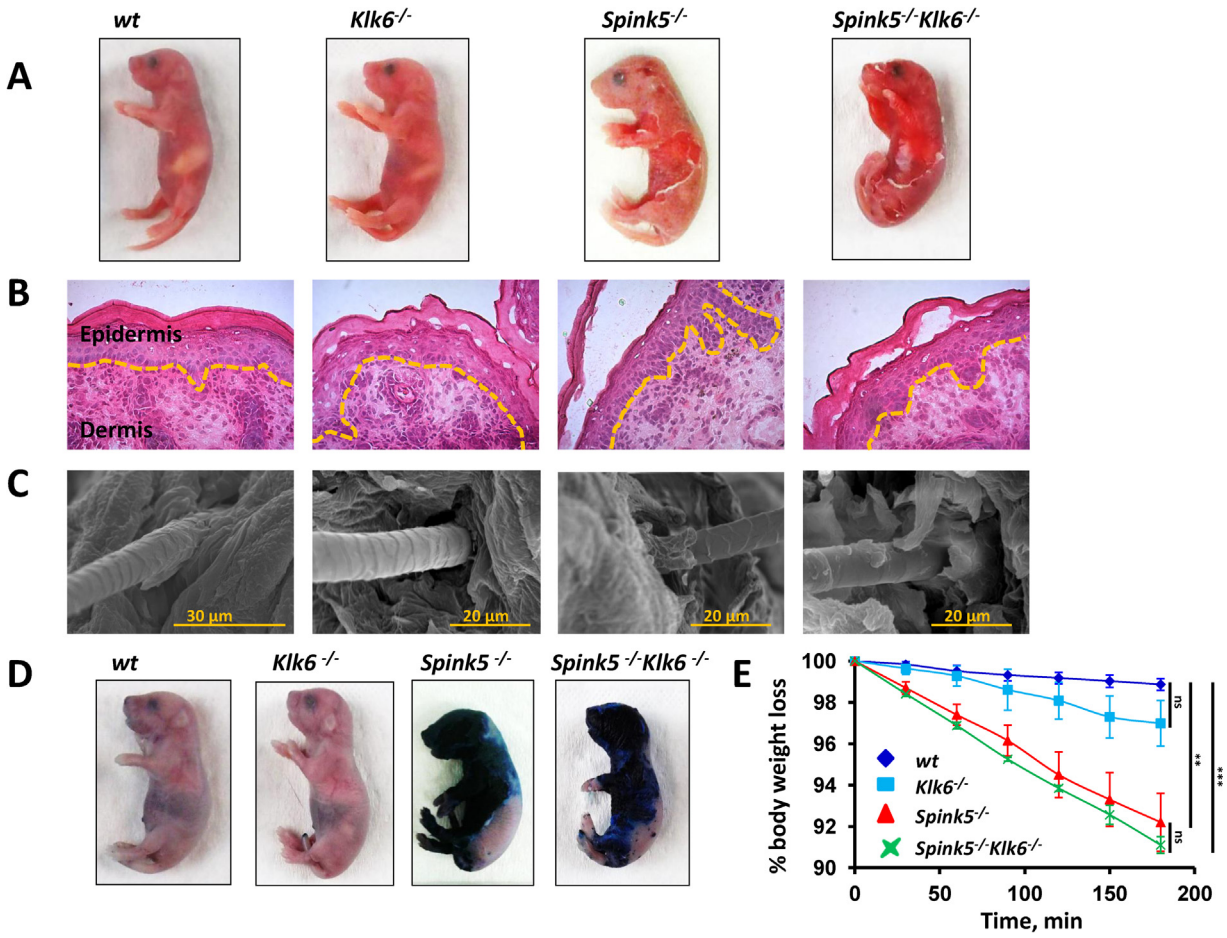


Fig. 1. Deletion of Klk6 in Lekti-deficient epidermis does not rescue the lethal NS phenotype. (A) Macroscopic appearance of newborn wt, *Klk6*$^{-/-}$, *Spink5*$^{-/-}$, and *Spink5*$^{-/-}$*Klk6*$^{-/-}$ mice. *Spink5*$^{-/-}$*Klk6*$^{-/-}$ appear macroscopically indistinguishable from the *Spink5*$^{-/-}$ displaying, detachment of the stratum corneum from the stratum granulosum and extensive desquamation. (B) H/E staining of skin cryosections from wt, *Klk6*$^{-/-}$, *Spink5*$^{-/-}$, *Spink5*$^{-/-}$*Klk6*$^{-/-}$ newborn mice shows detachment of the stratum corneum in *Spink5*$^{-/-}$*Klk6*$^{-/-}$ as in *Spink5*$^{-/-}$. (C) Whisker structure by scanning electron microscopy (SEM) shows that *Spink5*$^{-/-}$ and *Spink5*$^{-/-}$*Klk6*$^{-/-}$ display vibrissae defects. (D) Whole body toluidine blue staining of newborns shows that epidermal barrier in not restored by deletion of *Klk6* in *Spink5*-null background. (E) Water loss assay shows comparable dehydration rates between the *Spink5*$^{-/-}$*Klk6*$^{-/-}$ and *Spink5*$^{-/-}$ mice. Data are shown as mean ± SEM; ns not significant; ** $p \leq 0.01$; *** $p \leq 0.001$; n = 4 mice per group.

Please cite this article in press as: E. Zingkou, et al., A proinflammatory role of KLK6 protease in Netherton syndrome, J Dermatol Sci (2019), https://doi.org/10.1016/j.jdermsci.2019.06.004

2.4. PCR genotyping

DNA was extracted from mouse tail with the mammalian genomic DNA extraction kit (Sigma) and PCR genotyping was conducted as described [21].

2.5. Histology-Immunohistochemistry

Skin biopsies were stored in OCT at −70 °C or formaldehyde fixed and embedded in paraffin. 5 μm skin sections were cut with a cryotome or a microtome respectively. Sections were stained with hematoxylin-eosin or used for immunohistochemistry (IHC). IHC on cryosections was carried out as described [13]. For IHC on paraffin sections epitope retrieval was carried out by boiling the sections in 0.01 M sodium citrate-0.05% Tween-20, pH 6.0 for 20 min. Then the samples were processed as the cryosections. Mast cells were quantified after staining of cryosections with toluidine blue as described [13].

2.6. Scanning electron microscopy

Skin biopsies were fixed in 4% formaldehyde in PBS for 24 h, washed and dehydrated in a series of ethanol baths. Ethanol was replaced with acetone, samples were dried, gold sputtered and observed in field emission SEM.

2.7. Assessment of epidermal barrier

Epidermal barrier integrity was evaluated with whole-body toluidine blue staining and with monitoring the reduction of body weight *versus* time at 37 °C due to water loss.

2.8. RNA extraction

Total RNA extraction was carried out with Nucleospin RNA (Macherey-Nagel). The quality of RNA was checked with agarose electrophoresis and its concentration was measured by reading the absorbance at 260 nm.

2.9. RT-qPCR

Reverse transcription was carried out with the Superscript kit (Invitrogen). cDNAs were subjected to real-time PCR with the SYBR Select Master Mix (Invitrogen). For Il-6, the Kapa SYBR FAST Universal One Step qRT-PCR kit (Kappa Biosystems) was used. The primers have been described in our previous publication [13].

2.10. Western blot analysis

Total protein extracts from total skin or keratinocyte cell lines were electrophoresed on SDS-PAGE and then transferred onto

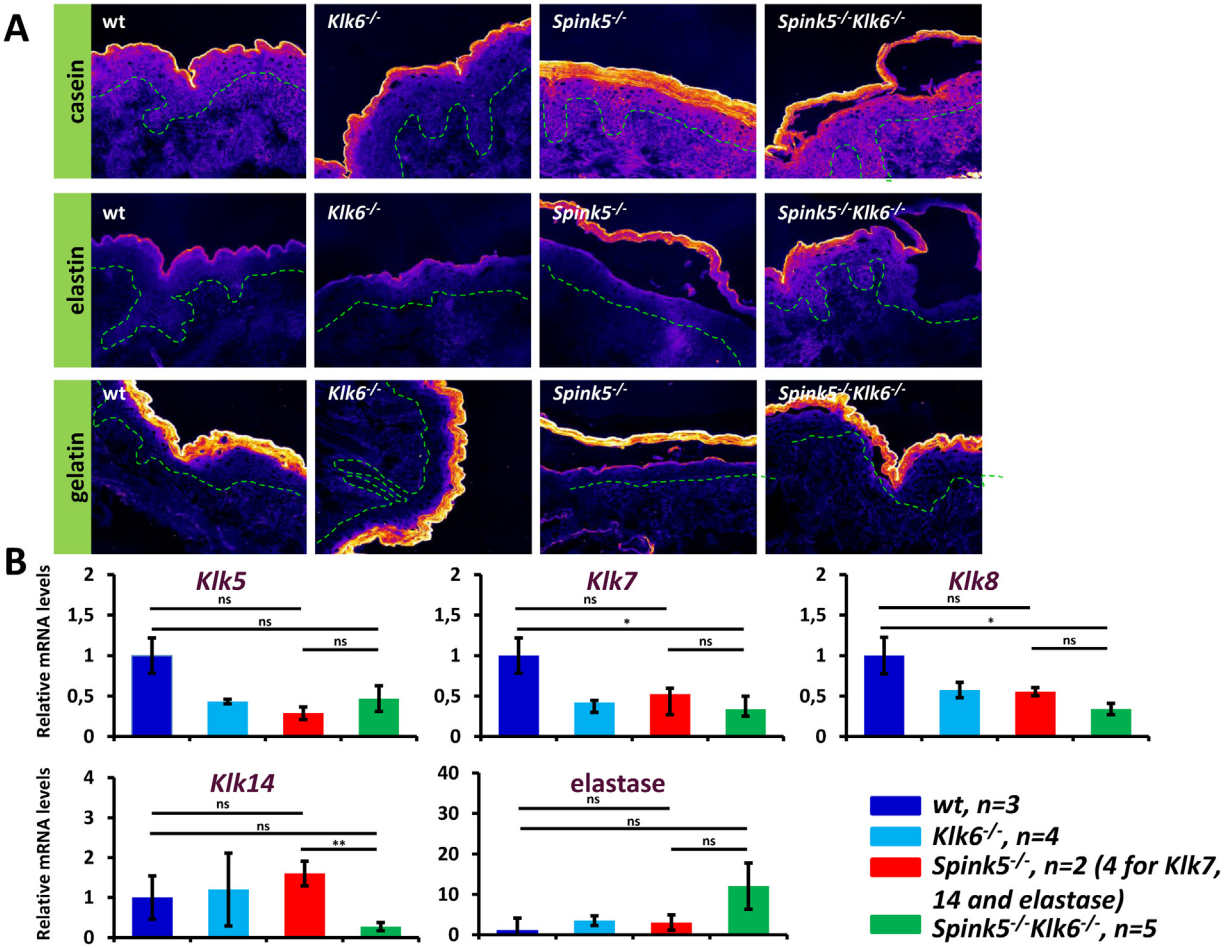


Fig. 2. Elimination of *Klk6* does not reduce epidermal proteolysis in *Spink5*$^{-/-}$. (A) *Spink5*$^{-/-}$*Klk6*$^{-/-}$ skin shows increased caseinolytic and elastinolytic activities in the stratum corneum comparable to *Spink5*$^{-/-}$ mice with *in situ* zymography. (B) RT-qPCR for *Klk5*, *Klk7*, *Klk8*, and *Klk14* (major serine proteases of the epidermis) reveals no significant differences in their gene expression. However, an increased expression of elastase in *Spink5*$^{-/-}$*Klk6*$^{-/-}$ was observed. Data are shown as mean ± SEM; ns not significant; * $p < 0.05$; ** $p < 0.01$.

Please cite this article in press as: E. Zingkou, et al., A proinflammatory role of KLK6 protease in Netherton syndrome, J Dermatol Sci (2019), https://doi.org/10.1016/j.jdermsci.2019.06.004

PVDF membrane. Membrane was blocked for 1 h in 5% non-fat dry milk in PSB, and then incubated with the appropriate primary antibody in 1% non-fat dry milk in PBS-0.05% Tween-20 overnight at 4 °C. Membrane was washed and incubated with the appropriate secondary HRP-conjugated antibody in PBS-0.05% Tween-20 for 1 h. Finally, the membrane was washed and the bands were visualized with enhanced chemiluminescence (Thermo).

2.11. Transfection of keratinocytes

Primary keratinocytes were isolated from the skin of healthy donors and cultivated in serum-free keratinocyte growth medium (Gibco) in the presence of 5% CO_2 at 37 °C. After immortalization with standard protocol stable cell line overexpressing KLK6 were generated by BIOSS Center for Biological Signalling Studies (Universität Freiburg, Germany). Briefly, lentiviral infection was used to overexpress KLK6 and infected were selected with puromycin. KLK6 overexpression was confirmed by qPCR and western blot analyses on cell lysates and conditioned media (data not shown).

2.12. 3D culture

3D-skin model was prepared using normal human keratinocytes or human keratinocytes overexpressing KLK6 and normal human fibroblasts cultivated in Keratinocyte Growth Medium (Gibco) and DMEM with 10% FCS, respectively. Construction of the 3D-skin cultures was performed as previously [22].

3. Results

3.1. Spink5$^{-/-}$Klk6$^{-/-}$ newborn mice display similar skin phenotype to Spink5$^{-/-}$ mice

Spink5$^{+/-}$ mice were crossed with *Klk6*$^{-/-}$ mice to generate the *Spink5*$^{+/-}$*Klk6*$^{+/-}$ and then the *Spink5*$^{+/-}$*Klk6*$^{-/-}$. Subsequently, *Spink5*$^{+/-}$*Klk6*$^{-/-}$ were intercrossed to generate the *Spink5*$^{-/-}$*Klk6*$^{-/-}$ double knockout (DKO) mice. DKOs appeared indistinguishable from the *Spink5*$^{-/-}$, exhibiting severe desquamation and stratum corneum (SC) detachment, and vibrissae defects (Fig. 1A–C). These

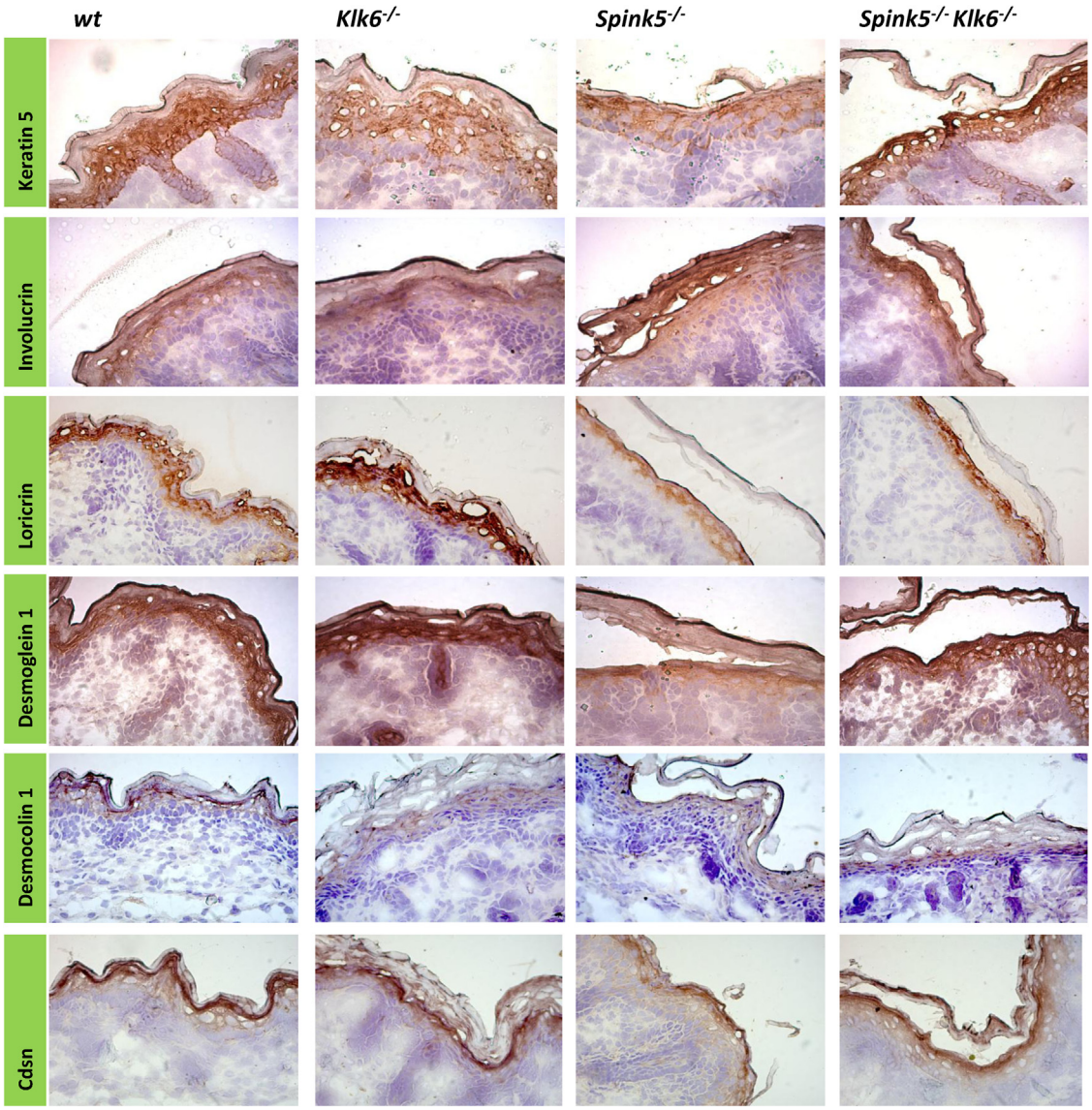


Fig. 3. Epidermal differentiation is normalized in *Spink5*$^{-/-}$*Klk6*$^{-/-}$. Expression of differentiation markers (keratin 5, involucrin, loricrin, desmoglein1, desmocollin 1, corneodesmosin) is normalized in *Spink5*$^{-/-}$*Klk6*$^{-/-}$ compared with *Spink5*$^{-/-}$.

Please cite this article in press as: E. Zingkou, et al., A proinflammatory role of KLK6 protease in Netherton syndrome, J Dermatol Sci (2019), https://doi.org/10.1016/j.jdermsci.2019.06.004

evidences suggest that deletion of *Klk6* does not prevent skin desquamation.

3.2. Spink5$^{-/-}$Klk6$^{-/-}$ mice die from severe epidermal barrier defect

DKO mice managed to survive for up to 5 h as the *Spink5*$^{-/-}$ mice. Since DKOs showed signs of severe desquamation we hypothesized that the cause of death was the epidermal barrier defect. As shown in Fig. 1D, the bodies of DKO mice were completely stained with toluidine blue in a manner identical to *Spink5*$^{-/-}$. Also, the DKO mice tend to lose water with the same rate as the *Spink5*$^{-/-}$ (Fig. 1E). Wild-type and *Klk6*$^{-/-}$ epidermis display almost identical levels of epidermal proteolysis (Fig. 2). Absence of Klk6 expression did not reduce the increase epidermal over-proteolysis observed in *Spink5*$^{-/-}$ mice as revealed by *in situ* zymography (Fig. 2A) neither it changed the expression of major epidermal proteases (Fig. 2B). In conclusion, it appears that Klk6 does not participate in the control of the overall epidermal proteolysis.

3.3. Absence of Klk6 restores normal epidermal differentiation

Differentiation markers were analyzed with IHC and Western blot. As shown in Fig. 3 and Fig. S1, *Klk6*$^{-/-}$ mice appear to have a normal differentiation pattern. Deletion of *Klk6* on *Spink5*$^{-/-}$ background restores the normal epidermal differentiation although this was not accompanied by rescue of neonatal lethality.

3.4. Elimination of Klk6 suppresses the expression of Tslp and other inflammatory cytokines

We investigated whether in the absence of *Klk6* the expression of the major pro-Th2 cytokine Tslp, is affected. Tslp is not expressed in wt or *Klk6*$^{-/-}$ skin but it was highly induced in the *Spink5*$^{-/-}$ while in the absence of *Klk6* its expression was highly reduced to normal levels (Fig. 4A). It has been reported that increased proteolytic activity in the epidermis is responsible for the production of Tslp [23]. Since the absence of Klk6 does not reduce the overall epidermal proteolysis, it appears that it controls a specific pathway upstream of Tslp that is not related to desquamation. To investigate whether Klk6 reduces the expression of other inflammatory cytokines we analyzed the expression of *Tnfα*, *Il-1β*, *Il-18*, *Il-6*, *Il-13*, *Il-4* and the Th17 cytokines *Il-17a* and *Il-23*. As shown in Fig. 4A we found reduced levels for most of the molecules tested in DKO skin compared to *Spink5*$^{-/-}$. In addition, we found reduction of mast cell infiltration in *Spink5*$^{-/-}$*Klk6*$^{-/-}$ epidermis compared to *Spink5*$^{-/-}$ to levels comparable to wild-type epidermis consistent with reduced inflammation (Fig. 4B).

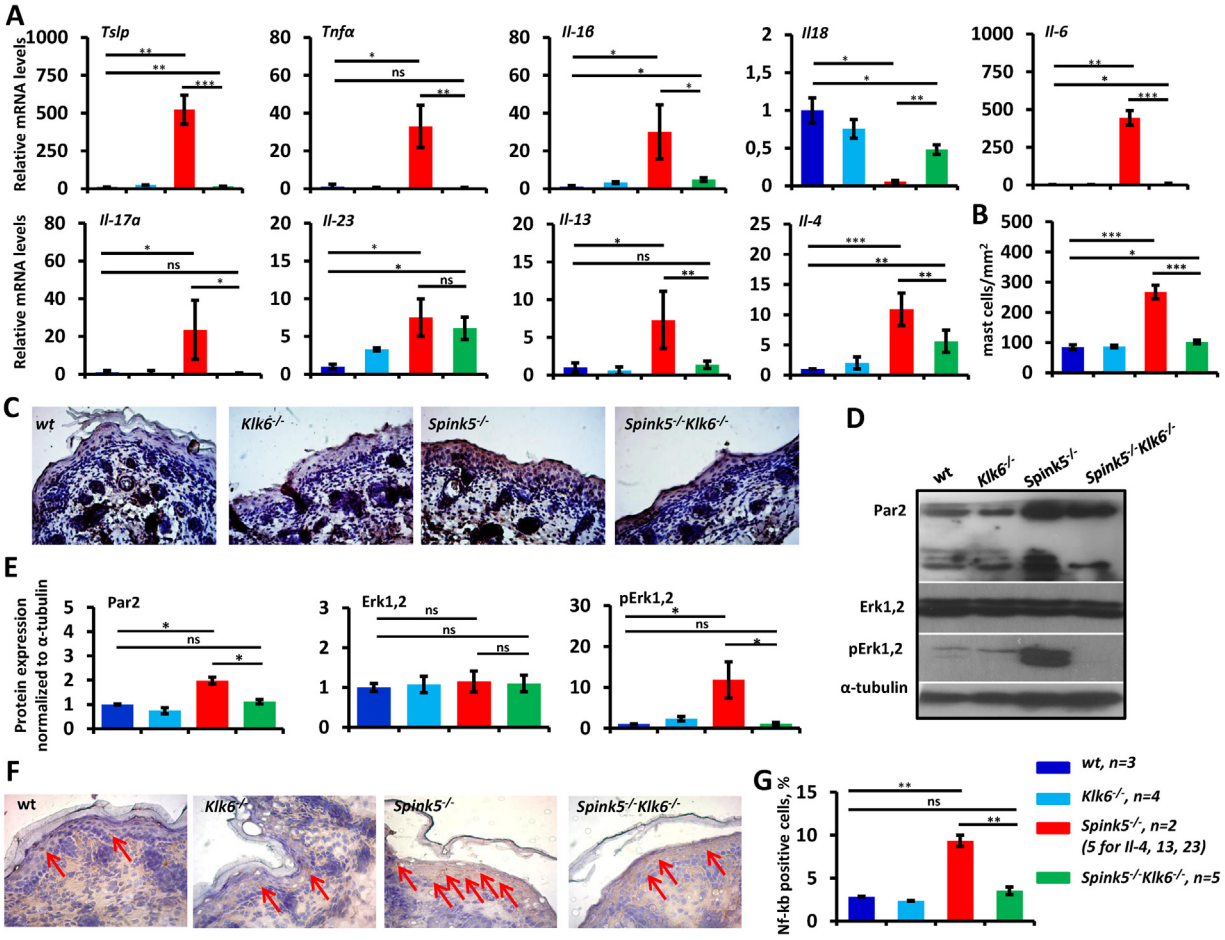


Fig. 4. Ablation of Klk6 suppresses cutaneous inflammation and infiltration of mast cells in *Spink5*$^{-/-}$*Klk6*$^{-/-}$. (A) RT-qPCR quantification of proinflammatory cytokine expression in mouse epidermis shows that *Klk6* inactivation improves cutaneous inflammation in *Spink5*$^{-/-}$ skin. (B) *Spink5*$^{-/-}$*Klk6*$^{-/-}$ shows similar number of mast cells to wt, while *Spink5*$^{-/-}$ skin shows approximately 3-times higher number. Quantification of mast cells was based on toluidine blue staining. (C) IHC staining for Par2 expression shows staining in *Spink5*$^{-/-}$ and *Spink5*$^{-/-}$*Klk6*$^{-/-}$ mice. (D) Western blot analysis of Par2, Erk1/2, phosphorylated Erk1/2 (pErk1/2) and a-tubulin expression in skin extracts from wt, *Klk6*$^{-/-}$, *Spink5*$^{-/-}$ and *Spink5*$^{-/-}$*Klk6*$^{-/-}$ mice. (E) Quantification of data from (D). (F) IHC staining for p65 subunit of Nf-kb shows activation (nuclear staining) mainly in *Spink5*$^{-/-}$ epidermis. Red arrows indicate positive staining in nucleus. (G) Quantification of data from (F). Data are shown as mean ± SEM; ns not significant; * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Please cite this article in press as: E. Zingkou, et al., A proinflammatory role of KLK6 protease in Netherton syndrome, J Dermatol Sci (2019), https://doi.org/10.1016/j.jdermsci.2019.06.004

Therefore, deletion of *Klk6* reduces the epidermal inflammation observed in NS.

Par2 mediated activation of Nf-κb induces the expression of pro-inflammatory cytokines in NS [3]. In *Spink5*$^{-/-}$, Par2 is highly expressed accompanied by increased nuclear staining for p65, a major subunit of Nf-κb. Indeed we found that the levels of Par2 were reduced in *Spink5*$^{-/-}$*Klk6*$^{-/-}$ relative to *Spink5*$^{-/-}$ epidermis (Fig. 4C–E). Further we found reduction in the levels of phosphorylated Erk1/2 in *Spink5*$^{-/-}$*Klk6*$^{-/-}$ epidermis (Fig. 4D–E). In *Spink5*$^{-/-}$*Klk6*$^{-/-}$ p65 staining is reduced suggesting that Klk6 drives the nuclear localization and, thus, activation of Nf-κb (Fig. 4F–G). However, recent *in vivo* findings suggested that Klk6 promoted skin inflammation and psoriasis-like symptoms induced by imiquimod in a Par2-independent manner [16].

Then, we overexpressed KLK6 in normal human keratinocytes by transfecting the cDNA encoding *KLK6* in keratinocytes (NHK). As shown in Fig. 5 the KLK6-overexpressing keratinocytes (NHK-KLK6) exhibited increased ERK1/2 phosphorylation and increased TSLP expression. When cultured in three dimensional system, the NHK-KLK6 differentiated and produced an *in vitro* epidermis model that showed increased NF-kB nuclear staining compared to the NHK controls (Fig. 5). Finally, KLK6 expression is increased in the epidermis of human NS patient, especially in regions of stratum corneum detachment. High KLK6 is accompanied by increased nuclear localization of NF-kB in NS compared to normal epidermis (Fig. 6), in agreement with the mice data. Conclusively, KLK6 seems to be involved in NS inflammation.

4. Discussion

KLK6 was originally identified as being overexpressed in a primary breast tumor and inactivasted in the lung metastases of the same breast cancer patient [14]. It was later found to display high levels of expression in the nervous system and it is expressed also in the skin. *In vitro* experiments showed that KLK6 can cleave protein components of desmosomes [18], thus, it could be

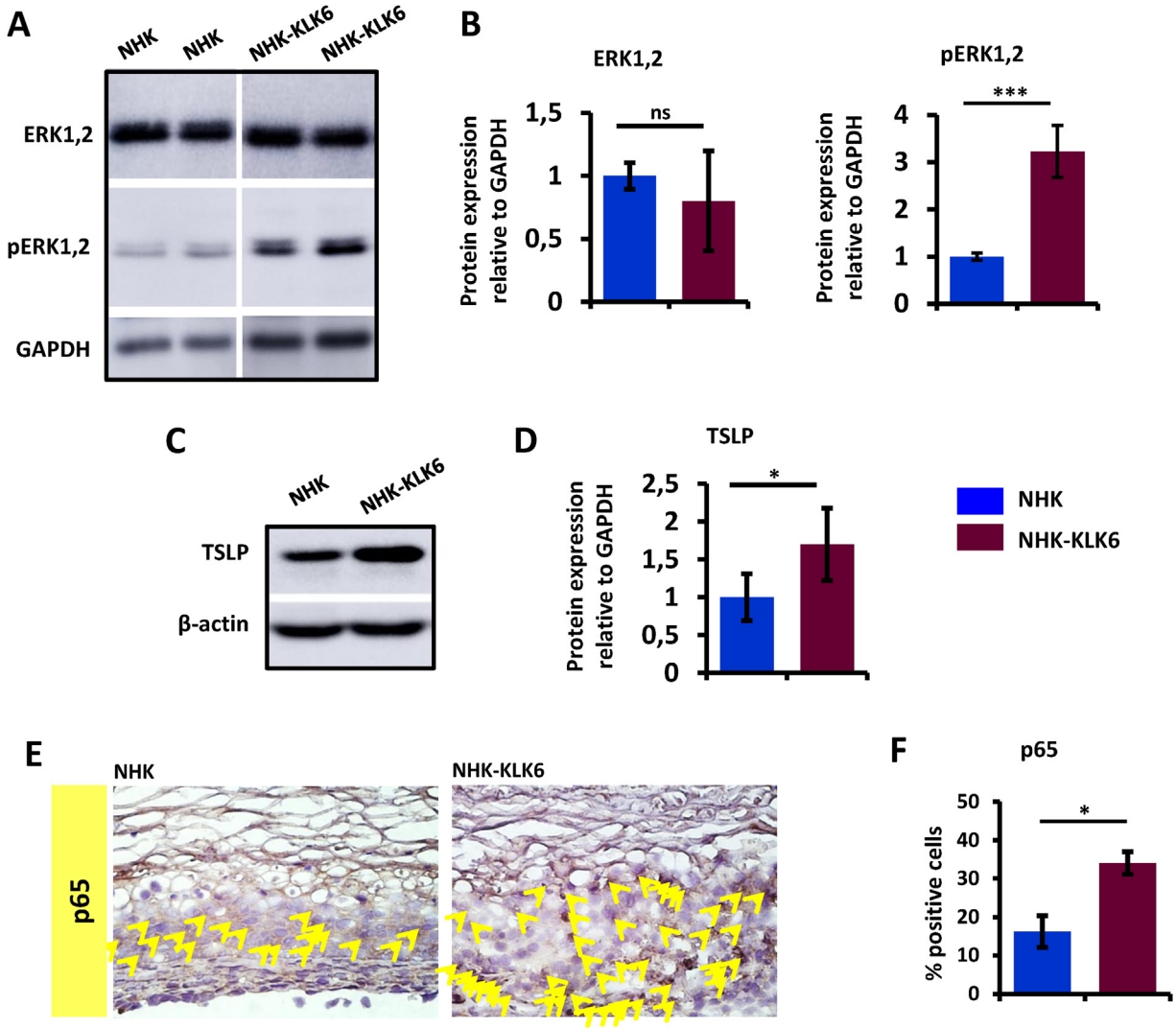


Fig. 5. Overexpression of KLK6 in normal human keratinocytes (NHK) activates pERK1,2-NFkB axis resulting in TSLP upregulation. (A) Western blots of cell lysates extracted from NHK and NHK overexpressing KLK6 (NHK-KLK6) shows that ectopic KLK6 expression induces ERK1/2 phosphorylation (pERK1/2) without changing the levels of total ERK1/2 proteins. (B) Quantification of total ERK1/2 and pERK1/2 by ImageJ. Expression was normalized to GAPDH and results are presented as fold increase relative to NHK. (C) Western blot analysis of cell lysates extracted from NHK and NHK-KLK6 shows that TSLP expression is elevated in NHK-KLK6. (D) Quantification of TSLP by ImageJ. Expression was normalized to β-actin and data are presented as fold increase relative to NHK. (E) Immunohistochemical analysis of p65 subunit of NF-kB in NHK and NHK-KLK6 skin 3D substitutes. Activation of NF-kB, represented by nuclear localization of p65, is increased upon overexpression of KLK6. Nuclear localization of p65 subunit is highlighted with yellow arrows. (F) Quantification of data from (E). Data are shown as mean ± SD; ns not significant; * $p < 0.05$; *** $p \leq 0.001$; n = 3 per group.

Please cite this article in press as: E. Zingkou, et al., A proinflammatory role of KLK6 protease in Netherton syndrome, J Dermatol Sci (2019), https://doi.org/10.1016/j.jdermsci.2019.06.004

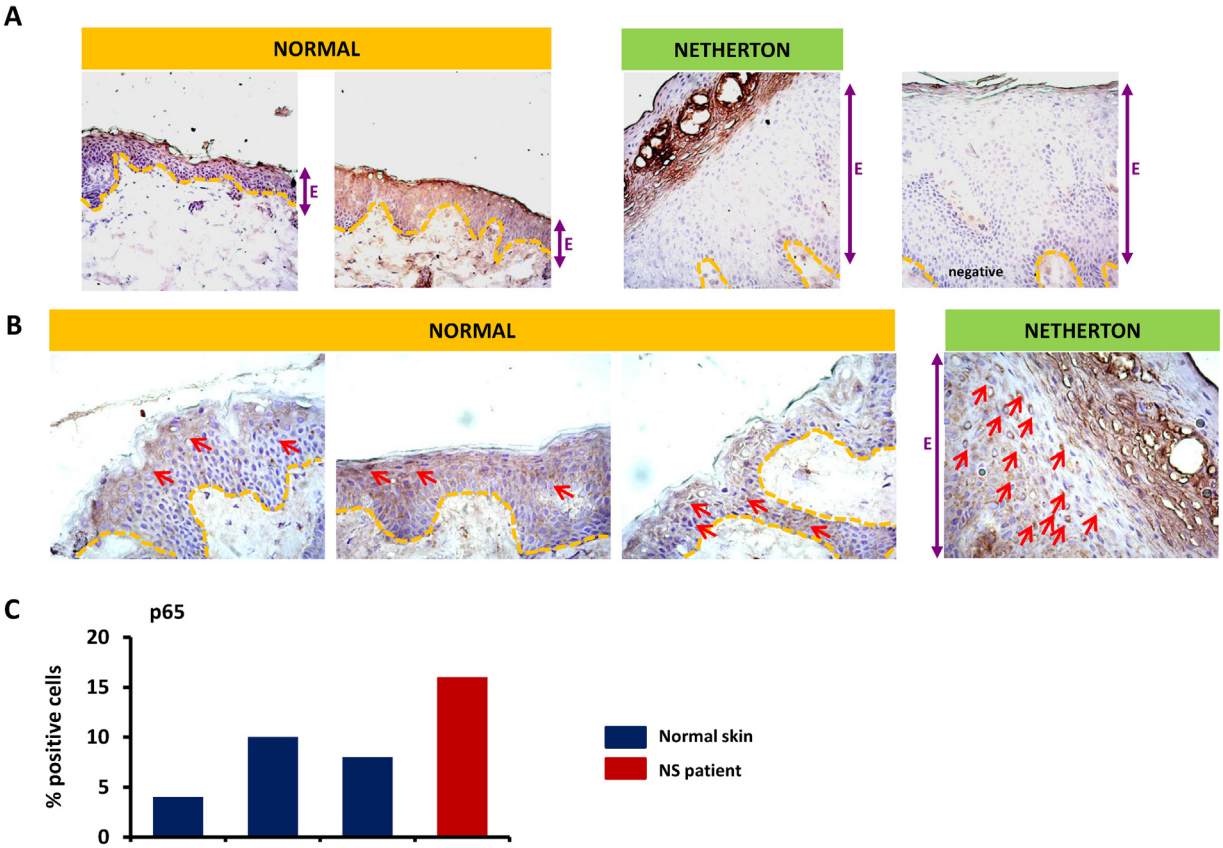


Fig. 6. KLK6 and NF-kB expression in human NS skin. (A) IHC staining for KLK6 shows higher expression in the NS patient than in normal skin. Further, KLK6 expression is diffused throughout the epidermis as opposed to normal samples where it is localized in the upper layers (mainly SG). (B) IHC staining for NF-kB shows increased nuclear staining in NS compared to normal (red arrows). (C) Quantification of data from (B). E, epidermis.

implicated in the process of pathological skin overdesquamation observed in NS and its elimination could rescue or ameliorate the NS phenotype. We tested this hypothesis *in vivo* by generating *Spink5*$^{-/-}$*Klk6*$^{-/-}$ mice. However, both *Spink5*$^{-/-}$ and *Spink5*$^{-/-}$*Klk6*$^{-/-}$ displayed the same desquamating severity and died within 5 h from birth from severe dehydration. Further, if Klk6 participated in the process of physiological desquamation, we would expect *Klk6*$^{-/-}$ mice to have hyperkeratosis that was not observed. Taken together, our data suggest that KLK6 is not involved in the desquamation process.

Deletion of *Klk6* restored normal epidermal differentiation patterns in *Spink5*$^{-/-}$ epidermis. Previously, it was shown that deletion of *Klk7* in *Spink5*$^{-/-}$ mice did not prevent epidermal overdesquamation but it normalized epidermal differentiation and inflammation [24], suggesting that Klk6 and Klk7 may have common functions in skin. Since KLK7 is not able to activate PAR2 [25] this finding indicates that, probably, there are other yet unknown PAR2-independent pathways that regulate the induction of inflammation by KLKs in the epidermis.

Interestingly, deletion of Klk6 reduced the expression of proinflammatory cytokines including Tslp that is also well-known to play important roles in atopic dermatitis. Our study adds a new proinflammatory axis in skin pathophysiology, the KLK6-ERK1/2-NF-kB-TSLP axis. Similarly, in keratin-deficient keratinocytes, it was found that the expression of TSLP is mediated through MEK1/2-ERK1/2 activation [26]. Although it has been reported that KLK5 drives skin inflammation *via* activation of PAR2-NF-κB-TNFα-TSLP pathway [3], a later study showed that deletion of *Par2* in *Spink5*$^{-/-}$ mice failed to rescue epidermal inflammation [27]. Further, a recent study pointed into the KLK5-dependent activation of alternative proinfammatory pathway(s) *in vivo*. Specifically, it was shown that persistent inflammation and TSLP production in atopic dermatitis (AD) is induced by KLK5 in a PAR2-independent manner [28]. On the other side, the latter study [28] confirmed that KLK5 plays an important role in DSG1 degradation as has been shown previously [13,24] further substantiating the key role of KLK5 in the desquamation process.

In the same line with the findings described here, we recently found that KLK6 regulates chronic epidermal inflammation induced by TPA. Specifically, epidermal application of TPA in *Klk6*$^{-/-}$ mice led to substantially reduced inflammatory response as evident by the strong suppression of the expression of proinflammatory cytokines compared to wt mice [29]. This strong attenuation of epidermal inflammation is associated with high resistance of *Klk6*$^{-/-}$ mice to chemically induced skin carcinogenesis [29]. Collectively, our previous study [29] and this study suggest that KLK6 is a major regulator of skin inflammation in two clearly distinct pathological contexts (*i.e.* non-melanoma skin cancer and Netherton syndrome). Revealing the underlying molecular mechanisms will require in future studies.

Given that *SPINK5* variations have been identified in atopic dermatitis [5–7] and that *Spink5*$^{-/-}$ mice are a model for atopic dermatitis [3], it is possible that KLK6 inhibition will be beneficial for the treatment of atopic dermatitis. Indeed, anti-inflammatory therapies with anti-TNFα agents have been used in severe cases of atopic dermatitis [30,31] and for alleviating the inflammatory phenotype in NS patients [32,33]. Our results presented here pinpoint KLK6 as an alternative pharmacological target. Recent findings suggest that KLK6 could be involved in psoriasis. Its expression was found highly increased in the skin of the KC-Tie2 psoriasis mouse model and in lesional psoriatic skin [15]. Psoriatic patients display increased serum KLK6 that correlates with

psoriasis area and severity index score [34]. KLK6 is highly expressed after induction of psoriasis-like symptoms in mice with imiquimod [16]. Therefore, the development of anti-inflammatory therapies based on targeting KLK6 could be beneficial for psoriasis as well.

Funding

Funded by the grant Skink5 (LS4-2139) implemented within the framework of the Action «Supporting Postdoctoral Researchers» of the Operational Program "Education and Lifelong Learning" (Action's Beneficiary: General Secretariat for Research and Technology) and co-financed by the European Social Fund (ESF) and the Greek State.

Acknowledgements

We would like to thank Prof Andriew McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK) for kindly providing the *Spink5*$^{-/-}$ mice. We also thank Dr. Andreas Seferlis (Laboratory of Electron Microscopy and Microanalysis, University of Patras, Greece) for technical assistance with scanning electron microscopy. Finally, we would like to thank Dr. Manthoula Valari, MD (First Department of Pediatrics, National and Kapodistrian University of Athens Medical School, "Aghia Sophia" Children's Hospital, Athens 11527, Greece) for performing all human biopsies and kindly providing the corresponding specimens.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.06.004.

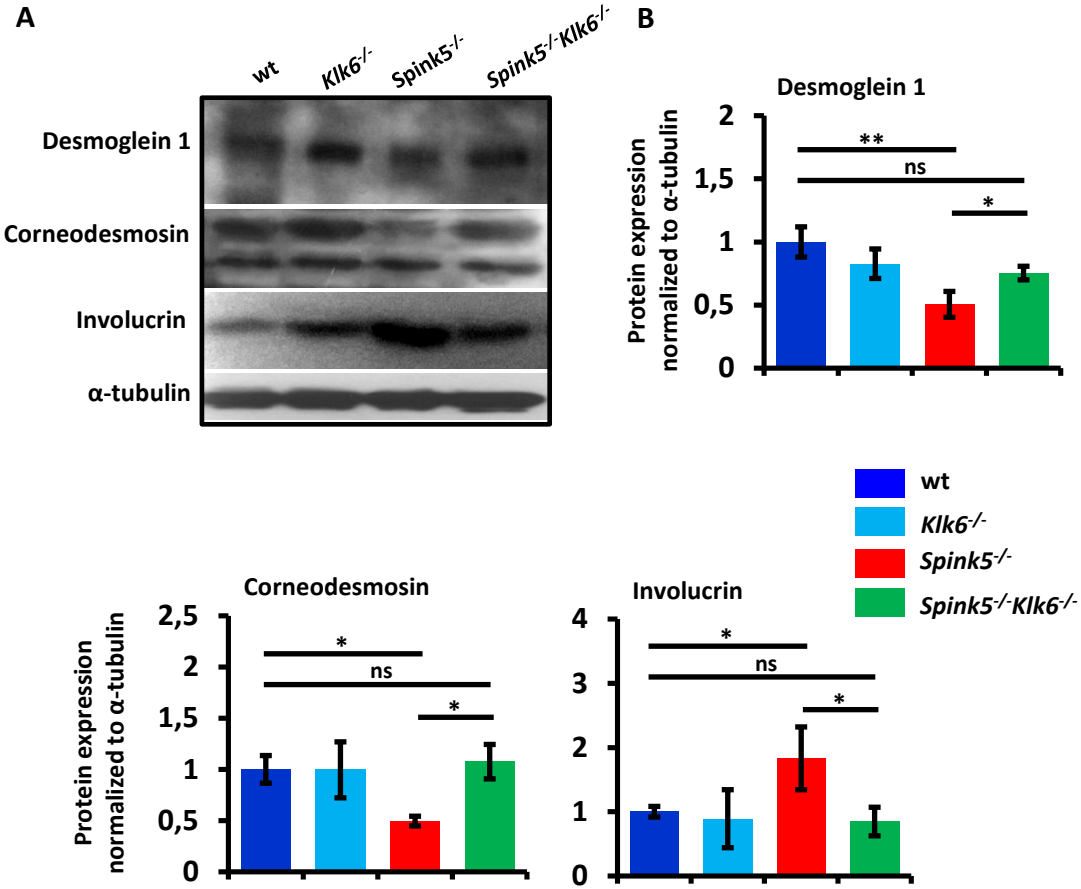
References

[1] S. Chavanas, C. Bodemer, A. Rochat, et al., Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome, Nat. Genet. 25 (2000) 141–142.
[2] P. Descargues, C. Deraison, C. Bonnart, et al., Spink5-deficient mice mimic Netherton syndrome through degradation of desmoglein 1 by epidermal protease hyperactivity, Nat. Genet. 37 (2005) 56–65.
[3] A. Briot, C. Deraison, M. Lacroix, et al., Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome, J. Exp. Med. 206 (2009) 1135–1147.
[4] K. Yamasaki, A. Di Nardo, A. Bardan, et al., Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea, Nat. Med. 13 (2007) 975–980.
[5] S. Morizane, M. Ouchida, K. Sunagawa, et al., Analysis of all 34 exons of the SPINK5 gene in Japanese atopic dermatitis patients, Acta Med. Okayama 72 (2018) 275–282.
[6] K. Dežman, P. Korošec, H. Rupnik, M. Rijavec, SPINK5 is associated with earlyonset and CHI3L1 with late-onset atopic dermatitis, Int. J. Immunogenet. 44 (2017) 212–218.
[7] P. Fortugno, L. Furio, M. Teson, et al., The 420K LEKTI variant alters LEKTI proteolytic activation and results in protease deregulation: implications for atopic dermatitis, Hum. Mol. Genet. 21 (2012) 4187–4200.
[8] S.R. Wilson, L. Thé, L.M. Batia, et al., The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch, Cell 155 (2013) 285–295.
[9] Y.J. Liu, Thymic stromal lymphopoietin: master switch for allergic inflammation, J. Exp. Med. 203 (2006) 269–273.
[10] J. Yoo, M. Omori, D. Gyarmati, et al., Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin, J. Exp. Med. 202 (2005) 541–549.
[11] Z. Zhang, P. Hener, N. Frossard, et al., Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 1536–1541.
[12] G. Sotiropoulou, G. Pampalakis, E.P. Diamandis, Functional roles of human kallikrein-related peptidases, J. Biol. Chem. 284 (2009) 32989–32994.
[13] L. Furio, G. Pampalakis, I.P. Michael, et al., KLK5 inactivation reverses cutaneous hallmarks of Netherton syndrome, PLoS Genet. 11 (2015)e1005389.
[14] A. Anisowicz, G. Sotiropoulou, G. Stenman, et al., A novel protease homolog differentially expressed in breast and ovarian cancer, Mol. Med. 2 (1996) 624–636.
[15] K.C. Lundberg, Y. Fritz, A. Johnston, et al., Proteomics of skin proteins in psoriasis: from discovery and verification in a mouse model to confirmation in humans, Mol. Cell Proteomics 14 (2015) 109–119.
[16] S. Iinuma, M. Kishibe, N. Saito, et al., Kallikrein-related peptidase 6 promotes psoriasiform skin inflammation through a protease-activated receptor 2independent mechanism, Exp. Dermatol. 26 (2016) 289–291.
[17] B. Klucky, R. Mueller, I. Vogt, et al., Kallikrein 6 induces E-cadherin shedding and promotes cell proliferation, migration, and invasion, Cancer Res. 67 (2007) 8198–8206.
[18] C.A. Borgoño, I.P. Michael, N. Komatsu, et al., A potential role for multiple tissue kallikrein serine proteases in epidermal desquamation, J. Biol. Chem. 282 (2007) 3640–3652.
[19] G. Sotiropoulou, G. Pampalakis, E. Prosnikli, et al., Development and immunochemical evaluation of a novel chicken IgY antibody specific for KLK6, Chem. Cent. J. 6 (2012) 148.
[20] G. Pampalakis, V.S. Sykioti, M. Ximerakis, et al., KLK6 proteolysis is implicated in the turnover and uptake of extracellular alpha-synuclein species, Oncotarget 8 (2017) 14502–14515.
[21] D.R. Hewett, A.L. Simons, N.E. Mangan, et al., Lethal, neonatal ichthyosis with increased proteolytic processing of filaggrin in a mouse model of Netherton syndrome, Hum. Mol. Genet. 14 (2005) 335–346.
[22] V.R. Mittapalli, J. Madl, S. Löffek, et al., Injury-driven stiffening of the dermis expedites skin carcinoma progression, Cancer Res. 76 (2016) 940–951.
[23] H. Kouzaki, S.M.O.' Grady, C.B. Lawrence, H. Kita, Protease induce production of thymic stromal lymphopoietin by airway epithelial cells through proteaseactivated receptor 2, J. Immunol. 183 (2009) 1427–1434.
[24] P. Kasparek, Z. Ileninova, O. Zbodakova, et al., KLK5 and KLK7 ablation fully rescues lethality of Netherton syndrome-like phenotype, PLoS Genet. 13 (2017) e1006566.
[25] K. Stefansson, M. Brattsand, D. Roosterman, et al., Activation of proteinase-activated receptor-2 by human kallikrein-related peptidases, J. Invest. Dermatol. 128 (2008) 18–25.
[26] V. Kumar, M. Behr, D. Kiritsi, et al., Keratin-dependent thymic stromal lymphopoitein expression suggests a link between skin blistering and atopic disease, J. Allergy Clin. Immunol. 138 (2016) 1461–1464.
[27] A. Briot, M. Lacroix, A. Robin, M. Steinhoff, et al., Part 2 inactivation inhibits early production of TSLP, but not cutaneous inflammation, in Netherton syndrome adult mouse model, J. Invest. Dermatol. 130 (2010) 2736–2742.
[28] Y. Zhu, J. Underwood, D. Mackmillan, et al., Persistent kallikrein 5 activation induces atopic dermatitis-like skin architecture independent of PAR2 activity, J. Allergy Clin. Immunol. 140 (2017) 1310–1322.
[29] N. Khoury, E. Zingkou, G. Pampalakis, et al., KLK6 protease accelerates skin tumor formation and progression, Carcinogenesis 39 (2018) 1529–1536.
[30] N. Cassano, F. Loconsole, C. Coviello, G.A. Vena, Infliximab in recalcitrant severe atopic eczema associated with contact allergy, Int. J. Immunopathol. Pharmacol. 19 (2006) 237–240.
[31] A. Jacobi, C. Antoni, B. Manger, et al., Infliximab in the treatment of moderate to severe atopic dermatitis, J. Am. Acad. Dermatol. 52 (2005) 522–526.
[32] Â. Roda, M. Mendonca-Sanches, A.R. Travassos, et al., Infliximab therapy for Netherton syndrome: a case report, JAAD Case Rep. 3 (2017) 550–552.
[33] L. Fontao, E. Laffitte, A. Briot, et al., Infliximab infusions for Netherton syndrome: sustained clinical improvement correlates with a reduction of thymic stromal lymphopoietin levels in the skin, J. Invest. Dermatol. 131 (2011) 1947–1950.
[34] N. Komatsu, K. Saijoh, C. Kuk, et al., Aberrant human tissue kallikrein levels in the stratum corneum and serum of patients with psoriasis: dependence on phenotype, severity and therapy, Br. J. Dermatol. 156 (2007) 875–883.

Supplementary Figures

Fig. S1. Expression of differentiation markers (A) Western blot analysis of differentiation markers. (B) Quantification of data from (A). Expression of differentiation markers is normalized in *Spink5*$^{-/-}$ *Klk6*$^{-/-}$ compared with *Spink5*$^{-/-}$. Data are shown as mean±SD; ns not significant; * $p<0.05$; ** $p\leq0.01$.

Figure S1


A
wt
Klk6-/-
Spink5-/-
Spink5-/-Klk6-/-
Desmoglein 1
Corneodesmosin
Involucrin
α-tubulin
B
Desmoglein 1
Protein expression normalized to α-tubulin
2
1,5
1
0,5
0
**
ns
*
Corneodesmosin
2,5
2
1,5
1
0,5
0
*
ns
*
Involucrin
4
3
2
1
0
*
ns
*
wt
Klk6-/-
Spink5-/-
Spink5-/-Klk6-/-