

Kallikrein 5 induces atopic dermatitis–like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome

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Netherton syndrome (NS) is a severe genetic skin disease with constant atopic manifestations that is caused by mutations in the *serine protease inhibitor Kazal-type 5 (SPINK5)* gene, which encodes the protease inhibitor lymphoepithelial Kazal-type–related inhibitor (LEKTI). Lack of LEKTI causes stratum corneum detachment secondary to epidermal proteases hyperactivity. This skin barrier defect favors allergen absorption and is generally regarded as the underlying cause for atopy in NS. We show for the first time that the pro-Th2 cytokine thymic stromal lymphopoietin (TSLP), the thymus and activation-regulated chemokine, and the macrophage-derived chemokine are overexpressed in LEKTI-deficient epidermis. This is part of an original biological cascade in which unregulated kallikrein (KLK) 5 directly activates proteinase-activated receptor 2 and induces nuclear factor κ B–mediated overexpression of TSLP, intercellular adhesion molecule 1, tumor necrosis factor α , and IL8. This proinflammatory and proallergic pathway is independent of the primary epithelial failure and is activated under basal conditions in NS keratinocytes. This cell–autonomous process is already established in the epidermis of *Spink5*^{−/−} embryos, and the resulting proinflammatory microenvironment leads to eosinophilic and mast cell infiltration in a skin graft model in nude mice. Collectively, these data establish that uncontrolled KLK5 activity in NS epidermis can trigger atopic dermatitis (AD)–like lesions, independently of the environment and the adaptive immune system. They illustrate the crucial role of protease signaling in skin inflammation and point to new therapeutic targets for NS as well as candidate genes for AD and atopy.

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Abbreviations used: AD, atopic dermatitis; GR, granular layer; HRP, horseradish peroxidase; ICAM1, intercellular adhesion molecule 1; KLK, kallikrein; LC, Langerhans cell; LEKTI, lymphoepithelial Kazal-type–related inhibitor; *Mdc*, macrophage-derived chemokine; NHK, normal human primary keratinocytes; NS, Netherton syndrome; PAR2, proteinase-activated receptor 2; SC, stratum corneum; *SPINK5*, serine protease inhibitor Kazal-type 5; *Tarc*, thymus and activation-regulated chemokine; TSLP, thymic stromal lymphopoietin.

The epidermis is a stratified epithelium providing a first line of defense against the harsh external environment. The skin also maintains the body's integrity by sequestering the internal milieu and impeding transcutaneous water loss through the formation of an impermeability barrier. This protective barrier is conferred by the outermost layer of the epidermis, the stratum corneum (SC), which results from a finely regulated terminal cell differentiation process from the basal layer of the epidermis to the granular layers (GRs), through the spinous compartment. The SC consists of dead and keratin-filled cells, corneocytes, which

are attached to each other by corneodesmosomes and embedded in a lipid matrix (1–3). Proteolytic degradation of corneodesmosomes by epidermal proteases leads to the shedding of the most superficial corneocytes (4). This desquamation process allows the regulation of the skin thickness. Alterations in these processes, as well as mutations in genes encoding proteins involved in terminal differentiation, can lead to abnormal stratification and keratinization, as seen in ichthyoses (5).

A. Briot and C. Deraison contributed equally to this paper.

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In this respect, the filament-aggregating protein FILAGGRIN is a key protein that plays an important role in the formation of the barrier function. Recent genetic studies have shown that loss-of-function mutations in the gene encoding FILAGGRIN underlie ichthyosis vulgaris (6), a very common genetic disorder of keratinization. The same *FILAGGRIN* mutations have also been identified as a major risk factor for atopic dermatitis (AD) and are strongly associated with asthma in AD patients (7, 8). These results linked for the first time a primary skin disease to systemic allergic manifestations, through defective skin barrier function.

AD is a chronic inflammatory skin disease characterized by eczema, pruritus, and cutaneous hyperreactivity to environmental factors that are innocuous to normal nonatopic individuals (9). AD has a complex etiology that results from interactions between environment and several susceptibility genes involved in skin barrier function and systemic and local immunological responses (10, 11). AD is often the initial step in the so-called “atopic march,” which leads to asthma and allergic rhinitis in the majority of afflicted patients (12–14). These conditions are all characterized by elevated serum IgE levels and peripheral eosinophilia (9). Clinically unaffected skin in AD manifests impaired skin barrier function that could favor the penetration of microbes and allergens (15), leading to the development of cutaneous and systemic allergies through activation of antigen-presenting cells such as Langerhans cells (LCs) (16). However, keratinocytes are immunologically active cells. Indeed, traumatic barrier disruption alone stimulates both keratinocyte proliferation and their cytokine and chemokine production (IL-1 α , IL-1 β , TNF- α , and GM-CSF), characteristic features of skin inflammatory diseases (17, 18). Thus, the skin should be considered as an important organ of innate immunity. In fact, keratinocytes from lesional skin of AD patients express a proallergic cytokine, thymic stromal lymphopoietin (TSLP). Notably, the local increase of TSLP is directly associated with LC activation and migration to skin draining lymph node, where they trigger the differentiation of naive CD4⁺ T cells into proallergic CD4⁺ Th2 cells (19, 20). TSLP overexpression is also sufficient in mouse skin to induce an inflammatory Th2 microenvironment and an AD-like skin phenotype (21). Keratinocytes are therefore important orchestrators of the innate immunity through the secretion of a wide range of proinflammatory and proallergic molecules inducing and supporting cutaneous acute and chronic inflammatory responses.

One of the most severe ichthyoses of children and young adults is Netherton syndrome (NS; Online Mendelian Inheritance of Man reference number 256500). NS is a rare (1 in 100,000 newborns) autosomal recessive skin disorder characterized by generalized exfoliative erythroderma, a specific hair shaft defect (trichorrhexis invaginata) and severe atopic manifestations, which distinguish NS from the other ichthyoses (22, 23). Indeed, NS patients suffer from recurrent AD with elevated serum IgE levels, asthma, and multiple food allergies (24, 25). Bacterial infection, hypernatraemic dehydration, hypothermia, and extreme weight loss are frequent complications during the neonatal period, resulting in high

postnatal mortality, and are probably favored by the severe alteration of the skin barrier function.

We previously identified *serine protease inhibitor Kazal-type 5* (*SPINK5*) as the defective gene in NS (26). *SPINK5* encodes the multidomain serine protease inhibitor lymphoepithelial Kazal-type-related inhibitor (LEKTI), whose tissue distribution pattern is restricted to the most differentiated viable layers of stratified epithelial tissues and the Hassall's corpuscles in the thymus (27). In the epidermis, LEKTI is mainly restricted to the GR. It is expressed as high molecular mass precursors, which are rapidly processed into several proteolytic fragments secreted in the intercellular space (27, 28). It has been shown that LEKTI fragments can efficiently and specifically inhibit the epidermal kallikrein (KLK) 5, KLK7, and KLK14 (28–30).

Using *Spink5* KO mice (*Spink5*^{-/-}), which faithfully reproduce key features of NS (31–33), we deciphered the biological functions of LEKTI and identified cutaneous pathophysiological pathways of the disease. We showed that epidermal LEKTI deficiency results in KLK5 and KLK7 hyperactivity and dysregulation of a new epidermal protease, which is under characterization. These unrestricted protease activities lead to abnormal desmosome cleavage in the upper GR, resulting in accelerated SC shedding and consequent loss of skin barrier function. This work identified LEKTI as a key regulator of epidermal protease activity and skin barrier integrity (32).

Because NS patients suffer from severe atopic manifestations, such as AD, genetic association between *SPINK5* single nucleotide polymorphisms and severe AD has been tested by several groups (34–41). Positive association was found in several studies in patients with different ethnical background (34, 36). Although the skin barrier defect is considered to be the key event leading to skin inflammation and allergy (42), it is likely that more specific and intrinsic signals could be directly induced by *SPINK5*^{-/-} keratinocytes. In this paper, we therefore explored the molecular mechanisms leading to the recruitment of inflammatory cells in NS skin. We show that in LEKTI-deficient keratinocytes, KLK5 hyperactivity activates proinflammatory signaling, leading to the recruitment of eosinophilic and mast cells, which is reminiscent of AD-like skin lesions in NS, independent from skin barrier defect, environmental stimuli, and the adaptive immune system.

RESULTS

Spink5^{-/-} grafted skin reproduces the histological and ultrastructural anomalies of NS

As newborn *Spink5*^{-/-} (KO) mice died a few hours after birth (32), total dorsal skin of embryonic day (E) 19.5 embryos was grafted on nude mice to analyze the consequences of *Spink5* inactivation in adult skin. 8 wk after grafting, WT skin (WTg) presented normal hair development (Fig. 1 A), whereas the KO graft was alopecic, scaly, and erythematous (Fig. 1 B). Histological analysis showed that WT grafted skin was similar to adult WT skin (Fig. 1 C). In contrast, the KO epidermis was hyperplastic (acanthosis), with epidermal invaginations in the dermis (papillomatosis), and nuclei were seen to persist in

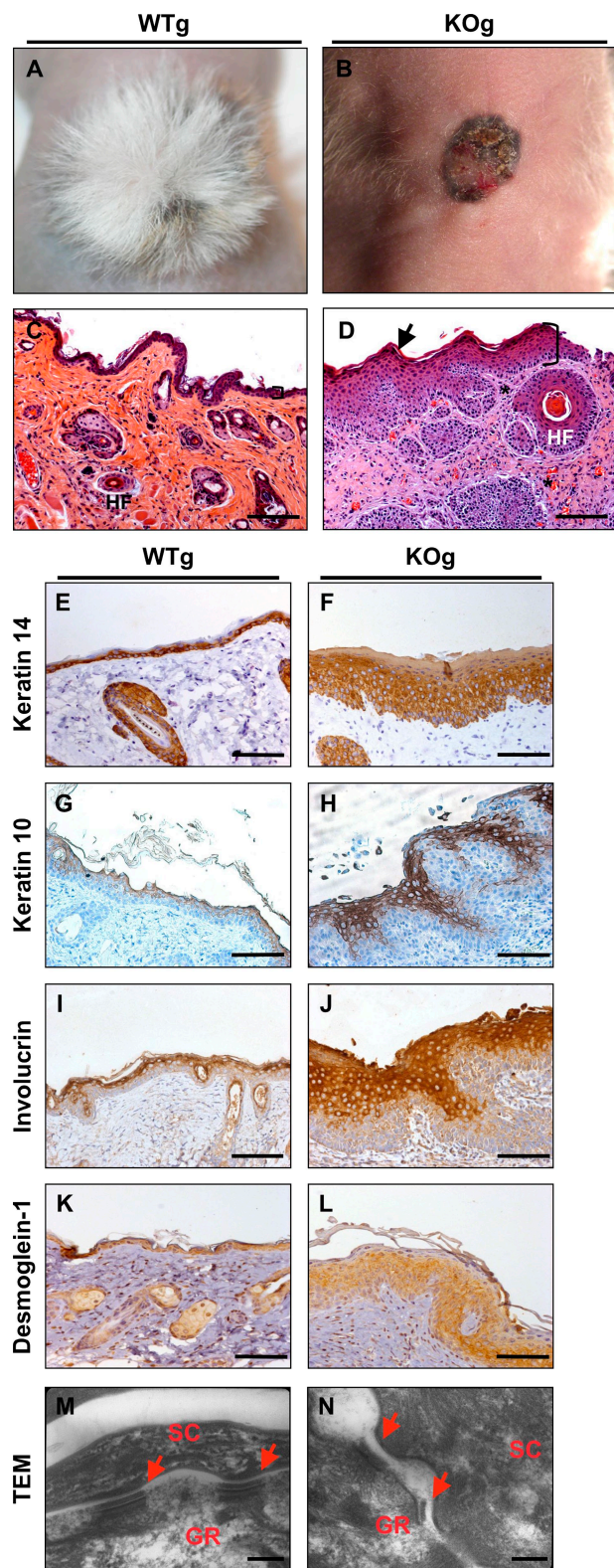


Figure 1. Ichthyosiform phenotype, abnormal differentiation, and asymmetrical desmosomal split in *Spink5*^{-/-} grafted epidermis. (A and B) Macroscopic observation of WT (WTg) or *Spink5* KO (KOg) mice skin 8 wk after grafting on nude recipient mice. (C and D) Hematoxylin/eosin staining of skin graft cross sections. The KOg epidermis shows hyper-

the SC (parakeratosis), which appeared focally detached from the GR. In addition, a massive inflammatory infiltrate was observed in the dermis and the epidermis associated with spongiosis in the basal layers (Fig. 1 D). The hair follicles were hyperplastic and cystic in KO graft, concordant with the alopecic phenotype (Fig. 1, B and D). Analysis of the differentiation program revealed that keratin 14 was only expressed in the basal layer of the WT graft epidermis, whereas all layers were stained in the KO epidermis (Fig. 1, E and F; and Fig. S5). Keratin 10 and involucrin, which are normally expressed in the suprabasal and GR of WT epidermis, respectively, extended to more profound spinous layers in the KO graft (Fig. 1, G–J; and Fig. S5). We previously demonstrated in newborn KO mice that premature desmosome cleavage leading to SC detachment was a key event of NS (32). As SC cohesion was also affected in KO graft, desmosomes were examined by electron microscopy. Well-structured desmosomes were seen at the interface between the GR and the SC (GR–SC) of the WT grafted epidermis, whereas asymmetrical split of desmosomes was observed in KO graft resulting in the loss of GR–SC cohesion and increase of intercellular space (Fig. 1, M and N). The desmosomal cadherin desmoglein-1 was not detected in these superficial layers of the KO epidermis (Fig. 1 L and Fig. S5), which is in agreement with its degradation as previously described in newborn KO mice and NS skin (32, 43). In summary, the abnormal differentiation pattern observed in KO graft, together with the desmosomal cleavage, confirmed that the KO grafted skin reproduces all major histological and ultrastructural anomalies seen in NS patients skin (43), thus providing a suitable model to understand the long-term effect of LEKTI deficiency in the skin.

Spink5^{-/-} grafted skin shows a marked inflammatory infiltrate

Skin inflammation is one of the major features of NS, and *Spink5*^{-/-} skin grafts exhibited a strong inflammatory infiltrate. Polynuclear eosinophils were observed in the upper dermis and in the basal epidermal layer in KO skin graft (Fig. 2, A–C). Eosinophilic cells were six times more numerous in KO than in WT dermis (Fig. S1). Toluidine blue coloration revealed the presence of a high number of mast cells in the dermis of KO graft (fourfold increase) compared with WT (Fig. 2, D–F; and Fig. S1). Immunodetection of β 1-tryptase

plasia with papillomatosis (bracket) and spongiosis, nuclei are seen in corneocytes (parakeratosis; arrow), and an inflammatory infiltrate is present in the dermis (asterisks). Pictures are representative of 14 WT and 17 KO independent grafts on nude mice, performed in five independent graft series. (E–J) Immunohistochemistry of epidermal differentiation markers performed on WTg and KOg grafted skin. (K and L) Desmoglein-1, detected by immunohistochemistry in the whole epidermis of WTg, is markedly decreased in the GR of KOg epidermis. (M and N) Desmosomes analyzed by transmission electron microscopy in the GR–SC intercellular space (red arrows). Asymmetrical split desmosomes were observed in KOg epidermis. Pictures are representative of two independent experiments, each including three WTg and three KOg. HF, hair follicle. Bars: (C and D) 29.4 μ m; (E–L) 23.5 μ m; (M and N) 0.1 μ m.

showed that this proinflammatory protease was detected in mast cells in the dermis as well as in the basal and suprabasal layers of the KOg epidermis (Fig. 2 H and Fig. S5). Western blot analysis confirmed overproduction of 34-kD β 1-tryptase monomer in KO grafted skin relative to WT (Fig. 2 I). In addition to the protease hyperactivities at 20 and 30 kD, which has been previously described in embryos (32), casein gel zymography experiments showed a KO graft-specific activity migrating at 34 kD. This activity was inhibited by leupeptin, which is consistent with monomeric β 1-tryptase activity (Fig. S2, B and C). Collectively, these results support mast cells activation and secretion of β 1-tryptase in KO grafted skin contrary to WT. Our results show that KO grafted skin exhibits a massive skin inflammation in athymic nude recipient mice, providing evidence that lack of LEKTI in the epidermis alone is sufficient to induce cutaneous inflammation in the absence of mature T lymphocytes.

***Spink5*^{-/-} grafted epidermis overexpresses proinflammatory mediators**

The proinflammatory cytokine IL-1 β is known to be secreted by keratinocytes after SC cohesion disruption (18). IL-1 β immunostaining was scant and localized at the GR-SC interface in WT epidermal grafts. In the KO epidermis, IL-1 β staining was increased and extended to the basal and suprabasal layers (Fig. 3, A and B; and Fig. S5). Western blot analysis confirmed overexpression of the precursor (31 kD)

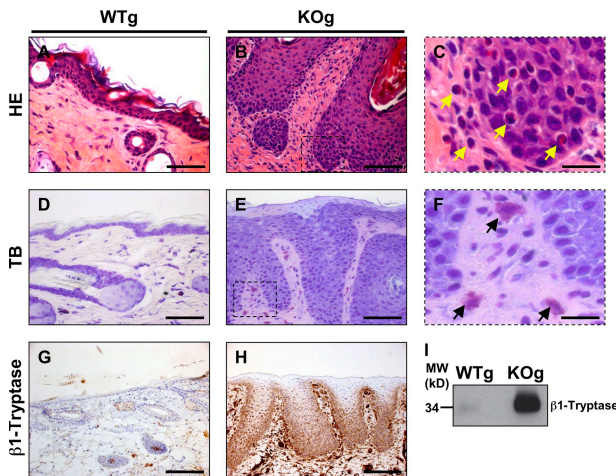


Figure 2. Inflammatory cells infiltrate in *Spink5*^{-/-} grafted skin. (A–C) Hematoxylin/eosin (HE) staining shows a high number of polynuclear eosinophilic cells (yellow arrows) in the upper dermis and the basal layer of KO grafted epidermis (KOg). (D–F) Numerous mast cells are colored by Toluidine blue (TB) in KOg dermis (black arrows) contrary to WTg dermis. (G–I) Immunohistochemistry staining and Western blotting confirms increased production of the 34-kD β 1-tryptase monomer in KOg compared with WTg. Three independent stainings were performed on four WTg and four KOg. Western blotting is representative of two independent experiments, each including two WTg and two KOg. C and F are enlargements of B and E, respectively. Bars: (A and B) 9.4 μ m; (C) 2 μ m; (D and E) 11.75 μ m; (F) 2.5 μ m; (G and H) 23.5 μ m.

and active forms (17 kD) of IL-1 β , which were detected in the KO sample only (Fig. 3 C).

Several studies have identified the proteinase-activated receptor 2 (PAR2) as a major effector of the cutaneous inflammatory response through its proteolytic activation by serine proteases (44–46). PAR2 is expressed in keratinocytes, and we therefore investigated its possible role in the development of the KO graft phenotype by immunohistochemistry analysis. In WT epidermis, PAR2 was restricted to the GR, whereas PAR2 staining was enhanced in the GR and extended to the suprabasal and basal layers in KO grafted epidermis (Fig. 3, D and E; and Fig. S5). The intercellular adhesion molecule 1 (ICAM1), which has been described as a direct target gene of PAR2 activation (47), was restricted to basal cells in the WT epidermis. In the KO grafted epidermis, ICAM1 plasma membrane staining was observed from the basal to the GR (Fig. 3, F and G; and Fig. S5), suggesting PAR2 activation. Increased expression of ICAM1 in KO grafted skin was confirmed by Western blot analysis (Fig. 3 H).

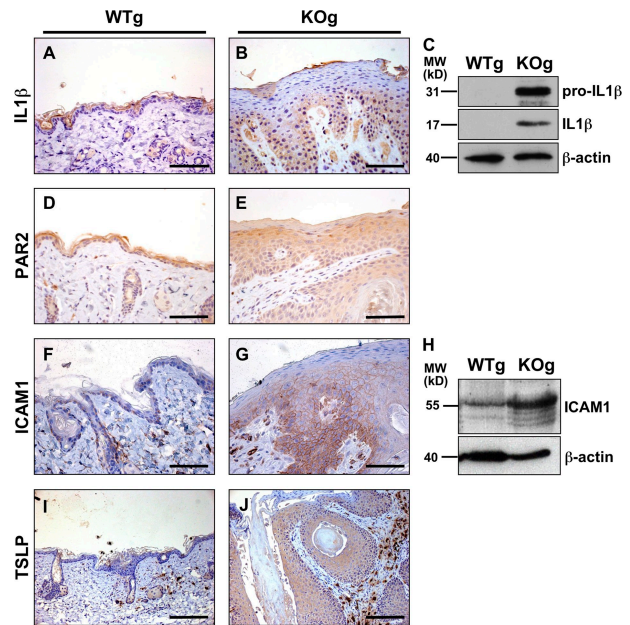


Figure 3. Proinflammatory mediators in *Spink5*^{-/-} graft. (A and B) IL-1 β immunohistochemistry staining is increased in *Spink5*^{-/-} grafted epidermis (KOg) compared with WTg. (C) Western blotting reveals the presence of high amounts of the proform (31 kD) and active form (17 kD) of IL-1 β in KOg epidermis. (D–J) Immunohistochemistry experiments on skin graft cross sections. (D and E) In WTg epidermis, PAR2 is localized in the GR. In KOg, PAR2 staining is increased in the GR and is extended to the basal layer. (F and G) ICAM1 is restricted to the basal cells in the WTg epidermis, whereas in the KOg it is extended to the membrane of suprabasal and GR cells. (H) Western blotting confirms the increase of ICAM1 expression in KOg epidermis. (I and J) TSLP is expressed in all epidermal layers of KOg but is not detected in WTg epidermis. Immunostaining panels and Western blotting are representative of two independent experiments, each including three WTg and three KOg, and two WTg and two KOg, respectively. Bars: (A, B, and D–G) 11.5 μ m; (I and J) 23 μ m.

Recently, the major role of the epithelial cell-derived cytokine TSLP was highlighted in the induction of cutaneous allergic inflammation and in AD epidermis (19), a key feature of NS. We therefore analyzed the expression of this cytokine in the epidermis of grafted skin. TSLP was not detected in WT grafted epidermis, whereas it was highly expressed by KO keratinocytes throughout the entire epidermis (Fig. 3, I and J; and Fig. S5). Positive cells in the dermis of WTg and KOg skin could be unspecific but could also correspond to activated mast cells that are known to express the TSLP receptor (Fig. 2, B and C; and Figs. S1 and S5). These results illustrated that LEKTI-deficient keratinocytes, through the production of proinflammatory mediators and TSLP expression, play a central role in the activation of innate immunity.

Spink5^{-/-} mice epidermis at E19.5 exhibits early proinflammatory signals

To investigate whether the inflammation in KO adult skin was the result of an intrinsic property of *Spink5*^{-/-} epidermis and not secondary to environmental stimuli resulting from a skin barrier defect, we analyzed skin from KO mice at E19.5. Histological analysis of E19.5 KO skin cross section did not reveal any epidermal infiltrate of eosinophilic cells (not depicted), nor did it reveal β 1-tryptase activity in KO embryo (Fig. S2 B), thus excluding the presence of activated mast cells at this stage of development.

IL-1 β immunostaining was faint and diffuse in the superior layers of the WT epidermis (Fig. 4 A and Fig. S5). In the KO epidermis, the staining was more intense and sharp in the area of SC detachment (Fig. 4, B and C; and Fig. S5). RT-PCR analysis confirmed that *Il-1 β* mRNA expression was highly enhanced in the epidermis and the dermis of KO embryos compared with WT (Fig. 4 M).

In addition, we investigated the induction of the proinflammatory cytokine TNF- α , which is also produced in response to cutaneous barrier disruption (17) and tightly associated with inflammatory deviation of the immune response, such as asthma and probably allergic inflammation (48, 49). Despite unsuccessful immunostaining of TNF- α on skin cross sections, we analyzed its transcriptional expression and showed that *Tnf- α* mRNA was strongly overexpressed in the KO epidermis and also slightly induced in the dermis of KO embryos compared with WT (Fig. 4 M).

To assess whether the PAR2 signaling pathway was activated at this stage of development, expression and localization of the receptor and the product of its target gene ICAM1 were studied by immunohistochemistry and RT-PCR analysis. PAR2 was detected in suprabasal layers of WT epidermis (Fig. 4 D and Fig. S5). In KO epidermis, PAR2 staining was increased and juxtannuclear. This subcellular localization is consistent with the presence of intracytoplasmic vesicles and/or Golgi pool-containing PAR2 because of its internalization and/or neosynthesis after its activation by epidermal proteases (50) (Fig. 4, E and F; and Fig. S5). RT-PCR analysis showed that *Par2* mRNA was slightly overexpressed in KO epidermis compared with

WT, whereas no variation was detected between WT and KO dermis (Fig. 4 M).

ICAM1 immunolocalization was restricted to the membrane of basal keratinocytes in WT epidermis, whereas in KO epidermis the protein was also expressed at the cell surface of suprabasal keratinocytes (Fig. 4, G–I; and Fig. S5). Increased immunostaining was also observed in the dermis of KO mice. These results were in accordance with the RT-PCR experiments showing *Icam1* mRNA overexpression in the epidermis and in the dermis of KO embryos (Fig. 4 M). ICAM1 overproduction in KO epidermis was confirmed by Western blotting analysis (Fig. S3).

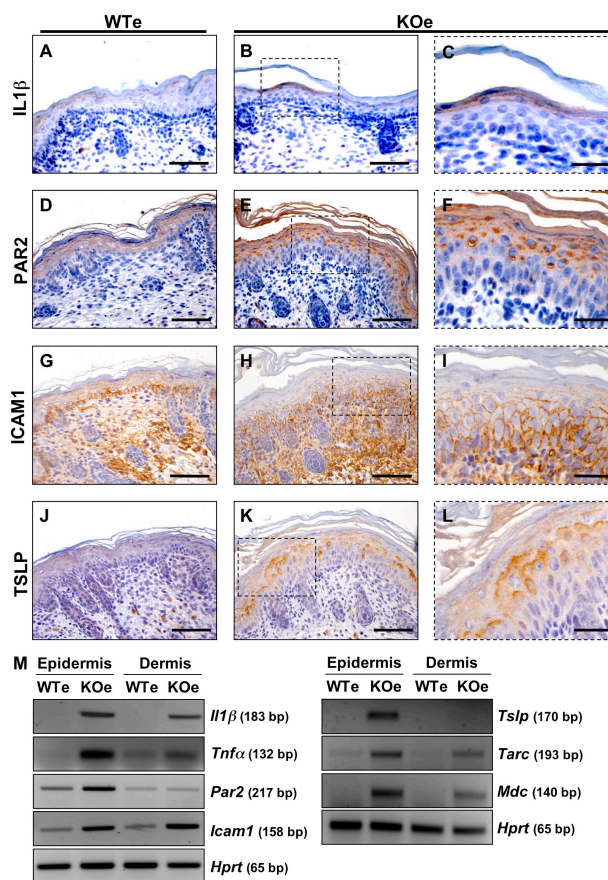


Figure 4. Early inflammatory events in *Spink5*^{-/-} embryos.

(A–L) Immunohistochemistry on E19.5 embryos skin cross sections. (A–C) IL-1 β immunostaining is more intense and sharp in the GR of *Spink5* KO embryo epidermis (KOe) compared with WT embryo (WTe). (D–F) Compared with WTe, PAR2 staining in KOe is increased and juxtannuclear in suprabasal keratinocytes. (G–I) In WTe, ICAM1 is restricted to the basal keratinocytes and to the dermis, whereas it is extended to suprabasal layers in KOe epidermis. (J–L) Contrary to WTe, TSLP staining is detected in the suprabasal and GR of KOe epidermis. Immunostaining panels are representative of two independent experiments, each including three WTe and three KOe. (M) mRNA expression level of *Il-1 β* , *Tnf- α* , *Par2*, *Icam1*, *Tslp*, *Tarc*, *Mdc*, and *Hprt* were measured by quantitative RT-PCR from WTe and KOe epidermis and dermis. Data are representative of two independent experiments in which two WTe and two KOe were analyzed. C, F, I, and L are enlargements of B, E, H, and K, respectively. Bars: (A, B, D, E, G, H, J, and K) 11.5 μ m; (C, F, I, and L) 4.9 μ m.

Finally, TSLP was highly expressed in the KO embryonic epidermis compared with WT (Fig. 4, J–L; and Fig. S5). TSLP immunolocalization appeared to be cytoplasmic and extracellular, suggesting that it was secreted by KO keratinocytes. mRNA analysis confirmed the absence of *Tslp* transcripts in WT skin and a marked overexpression in the epidermis of E19.5 KO mice (Fig. 4 M).

We also analyzed the expression of two pro-Th2 chemokines, *thymus and activation-regulated chemokine* (*Tarc*; *Ccl17*), which was found to be up-regulated in *Tslp*-transgenic mice skin (21), and *macrophage-derived chemokine* (*Mdc*; *Ccl22*). These two chemokines are known to preferentially attract CCR4⁺ Th2 cell (51). In the epidermis and the dermis of E19.5 KO mice, *Tarc* and *Mdc* were overexpressed at the transcriptional level (Fig. 4 M).

These results established that proinflammatory and Th2-polarizing mediators were overexpressed as early as E19.5 in the KO epidermis and could be secondary to PAR2 activation. These in utero events appear to be essential in explaining the inflammatory changes observed in the adult KO skin. These observations in *Spink5*^{-/-} embryos support the notion that LEKTI-deficient epidermis has the intrinsic property of inducing a proinflammatory cascade, even in the absence of immune cell infiltrate and environmental stimuli.

The pro-Th2 cytokine TSLP is overexpressed in the skin of NS patients

TSLP immunodetection on skin cross sections from two healthy controls (healthy 1 and 2) and four NS patients (NS1–NS4) revealed that the pro-Th2 cytokine was overexpressed in the patient skin, in accordance with the result obtained in *Spink5*^{-/-} mice epidermis (Fig. 5). In healthy control skin, no signal was detected in the epidermis (healthy 1 and 2). In NS patient epidermis, a cytoplasmic gradient of TSLP staining was observed from the granular to the spinous layer, the intensity of which was different between individuals (Fig. 5). Some patients also exhibited a dermal staining (NS1 and NS3) that could correspond to immune cell infiltrates (Fig. 5), possibly mast cells, or to unspecific staining of dermal structures as observed in mouse isotype control panel (Fig. S6 A). These results confirmed TSLP overproduction in LEKTI-deficient human skin and showed additional similarity with AD skin that could account for the recurrent atopic manifestations in NS.

KLK5 up-regulates TSLP and proinflammatory mediator expression through PAR2 and NF- κ B signaling in human keratinocytes

We next hypothesized that protease hyperactivity could lead to overexpression of proinflammatory mediators. Indeed, KLK5, but not KLK7, has previously been shown to cleave and activate PAR2 (52). Therefore, the unregulated KLK5 observed in NS and in *Spink5*^{-/-} mice could induce a proinflammatory cascade independently of external stimuli.

We analyzed two target genes of PAR2 activation in cultured keratinocytes, *ICAM1* (47) and the chemokine *IL8* (53). In addition, we assessed whether IL-1 β , TNF- α , and the pro-

Th2 cytokine TSLP and chemokines TARC and MDC, which we showed to be overexpressed in LEKTI-deficient epidermis, could be directly up-regulated by hyperactive KLK5.

Addition of recombinant human KLK5 in the conditioned medium of normal human primary keratinocytes (NHKs) led to an increase of *ICAM1* mRNA (mean of fivefold; Fig. 6 A), in accordance with previously published data, on NHK treated with trypsin (47), suggesting PAR2 cleavage and activation by KLK5 in our in vitro model. In KLK5-treated NHK, *TSLP* mRNA was significantly increased (mean of sevenfold; Fig. 6 A). TSLP overexpression was confirmed by immunocytofluorescence detection after KLK5 treatment and protein secretion blocking with brefeldin A (Fig. S4). Transcripts of the proinflammatory molecules *IL8* and *TNF- α* were increased as well, in contrast to *IL-1 β* , *TARC*, and *MDC* expression, which was not induced by KLK5 (Fig. 6 A). These data emphasized the role of KLK5 in cutaneous inflammation by regulating cytokine and chemokine expression in keratinocytes independently of environmental stimuli, inflammatory cells, and skin barrier defect.

To demonstrate a direct role of PAR2 signaling in up-regulation of these molecules, we knocked down *PAR2* mRNA

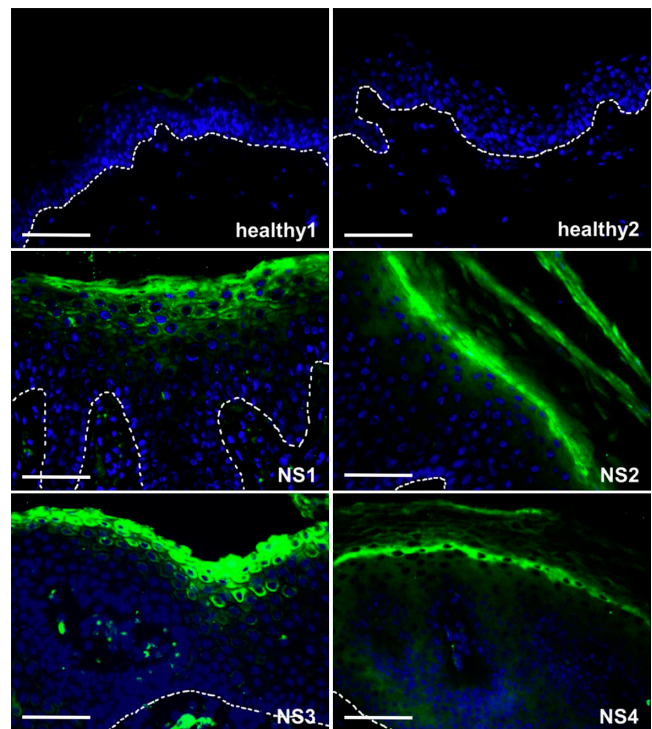


Figure 5. Overexpression of TSLP in skin of NS patients. Immunofluorescence experiments performed on skin cryosections using anti-TSLP antibody (green). TSLP expression is not detectable in two healthy control skins but, in contrast, this cytokine is strongly expressed in the skin of four NS patients (NS1–NS4). The signal is cytoplasmic in the granular keratinocytes and decreases through the spinous layers. The pictures are representative of two independent experiments in which two healthy and four NS patients were included. Dashed white lines represent epidermis/dermis junction. Blue staining (DAPI) indicates nuclei. Bars, 11.5 μ m.

using siRNA in NHK and applied a KLK5 on these cells. The *PAR2* mRNA knockdown was confirmed by quantitative RT-PCR (56% decreased; Fig. 6 B). In these conditions, *ICAM1* stimulation by KLK5 was significantly reduced (mean of 55% induction relative to control siRNA), confirming that the signaling pathway initiated by PAR2 cleavage was affected (Fig. 6 B). KLK5 was also considerably less efficient in inducing *TSLP* mRNA expression (mean of 48% induction relative to control siRNA) as well as *IL8* and *TNF- α* transcripts. Previous studies have shown that up-regulation of *ICAM1* by PAR2 signaling was mediated by NF- κ B pathway (47). Moreover, a functional NF- κ B binding site was previously identified in the *TSLP* promoter in airway epithelial cells (54, 55). We therefore assessed the involvement of NF- κ B signaling in *TSLP*, *IL8*, and *TNF- α* up-regulation by KLK5 using BAY11-7082, a specific inhibitor of the NF- κ B pathway. Pretreatment of NHK with BAY11-7082 decreased the effect of KLK5 on *ICAM1* mRNA expression (mean of 70% decreased; Fig. 6 C), which is in accordance with already published data

(47). Moreover, this treatment also strongly reduced KLK5 efficiency in inducing *TSLP*, *IL8*, and *TNF- α* transcription (mean of 81, 59, and 40% decrease, respectively; Fig. 6 C). Collectively, these in vitro experiments showed that KLK5 can directly induce cutaneous inflammation through PAR2 and NF- κ B pathway activation, leading to up-regulation of *TSLP*, *IL8*, and *TNF- α* mRNAs.

KLK5 hyperactivity induces TSLP overexpression in keratinocytes from NS patients

Positive staining of TSLP in NS skin (Fig. 5), together with in vitro data, supports the hypothesis that, in LEKTI-deficient keratinocytes, unregulated proteases can directly induce cytokine and chemokine secretion, including TSLP. To rule out indirect effects of epidermal barrier dysfunction and immune cells on TSLP expression, keratinocytes from three different NS patients (NSK) were cultivated under the same experimental conditions as NHK from three different healthy controls. *TSLP* mRNA quantification revealed that expression of this cytokine was significantly enhanced in NSK compared with NHK in baseline conditions in vitro (mean of fivefold; Fig. 7 A). *ICAM1*, *IL8*, and *TNF- α* mRNAs were also increased in NSK in comparison to NHK, although no variation was observed for IL-1 β (Fig. 7 A). Expression of *TARC* and *MDC* mRNAs was also enhanced in NSK compared with NHK (Fig. 7 A), even though these two genes encoding chemokines may not be direct target genes in the KLK5–PAR2 pathway (Fig. 6 A).

Except for *TSLP*, the other proinflammatory and pro-Th2 molecules were overexpressed in two out of three NSK compared with NHK (Fig. 7 A). This could reflect interindividual variations caused by multiple transcriptional regulations in a LEKTI-deficient context.

Immunofluorescence experiments on keratinocytes treated with brefeldin A confirmed that TSLP was produced at high level in NSK compared with NHK (Fig. 7 B). All these results confirm that production of TSLP arise from an intrinsic property of *SPINK5*^{-/-} cells.

To confirm that unregulated KLK5 secreted by keratinocytes could be directly responsible for TSLP and proinflammatory molecule overexpression, NSKs were transfected with siRNA against KLK5. Down-expression of *KLK5* mRNA and decreased synthesis of the protein were verified by quantitative RT-PCR in NSK (Fig. 7 C) and Western blot analysis on the conditioned medium and were quantified by ELISA assay (Fig. 7 D). The amount of the protease in the conditioned medium of NSK transfected with KLK5 siRNA was significantly decreased compared with control siRNA at 48 h (26 vs. 46 pg/ μ l) and 72 h (118 vs. 382 pg/ μ l). Concomitantly, *TSLP* expression was reduced by 42 and 54% at 48 and 72 h after transfection, respectively (Fig. 7 E). However, down-regulation of KLK5 in NSK was associated with a less efficient decrease of *IL8* and *TNF- α* mRNAs and had little effect on *ICAM1* mRNA expression (Fig. 7 E). The residual amount of *TSLP* mRNA and the weak effect on *ICAM1*, *IL8*, and *TNF- α* transcripts observed in NSK transfected with KLK5 siRNA could be the result of uncompleted knockdown

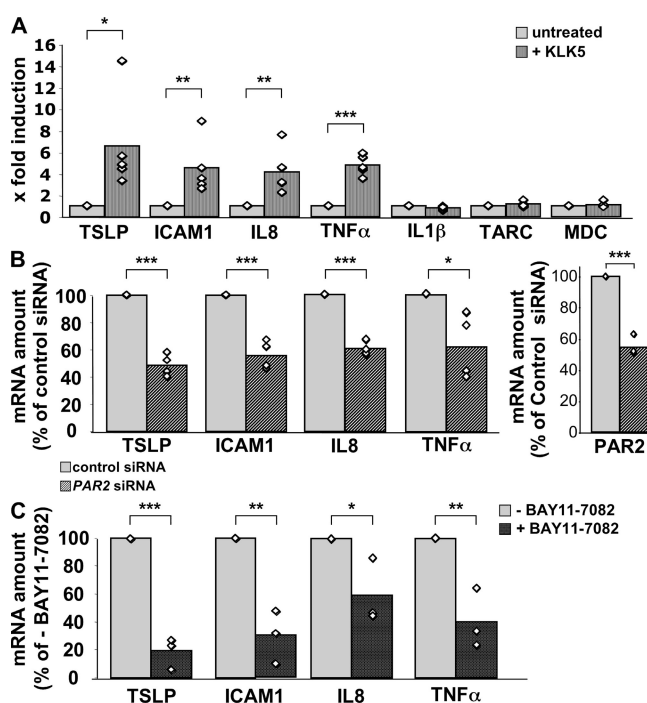


Figure 6. KLK5 up-regulates TSLP and proinflammatory molecules in keratinocytes. (A) NHKs were stimulated with 400 nM of recombinant KLK5 for 3 h. *TSLP*, *ICAM1*, *IL8*, and *TNF- α* transcripts are increased by seven-, five-, four-, and fivefold, respectively. No difference was observed for *IL-1 β* , *TARC*, and *MDC* ($n = 5$). (B) NHK transfected with 60 pmol *PAR2* siRNA and stimulated with KLK5 showed *PAR2* mRNA down-regulation (by 56%) 4 d after transfection. In *PAR2* siRNA-transfected cells, KLK5 is less efficient in inducing *TSLP*, *ICAM1*, *IL8*, and *TNF- α* mRNA (48, 55, 60, and 62% of control, respectively; $n = 4$). (C) In cells treated with 10 μ M NF- κ B pathway inhibitor (BAY11-7082), KLK5 efficiency to up-regulate *TSLP*, *ICAM1*, *IL8*, and *TNF- α* mRNA is decreased by 81, 70, 59, and 40%, respectively ($n = 3$). For each experiment, the mRNAs of interest were measured by quantitative RT-PCR. Each point is the mean of triplicate amplification for at least three independent experiments (n). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of *KLK5* mRNA but could also be the result of the activation of PAR2 by other hyperactivated proteases, such as *KLK14* (52) and/or other signaling pathway induction.

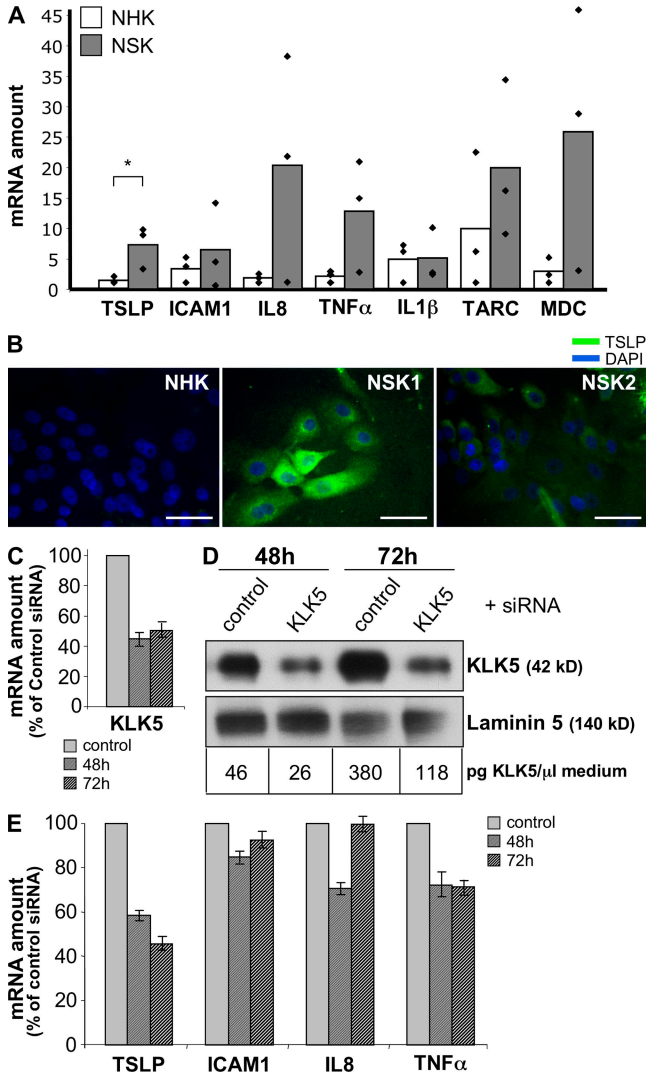


Figure 7. TSLP and proinflammatory molecules are induced by KLK5 in keratinocytes of NS patients. (A) Quantitative RT-PCR on cultured primary keratinocytes reveals that *TSLP* is significantly overexpressed by fivefold in three NS patients (NSK) compared with three healthy controls (NHK). *ICAM1*, *IL8*, *TNF- α* , *TARC*, and *MDC* mRNA are overexpressed in two out of three NSKs but not *IL-1 β* mRNA. Each point represents the mean of two independent experiments for each individual. *, $P < 0.05$. (B) NHK and NSK were treated with 10 μ g/ml brefeldin A, and TSLP was detected by immunofluorescence (green). Blue staining is DAPI. Pictures are representative of two independent brefeldin A treatments, each realized on two NHKs and two NSKs. (C and D) NSKs were transfected with KLK5 siRNA and its efficient down-regulation was confirmed by quantitative RT-PCR, Western blotting (laminin 5 used as loading control), and ELISA, 48 and 72 h after transfection. Results are representative of three independent experiments. (E) KLK5 knockdown induces a decrease of *TSLP* measured by quantitative RT-PCR. *ICAM1*, *IL8*, and *TNF- α* mRNAs were less efficiently reduced. Data are the mean \pm SD of one experiment, which is representative of three independent experiments. Bars, 49 μ m

These results highlight for the first time KLK5's direct role in TSLP up-regulation in NSK, which is independent of external stimuli including environment, microorganism penetration, or immune cell activation. KLK5 also induced the production of other inflammatory molecules that probably maintain and aggravate the proinflammatory microenvironment in the affected skin and favor a pro-Th2 microenvironment.

DISCUSSION

The recurrent atopic manifestations, which are constantly associated with NS, are generally regarded as resulting from premature SC detachment facilitating allergen penetration and inducing stress signal on keratinocytes. In this paper, we provide evidence that the epidermis plays a major and direct role in the modulation of the immune response. We show that in baseline culture conditions, keratinocytes from NS patients secrete major proinflammatory (*ICAM1*, *IL8*, and *TNF- α*) and Th2-polarizing (*TSLP*, *TARC*, and *MDC*) mediators in the absence of any external stimulation, thus excluding any effect of skin barrier disruption and/or pathogen and allergen penetration. We demonstrate for the first time the direct role of KLK5 hyperactivity in initiating a proallergic signaling cascade through PAR2 receptor cleavage and NF- κ B pathway activation, which results in *ICAM1*, *IL8*, *TNF- α* , and *TSLP* overexpression in keratinocytes (Fig. 8). This study disclosed a new role for KLK5 in skin inflammation and in the induction of allergic inflammatory actors. Therefore, KLK5 represents a major epidermal specific protagonist, which initiates TSLP overexpression without contribution of the adaptive immune system. In addition, the pro-Th2 mediators TARC and MDC were overexpressed in keratinocytes from NS patients independently of KLK5 and PAR2 activation. This up-regulation could be promoted by other uncontrolled proteases, such as KLK7 or KLK14 and/or secreted factors in the medium, sustaining the induction of multiple signaling pathways in LEKTI-deficient skin. These results provide evidence that the activation of this biological cascade leading to cutaneous inflammation in NS is an intrinsic property of LEKTI-deficient keratinocytes, i.e., is a cell-autonomous process.

Analysis of *Spink5* KO epidermis in E19.5 embryos revealed that this KLK5 biological cascade is an early event and, consequently, is relevant in vivo. In *Spink5*^{-/-} skin, no inflammatory infiltrate could be observed in WT and KO epidermis, whereas the proinflammatory (*Icam1*, *Il-1 β* , and *Tnf- α*) and pro-Th2 (*Tslp*, *Mdc*, and *Tarc*) molecules were already overproduced (Fig. 4). In addition, these molecules were induced in embryos that had been delivered by caesarian from gestate females maintained in a pathogen-free environment. That supports a major and direct role of KO keratinocytes in sensing and promoting inflammatory signal rather than the absorption of allergen by epidermal LC precursors (56), which in this condition will probably not encounter foreign particles.

Interestingly, we showed that the intracellular pool of PAR2 was increased in the more differentiated layers of the E19.5 KO epidermis and colocalized with TSLP and hyperactive KLK5 in the upper layers of the KO epidermis (28, 43).

Therefore, these early events observed in utero confirmed that deregulated KLK5 in the upper GR layers initiates the proinflammatory response through PAR2 cleavage and TSLP production in vivo.

IL-1 β was also increased in the epidermis of *Spink5*^{-/-} embryos in contrast to primary keratinocytes from NS patients in culture. Nonetheless, IL-1 β overexpression in *Spink5*^{-/-} embryos epidermis is likely to be a consequence of SC detachment that is already present before birth (17). IL-1 β can be proteolytically activated by KLK7 (57), which is hyperactivated in *Spink5*^{-/-} skin (32). Thus, this protease could participate in the inflammatory process in our model as observed in KLK7 transgenic mice (58).

These proinflammatory mediators already in place at a late developmental stage (E19.5) in the epidermis of KO embryos could contribute to the amplification of the proallergic microenvironment. In a context of immunocompetent mice, TARC and MDC could lead to the recruitment of Th2 CCR4⁺ cells at the site of inflammation (51). In addition, IL-1 β and TNF- α overexpression in *Spink5*^{-/-} epidermis may induce TSLP expression in keratinocytes (19, 54) and attract innate immune cells. These processes could explain

worsening of the cutaneous phenotype observed in LEKTI-deficient adult skin.

The *Spink5*^{-/-} skin grafts exhibit a marked inflammatory infiltrate in the absence of T cell-mediated immunity. This inflammatory infiltrate was essentially composed of polynuclear eosinophilic cells and activated mast cells that are usually present in allergic response. Interestingly, these features were associated neither with infection nor with the presence of mature T cells, as skin grafts were performed on athymic nude mice. It is of interest to note that elimination of T lymphocytes in *K5-Tslp* mice by crossing with TCR- β -deficient mice does not affect eosinophilic and mast cell skin infiltration (21), as observed in *Spink5* KO grafted skin. These data support the notion that, in our model, TSLP-mediated skin inflammation mainly involves the innate immune system and that this cytokine may directly recruit and activate mast cells that express TSLP receptor (59). TSLP, synergistically with IL-1 β and TNF- α , stimulates the production of pro-Th2 cytokines by activated mast cells (59). Massive production of active β 1-tryptase by activated mast cells in *Spink5*^{-/-} grafted skin could also directly participate in the inflammatory response and favor a Th2-permissive microenvironment

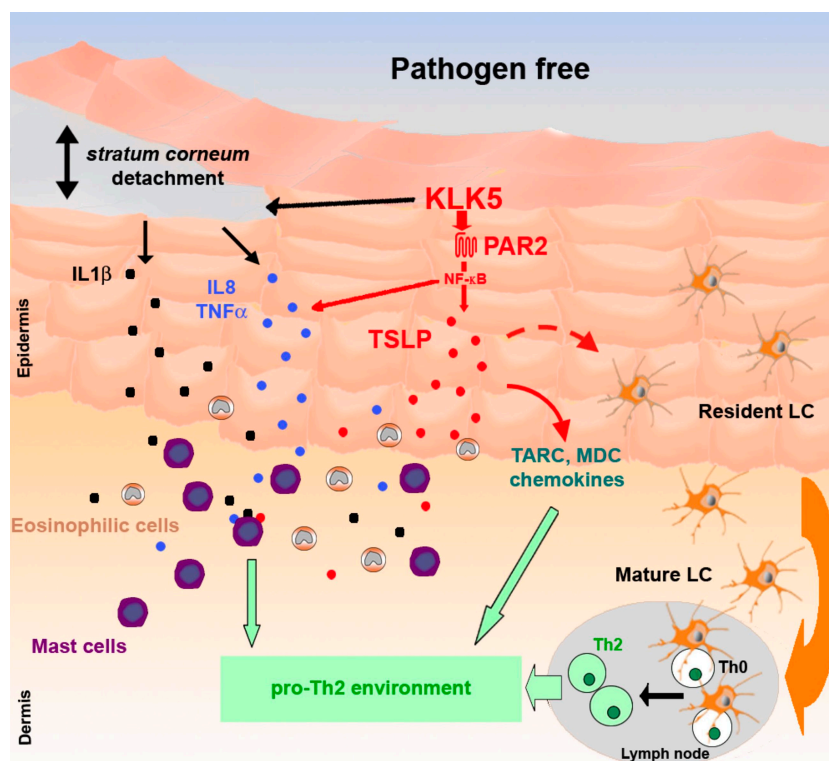


Figure 8. KLK5 triggers proinflammatory and proallergic microenvironment independently of external stimuli. Lack of KLK5 inhibition by LEKTI initiates proinflammatory and proallergic cascades independently of environmental factors. In LEKTI-deficient keratinocytes, hyperactive KLK5 directly induces TSLP, IL8, and TNF- α overexpression through PAR2 and NF- κ B pathway activation. Unregulated KLK5 also degrades desmosomes at the interface between the GR and the SC leading to defective SC adhesion and IL-1 β , IL8, and TNF- α secretion by mechanically stressed keratinocytes. In addition, all these cytokines could induce TARC and MDC chemokines secretion by keratinocytes and dermal fibroblasts. These proinflammatory mediators trigger eosinophilic and mast cell recruitment and activation. TSLP has been reported to activate resident LCs, which migrate to draining lymph nodes and promote the differentiation of naive T cells (Th0) into Th2 cells. Collectively, activated keratinocytes together with eosinophilic and mast cells induce pro-Th2 microenvironment favoring the development of an AD-like phenotype.

through PAR2 activation on mast cells (60), dendritic cells (61), and eosinophils (62, 63). In addition, in an immune-competent system, TSLP has the potential to activate dendritic cells and epidermal LCs in particular, triggering the generation of proallergic CD4⁺ Th2 cells (19, 20, 64).

Our present results established that *Spink5*^{-/-} epidermis by itself induces a cutaneous inflammatory response and the synthesis of proallergic mediators. This new mechanism could account for atopic manifestations in NS patients and is concordant with TSLP overproduction in the epidermis of NS patients (Fig. 5) who display KLK5 and PAR2 deregulation (43). Thus, TSLP provides an important link between the absence of LEKTI protease inhibitor and the AD-like phenotype constantly seen in NS patients. This notion supports the possibility that *SPINK5* could play a role in atopic disease. Several studies have reported significant genetic association of *SPINK5* polymorphisms with AD in different populations (34–38), whereas other studies did not confirm these results (39–41). This suggests that *SPINK5* genetic variations may account, to some extent, for predisposition to AD in some patients, depending on other genetic and/or environmental factors.

Therefore, expression of TSLP, TARC, and MDC by LEKTI-deficient keratinocytes could initiate and amplify the skin atopic inflammation as well as the systemic allergic response of NS patients. The abundant and epidermally restricted TSLP secretion could induce an AD-like phenotype associated with elevated serum levels of IgE, as illustrated in *Tslp* skin-specific transgenic mice (21, 65). Thereby, the effect of LEKTI deficiency on triggering PAR2 signaling could be a primary event inducing atopic manifestations in NS before skin barrier defect. In parallel, as the result of protease hyperactivities, FIL-AGGRIN is also decreased (agranulosis) and the SC is often detached from the underlying cell layers in the epidermis of NS patients (43). Both anomalies impair skin barrier and favor allergen and pathogen penetration in NS epidermis, thus contributing to the aggravation of the atopic phenotype.

These results identify KLK5 as a major actor in NS because up-regulation of this protease has a direct effect on pro-inflammatory signaling and on skin barrier disruption, both of which contribute to the development of atopic manifestations in NS. KLK5 and all the highlighted actors in this study constitute attractive candidates for therapeutic intervention, which are upstream from the IgE response and independent of allergen exposure. These new approaches based on blocking this biological cascade initiated in the epidermis could improve not only the allergic manifestations but also the skin barrier defect of NS patients. In addition, the results point out the importance of the control of protease/protease inhibitor balance in skin barrier function and in immune response orientation.

An emerging hypothesis regarding asthma and AD is that they are epithelial diseases initiated by the epithelial cells themselves through the production of TSLP (66). In the present study, we have identified a mechanism leading to the production of the major pro-Th2 cytokine TSLP by skin epithelial cells, despite the absence of microbe products or physical in-

jury. This new physiological pathway could also explain why the eczematous skin lesions in infants often start in the absence of specific IgE antibodies, indicating that IgE sensitization may occur secondary to eczema. This new mechanism, which is based on the control of epidermal serine protease activity, could be considered to be additional genetic regulation involved in AD.

MATERIALS AND METHODS

Skin sample and dermis/epidermis separation technique. This work was approved by the Commission de Génie Génétique (23 November 2003, agreement number 3987) and by the Local Ethical Committee (DEC 04005). All experiments were done in accordance with the relevant guidelines and regulations. Mice embryos were delivered by caesarean at E19.5.

For RNA analysis, dermis/epidermis separation was performed by incubating total dorsal skin into phosphate buffer saline containing 2.4 mg/ml dispase II (Roche) overnight at 4°C. For protein analysis, skin was incubated in PBS at 56°C for 45 min.

Skin grafting. Total dorsal skin from E19.5 mice was transplanted onto nude mice using the skin flap technique (67) and analyzed after 8 wk.

Histological and immunohistochemical analysis. Skin samples were fixed in 10% formalin and embedded in paraffin or directly frozen at -80°C in Tissue-Tek O.C.T. (Sakura). 4- μ m sagittal sections were stained with hematoxylin/eosin or Toluidine blue for histological examination.

For antigen retrieval, paraffin-embedded cross sections were boiled in 1 mM of citrate buffer, pH 6, for 20 min or treated with S1700 solution (Dako) for 45 min for Desmoglein-1 immunodetection. Skin cryosections were fixed with acetone for 10 min. Immunodetection was performed using appropriate horseradish peroxidase (HRP; diaminobenzidine)-conjugated polymers (EnVision system; Dako) or Alexa Fluor 488-coupled secondary antibody (Invitrogen).

Each NS patient was a homozygote or a compound heterozygote for *SPINK5* mutations, leading to the absence of detectable LEKTI on immunohistochemical analysis of skin sections as previously described (27). Informed consent was obtained from the patients. This study was approved by the medical ethical committee of the Purpan Hospital in Toulouse. The study was conducted in compliance with the ethical principles of the Declaration of Helsinki.

Transmission electron microscopy analysis. WT and *Spink5*^{-/-} grafted skin samples were prepared as described previously (32).

Western blotting. Epidermises were crushed in protein extraction buffer (150 mM NaCl, 50 mM Tris HCl, pH 8, 5 mM EDTA, pH 8, 0.1% Nonidet-P40, and protease inhibitor cocktail tablets [Complete; Roche]) with Ultra-Turrax. Equal amounts of soluble protein were added to Laemmli buffer (62.5 mM Tris HCl, pH 6.8, 5% β -mercaptoethanol, 2% SDS, 10% glycerol, and 0.002% bromophenol blue). Samples were separated by SDS-PAGE and transferred onto Hybond-C extra membrane (GE Healthcare). For mouse β 1-tryptase detection, samples in acetic acid buffer were used. Total amount of protein was quantified by Bradford assay before gel migration and then by coomassie blue staining of SDS-PAGE gel and measurement of the intensity of every band with Image software (version 1.63; National Institutes of Health; Fig. S2). To quantify secreted KLK5 and Laminin 5, 20 μ l of keratinocyte conditioned medium was used.

Quantitative real-time PCR. Total RNA from mouse dermis and epidermis and human keratinocytes, were isolated using the RNeasy mini kit (QIAGEN). Complementary DNA synthesis from 2–3 μ g of total RNA was performed using SuperScript III First-Strand Synthesis system (Invitrogen).

Quantitative real-time PCR was realized on an ABI prism 7000 Sequence Detection system (Applied Biosystems) using qPCR MasterMix Plus SYBR

Green I kit (Eurogentec). Results were normalized with the *HPRT* (human or mouse) gene and analyzed using Sequence Detection System version 1.2 (Applied Biosystems). P-values were calculated using the Student-Fischer test.

Human keratinocyte culture and siRNA transfection. NHKs were seeded on 6-well plates and grown in half EpiLife 0.06 mM CaCl₂ (Invitrogen) and half Green medium (68) until confluency. NHK were treated with 400 nM KLK5 (1 U trypsin-like equivalent) in serum-free Green medium for 3 h. For the NF-κB pathway inhibition test, cells were treated 1 h before activation with 10 μM (E) 3-[(4-methylphenyl) sulfonyl]-2-propenenitrile (BAY11-7082; EMD). For siRNA transfection, NHK or primary keratinocytes from NS patients (NSK) were grown until 60% confluency. Cells were transfected with 60 pmol of human *PAR2* siRNA or 20 pmol of human *KLK5* siRNA (Santa Cruz Biotechnology, Inc.) using the transfection reagent jetSI-ENDO (Ozyme) in serum-free and antibiotic-free EpiLife. Control siRNA fluorescein conjugate (Santa Cruz Biotechnology, Inc.) was used as described by the manufacturer. 5 d after human *PAR2* siRNA transfection, NHKs were treated with KLK5.

Immunocytofluorescence. NHK and NSK were seeded on 400-mm² slides. Cells were incubated with 10 μg/ml brefeldin A (eBioscience) for 3 h, fixed in 4% formaldehyde solution, and incubated overnight with human TSLP antibody at 4°C. Signal was detected with an FITC-conjugated secondary antibody (Dako) and nuclei were stained with DAPI (Vector Laboratories). Fluorescence signal was visualized with an inverted high-end microscope (Axiovert 200; Carl Zeiss, Inc.).

Immunofluorometric assay (ELISA) for human KLK5. MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated with 1 μg per well of Fc fragment-specific goat polyclonal anti-mouse IgG (Interchim). Saturation was performed with a solution containing 5% BSA and 5% sucrose. Mouse monoclonal anti-human KLK5 antibody (R&D systems) in 1% BSA, 0.05% Tween 20, and PBS was added to each well and incubated overnight at 4°C. 50 μl of undiluted or diluted cell-conditioned medium were incubated for 2 h at room temperature. KLK5 detection was performed using a biotinylated polyclonal goat anti-human KLK5 antibody (BAF1108; R&D systems). Signal was revealed using HRP-coupled streptavidin (N-100; Endogen; Interchim) and UptiLight HS ELISA HRP substrate (Interchim). Luminescence was recorded with Mithras (Berthold Technologies). Recombinant human KLK5 (R&D system) was used as standard range.

Online supplemental material. Table S1 provides information about primary antibodies and control antibodies used. Table S2 indicates primer sequences for mouse and human complementary DNA PCR amplification. Fig. S1 is a graphic representation of the increased number of eosinophilic and mast cells in the dermis of KO grafted skin. Fig. S2 shows a loading control for β1-tryptase Western blotting, β1-tryptase hyperactivity, and its inhibition by leupeptin in KO grafted skin by casein gel zymography. Fig. S3 shows increased expression of ICAM1 protein in E19.5 KO epidermis by Western blot analysis. Fig. S4 confirms by immunocytofluorescence that KLK5 treatment induces TSLP protein expression in NHK. Fig. S5 shows negative staining with rabbit and goat control antibodies on mouse skin sections. Fig. S6 shows negative staining with mouse isotype control and rabbit control antibody on human skin sections and cultured keratinocytes. A supplemental materials and methods is also provided for the casein gel zymography experiment. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20082242/DC1>.

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