

Par2 Inactivation Inhibits Early Production of TSLP, but Not Cutaneous Inflammation, in Netherton Syndrome Adult Mouse Model

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Netherton syndrome (NS) is a severe genodermatosis characterized by abnormal scaling and constant atopic manifestations. NS is caused by mutations in *SPINK5* (Serine Protease INhibitor Kazal-type 5), which encodes LEKTI (LymphoEpithelial Kazal Type-related Inhibitor). Lack of LEKTI causes stratum corneum detachment secondary to epidermal proteases hyperactivity. Whereas a skin barrier defect is generally regarded as a major cause for atopy, we previously identified a cell-autonomous signaling cascade that triggers pro-Th2 cytokine thymic stromal lymphopoietin (TSLP) production in LEKTI-deficient epidermis. This signaling is initiated by unrestricted kallikrein 5 (KLK5) activity, which directly activates proteinase-activated receptor 2 (PAR2)-mediated expression of TSLP and favors a cutaneous proallergic microenvironment independently of the environment and of the adaptive immune system. To further confirm these results *in vivo*, we generated *Spink5/Par2* double knockout (DKO) mice. At embryonic day 19.5, these mice display a dramatic decrease in TSLP expression, although stratum corneum detachment persists, confirming the role of the KLK5–PAR2 cascade in TSLP-mediated early proallergic signaling. However, deletion of *Par2* in adult DKO-grafted skin does not rescue the inflammatory phenotype probably resulting from stratum corneum detachment. We conclude that several mechanisms trigger and maintain the inflammatory phenotype in NS. These include skin barrier impairment, mechanical stress secondary to stratum corneum detachment, as well as protease-induced proinflammatory and proallergic pathways, including PAR2-mediated overexpression of TSLP.

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INTRODUCTION

The epidermis is a pluristratified epithelium, mainly composed of keratinocytes, which provides the first barrier protecting the body against penetration of harsh environmental agents. Skin injuries, danger signals, and epidermal barrier dysfunction lead to complex mechanisms triggering activation of keratinocytes, which produce a wide range of proinflammatory molecules (Nickoloff and Naidu, 1994). Loss of this skin barrier function is believed to favor the development of atopic dermatitis (AD) by facilitating allergen

penetration, as illustrated by patients with eczema due to null mutations in the filaggrin gene (O'Regan *et al.*, 2008), and by the spontaneous mice mutant bearing a stop mutation in the murin *Flg* gene, which develop severe dermatitis after exposition to experimental allergen (Fallon *et al.*, 2009).

Netherton syndrome (NS, OMIM 256500) is a rare and severe inherited skin disorder characterized by congenital scaly erythroderma, a specific hair shaft defect (*Trichorrhexis invaginata*), and constant atopic manifestations (Comel, 1949; Netherton, 1958). Allergic manifestations in NS include “atopic dermatitis”-like lesions associated with hyperimmunoglobulinemia E, allergic rhinitis, and food allergies (Traupe, 1989; Judge *et al.*, 1994). This is in sharp contrast with other ichthyoses, which exhibit skin barrier impairment but do not develop constant atopic manifestations. This observation suggests that specific mechanisms in NS underlie an allergic response. We previously showed that NS is caused by mutations in *SPINK5* (Serine Protease INhibitor Kazal-type 5), encoding the serine protease inhibitor LEKTI (LymphoEpithelial Kazal Type-related Inhibitor) (Chavanas *et al.*, 2000). LEKTI tissue distribution pattern is restricted to the most differentiated viable layers of stratified epithelial tissues and the thymic Hassall's corpuscles (Bitoun *et al.*, 2003). In the epidermis, LEKTI is

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Abbreviations: AD, atopic dermatitis; DKO, double knockout; KLK5, kallikrein 5; LEKTI, LymphoEpithelial Kazal Type-related Inhibitor; NS, Netherton syndrome; PAR2, proteinase-activated receptor 2; SPINK5, Serine Protease INhibitor Kazal-type 5; TNF α , tumor necrosis factor alpha; TSLP, thymic stromal lymphopoietin; WT, wild type

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mainly restricted to the granular layer where it is expressed as high-molecular-weight precursors, which are rapidly processed into several proteolytic fragments secreted in the intercellular space (Bitoun *et al.*, 2003; Deraison *et al.*, 2007). We along with others have shown that LEKTI fragments can efficiently and specifically inhibit epidermal kallikrein (KLK)5, KLK7, and KLK14 (Egelrud *et al.*, 2005; Schechter *et al.*, 2005; Deraison *et al.*, 2007).

Spink5 knockout mice (*Spink5*^{-/-}) faithfully reproduced key features of NS (Yang *et al.*, 2004; Descargues *et al.*, 2005; Hewett *et al.*, 2005), and provided good models to gain insights into the cutaneous pathophysiological pathways of the disease. We showed that epidermal LEKTI deficiency results in KLK5, KLK7, and elastase 2 (ELA2) unrestricted protease activity, leading to premature desmosome cleavage and abnormal filaggrin maturation in the upper granular layer (Descargues *et al.*, 2005; Bonnart *et al.*, 2010). This induces accelerated stratum corneum shedding and impaired epidermal barrier function (Descargues *et al.*, 2005; Bonnart *et al.*, 2010). More recently, we demonstrated that unrestricted KLK5 activity also directly triggers the expression of proinflammatory (tumor necrosis factor alpha (TNF α), ICAM1, and IL8) and proallergic (thymic stromal lymphopoietin, TSLP) molecules through proteinase-activated receptor 2 (PAR2) activation in NS keratinocytes (Briot *et al.*, 2009). The pro-Th2 cytokine TSLP is a major actor of allergic inflammation, overexpressed in the AD lesional skin (Soumelis *et al.*, 2002). TSLP is necessary and sufficient to induce eczematous lesions and a systemic Th2 response, in the absence of exogenous allergen (Yoo *et al.*, 2005). We have shown that TSLP induction, already present in *Spink5*^{-/-} embryos, is a cell-autonomous process, which is likely to have a key role in the development of AD-like lesions in NS.

To extend these results *in vivo*, we generated mice inactivated for both *Spink5* and *Par2* genes. This double knockout (DKO) mouse model allowed us to identify PAR2 as an essential actor of TSLP expression early in life, although *Par2* deletion is not sufficient to prevent inflammation in the adult skin where other mechanisms such as stratum corneum detachment could contribute to the inflammatory process.

RESULTS

Spink5/Par2 DKO newborn mice display cutaneous features similar to *Spink5*^{-/-} animals

To further explore the implication of the KLK5–PAR2 pathway in the *Spink5*^{-/-} phenotype, we generated DKO mice for both *Spink5* and *Par2* genes. We crossed heterozygous *Spink5*^{+/-} mice with *Par2*^{-/-} mice (Lindner *et al.*, 2000) and then intercrossed viable *Spink5*^{+/-}/*Par2*^{-/-} animals to obtain DKO mice. DKO newborn mice exhibited skin erosions and vibrissae abnormalities as seen in *Spink5*^{-/-} mice and underwent rapid postnatal lethality due to severe dehydration consequently to stratum corneum detachment (Figure 1a). These results suggested that *Par2* deletion, in a *Spink5*^{-/-} context, does not abrogate the severe cutaneous phenotype of *Spink5*^{-/-} newborn mice.

Par2 deficiency represses TSLP-induced expression in *Spink5*^{-/-} embryos

We next analyzed the skin from embryos delivered by Caesarean section at embryonic day 19.5 (E19.5) to disregard the effect of the environment on disease expression. Histological examination of skin cross-sections after hematoxylin–eosin coloration showed stratum corneum detachment and moderate acanthosis in both *Spink5*^{-/-} and DKO embryos. In contrast, the *Par2*^{-/-} skin appeared similar to the wild-type (WT) skin (Figure 1b). These observations suggested that the lack of PAR2 does not abolish epidermal protease hyperactivity leading to stratum corneum detachment and confirmed the severe cutaneous features observed in *Spink5*^{-/-} newborns (Figure 1a). We nevertheless explored the effect of PAR2 inactivation on TSLP and ICAM1 expression, which we previously showed to be induced by KLK5–PAR2 signaling as early as E19.5 in the context of *Spink5* deficiency (Briot *et al.*, 2009). We confirmed that TSLP was not detected in the WT or in the *Par2*^{-/-} epidermis, whereas it was strongly produced in the epidermis of skin sections from *Spink5*^{-/-} embryos (Figure 1c). In striking contrast, TSLP staining was poorly detected in the DKO epidermis (Figure 1c). Quantitative real-time-PCR analyses of the skin from E19.5 embryos showed that *Tslp* transcripts were highly overexpressed in the *Spink5*^{-/-} skin as observed previously (Briot *et al.*, 2009) and could be compared with an on/off induction relative to the WT mice skin. In the DKO skin, induction of *Tslp* mRNA reached only 10% of that observed in the *Spink5*^{-/-} skin (Figure 1e), confirming the immunohistological observation. ICAM1 staining, restricted to the basal layer in the WT and *Par2*^{-/-} skin, was extended to suprabasal layers in the *Spink5*^{-/-} and DKO epidermis (Figure 1d). Quantitative real-time-PCR confirmed that *Icam1* mRNA amount was similar between the *Spink5*^{-/-} and DKO embryo skin (data not shown). Finally, we analyzed transcriptional expression levels of *Tnf α* , which we previously identified as another target gene of KLK5–PAR2 signaling (Briot *et al.*, 2009). *Tnf α* mRNA increase in DKO was 60% of that seen in *Spink5*^{-/-} (15.8- and 26.6-fold induction, respectively, relative to WT) (Figure 1e). Taken together, these results demonstrate that, in the NS mouse model, TSLP expression is highly dependent on the KLK5–PAR2 pathway *in vivo* as early as E19.5.

Par2 inactivation does not improve cutaneous inflammation in the *Spink5*^{-/-} adult skin

To determine whether inhibition of the KLK5–PAR2 cascade is sufficient to reverse the inflammatory phenotype previously observed in the *Spink5*^{-/-} adult skin (Briot *et al.*, 2009), we grafted the E19.5 skin onto athymic immunodeficient nude mice. Six weeks after grafting, WT and *Par2*^{-/-} grafts were covered with normal hair, whereas *Spink5*^{-/-} and DKO-grafted skin appeared erythematous, scaly, and showed no hair (Figure 2a). Histological examination showed that both the *Spink5*^{-/-} and the DKO-grafted skin exhibit acanthosis with papillomatosis, hyperkeratosis (thickening of the stratum corneum), and parakeratosis (persistence of the nuclei in the stratum corneum) (Figure 2b). Luna staining of skin graft cross-sections showed rare polynuclear eosinophilic cells,

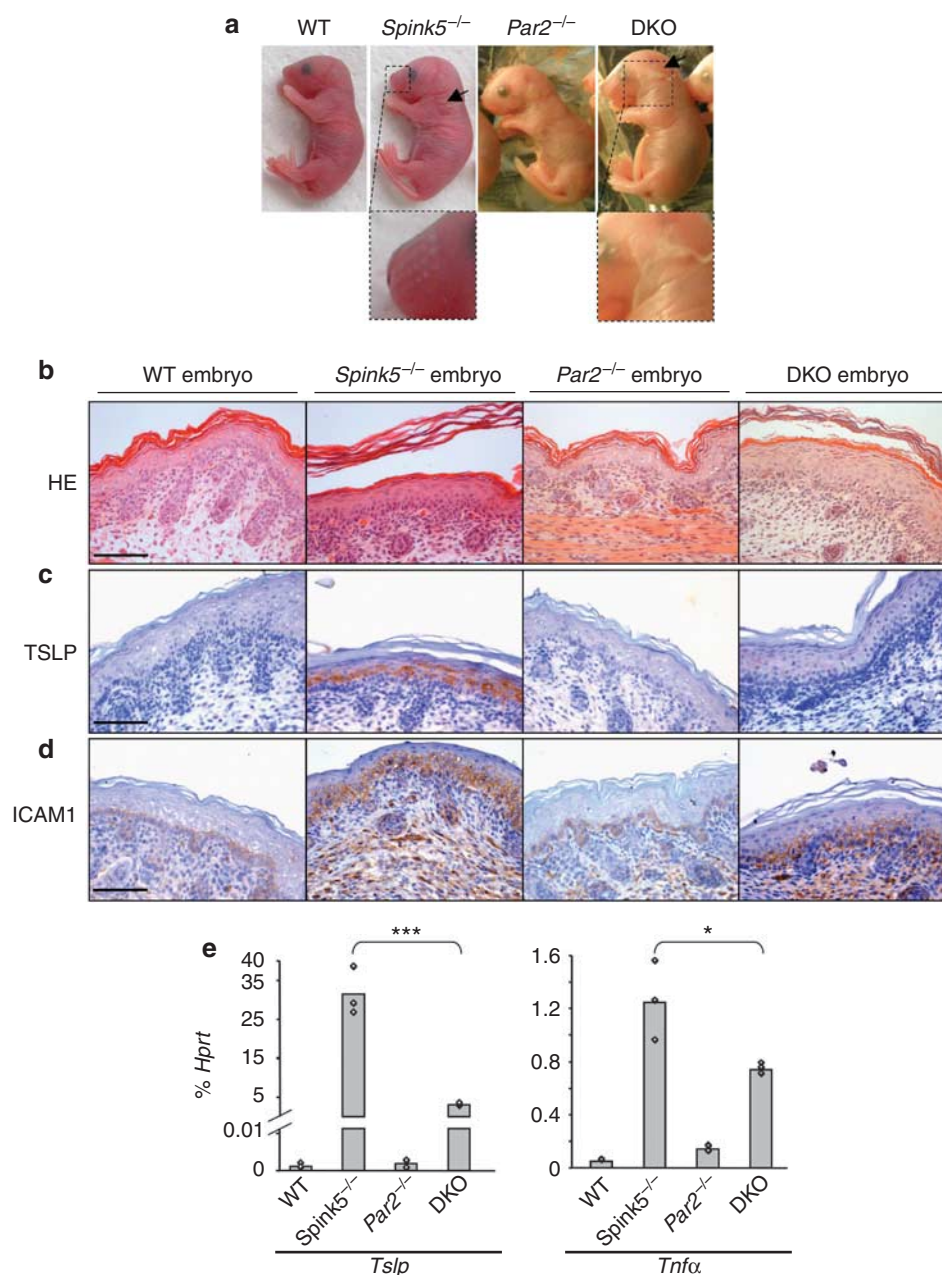


Figure 1. *Par2* inactivation does not rescue stratum corneum detachment but inhibits TSLP expression in *Spink5*^{-/-} mice embryos. (a) At birth, *Spink5*^{-/-} and double knockout (DKO) mice exhibit vibrissae abnormalities (left enlargement) and skin erosions (arrows and right enlargement). (b) At embryonic day 19.5 (E19.5), hematoxylin–eosin coloration reveals that the *Spink5*^{-/-} and DKO epidermis show slight acanthosis and stratum corneum detachment. (c) Immunostaining shows that TSLP is not detected in the WT and *Par2*^{-/-} epidermis and is poorly detected in the DKO epidermis in contrast to the *Spink5*^{-/-} epidermis. (d) ICAM1 staining, restricted to basal keratinocytes in the WT and *Par2*^{-/-} epidermis, is extended to suprabasal keratinocytes in the *Spink5*^{-/-} and DKO epidermis. (e) Quantitative RT-PCR was performed on RNA extracted from the E19.5 skin. mRNA amount is expressed as a percentage of *Hprt*. Graphs represent the mean of three independent embryos ($n=3$). * $P<0.05$; *** $P<0.01$. (Panels b–d) Bar = 9.2 μ m. *Par2*, proteinase-activated receptor 2; RT-PCR, real-time PCR; *Spink5*, Serine Protease INhibitor Kazal-type 5; TSLP, thymic stromal lymphopoietin; WT, wild type.

colored in red, in the dermis of WT and *Par2*^{-/-} grafts. In contrast, these cells were numerous in the dermis and infiltrated the epidermis of DKO- and *Spink5*^{-/-}-grafted skin (Figure 2c). Toluidin blue staining showed that mast cells were more numerous (4.5- and 3.8-fold increased, respectively) in *Spink5*^{-/-} and DKO grafts than in WT and *Par2*^{-/-} grafts dermis (Figure 2d and g).

We next analyzed TSLP and ICAM1 expression in the adult grafted skin. TSLP was not detected in the WT and *Par2*^{-/-} skin in contrast to the *Spink5*^{-/-} epidermis where it was strongly enhanced. TSLP was also detected in the DKO adult skin (Figure 2e). Immunostaining of ICAM1, restricted to the basal layer in WT or *Par2*^{-/-} adult epidermis, extended to suprabasal layers in *Spink5*^{-/-} and DKO (Figure 2f).

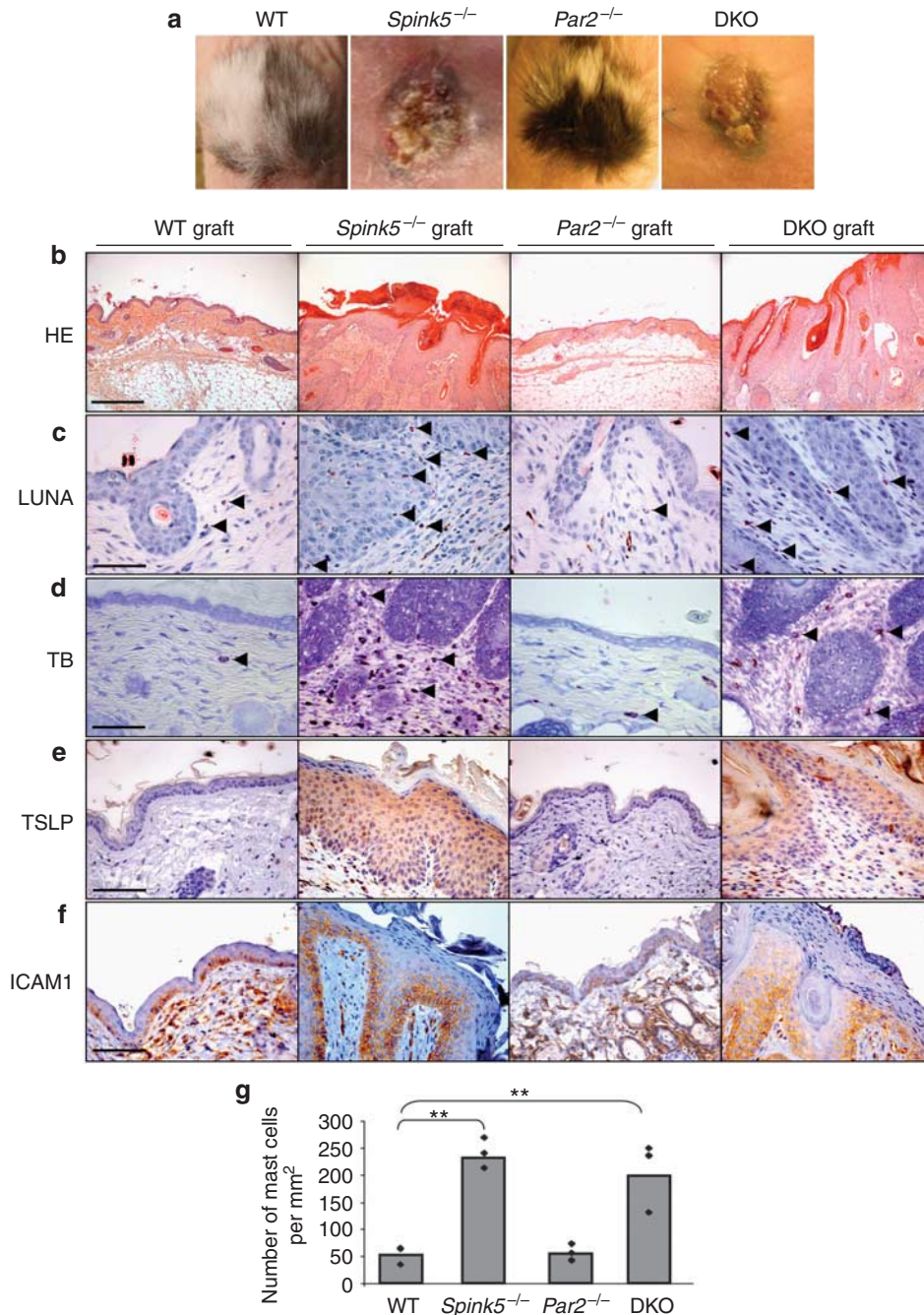


Figure 2. *Par2* deficiency does not improve the *Spink5*^{-/-} adult skin phenotype or inflammatory features. (a) The skin from embryonic day 19.5 (E19.5) mice was grafted onto nude mice for 6 weeks. (b) After hematoxylin-eosin coloration, WT- and *Par2*^{-/-}-grafted skin appears similar to the normal adult skin. In contrast, the *Spink5*^{-/-} and DKO-grafted epidermis exhibit acanthosis, papillomatosis, and detachment of the stratum corneum, which is hyperkeratotic and parakeratotic. Inflammatory infiltrates were present in the dermis. (c) Luna staining reveals rare polynuclear eosinophilic cells (arrowhead) in the dermis of WT and *Par2*^{-/-} grafts. In contrast, numerous polynuclear eosinophilic cells infiltrated the dermis and the epidermis of both *Spink5*^{-/-} and DKO grafts. (d) Mast cells, stained with toluidin blue (arrowhead), are rare in the dermis of WT and *Par2*^{-/-} grafts as compared with a higher number in the dermis of *Spink5*^{-/-} and DKO grafts. (e) Immunohistochemistry reveals no TSLP detection in WT and *Par2*^{-/-} adult-grafted epidermis, whereas it is induced in *Spink5*^{-/-} mice and also in DKO animals. (f) ICAM1 expression, restricted to basal keratinocytes in the WT and *Par2*^{-/-} epidermis, is extended to suprabasal keratinocytes in both the *Spink5*^{-/-} and the DKO epidermis. (g) Mast cells were counted per dermal surface unit. Graphs represent the mean of 3 independent mice (*n* = 3) for which 10 microscopic fields were analyzed. ***P* < 0.02. (Panel b) bar = 36.8 μm; (panel c) bar = 5.75 μm; (panels d-e) bar = 9.2 μm. DKO, double knockout; *Par2*, proteinase-activated receptor 2; *Spink5*, Serine Protease INhibitor Kazal-type 5; TSLP, thymic stromal lymphopoietin; WT, wild type.

Taken altogether, these data indicate that *Par2* deletion is not sufficient to abolish inflammation in the LEKTI-deficient adult skin.

DISCUSSION

Patients with NS exhibit abnormal desquamation and constant atopic manifestations. We previously demonstrated

that in the absence of LEKTI, unrestricted KLK5 activity causes both stratum corneum detachment (Descargues *et al.*, 2005) and expression of proallergic and proinflammatory molecules by keratinocytes (Briot *et al.*, 2009). Indeed, KLK5 is able to cleave and activate PAR2 (Oikonomopoulou *et al.*, 2006), leading to the induction of *TSLP*, *ICAM1*, and *TNF α* expression in human primary keratinocytes (Briot *et al.*, 2009). Thus, the KLK5–PAR2–TSLP signaling pathway seems to have a key role in atopic manifestations in NS. To further confirm these *in vitro* data, we developed *Spink5/Par2* DKO mice. At birth, DKO mice displayed skin erosions similar to *Spink5*^{−/−} mice and underwent rapid postnatal lethality. Histological examination of the skin at E19.5 confirmed abnormal detachment of the stratum corneum in DKO embryos as seen in *Spink5*^{−/−} embryos. This observation implied that PAR2 inactivation does not abolish epidermal protease hyperactivity and consequently does not prevent stratum corneum detachment. This is consistent with previous data showing that KLK5 is a direct target of LEKTI (Egelrud *et al.*, 2005; Schechter *et al.*, 2005; Deraison *et al.*, 2007). Thus, enhanced protease activities in *Spink5*^{−/−} newborn mice is due to the absence of LEKTI, with no increased transcriptional expression of these proteases (Descargues *et al.*, 2005).

To disregard an indirect effect of environmental stimuli secondary to skin barrier impairment, we studied the expression of proinflammatory and proallergic molecules in the skin of DKO E19.5 embryos. *Par2* deletion, in a context of LEKTI deficiency, dramatically inhibited *Tslp* induction at the mRNA and the protein levels, and decreased *Tnf α* expression by 40% in the skin of DKO embryos compared with *Spink5*^{−/−} embryos. However, *Icam1* expression seemed similar between the DKO and the *Spink5*^{−/−} embryo skin. Although *ICAM1* was demonstrated to be induced by PAR2 activation in human primary keratinocytes (Buddenkotte *et al.*, 2005; Briot *et al.*, 2009), this key molecule is probably activated through other various and numerous stimuli, leading to immune cell recruitment in our mouse model. Taken together, these results demonstrate that *Tslp* expression is highly dependent on the KLK5–PAR2 pathway *in vivo* and that *Tnf α* expression is less dependent on this pathway, at least in the initial steps of the inflammatory response. This supports our previous data on TSLP overexpression in the epidermis of E19.5 *Spink5*^{−/−} mice and in NS primary keratinocytes (Briot *et al.*, 2009). These data obtained *in vivo*, confirm the role of the KLK5/PAR2 axis in triggering a proallergic microenvironment through strong *Tslp* induction as early as E19.5, and consequently independently of the adaptive immune system and environmental stimuli.

To further explore whether inhibition of the KLK5–PAR2 cascade could reduce inflammation previously observed in the *Spink5*^{−/−} adult skin (Briot *et al.*, 2009), we grafted skin from E19.5 DKO onto athymic immunodeficient nude mice. Six weeks after grafting, the DKO adult skin exhibited a severe ichthyosiform phenotype similar to *Spink5*^{−/−} grafts. At the histological level, the skin from DKO grafts displayed epidermal acanthosis and stratum corneum detachment as seen in the *Spink5*^{−/−} skin, highlighting that these NS features are independent of PAR2. Moreover, although *Par2*^{−/−} mice

exhibit accelerated barrier recovery after tape stripping (Hachem *et al.*, 2006), *Par2* deletion was not sufficient to rescue the effects of premature desquamation in the DKO adult epidermis.

The adult DKO-grafted skin also displayed an inflammatory infiltrate composed of a high number of mast cells and polynuclear eosinophilic cells. This could be secondary to persistent SC detachment favoring sensitization by environmental allergens. However, DKO mice already suffer from stratum corneum detachment as early as E19.5. Considering the fact that our mice are maintained under specific pathogen free conditions and that other mouse models such as flaky tail mice do not exhibit skin inflammation without allergen challenge under this condition (Fallon *et al.*, 2009), it is likely that cutaneous inflammation in the DKO-grafted skin is also due to intrinsic signals secondary to LEKTI deficiency. Unregulated proteases activities, in the absence of LEKTI, could induce signals independently of PAR2, promoting expression of inflammatory molecules such as thymus and activation-regulated chemokine and macrophage-derived chemokine that are overproduced by NS primary keratinocytes cultured under basal conditions (Briot *et al.*, 2009). The role of other unrestricted protease activities in a LEKTI-deficient context is also supported by *Ela2* (Bonnart *et al.*, 2010) and *Klk7* (Hansson *et al.*, 2002) transgenic mice, which both exhibit spontaneous cutaneous inflammation. In addition, as demonstrated in the literature, other mechanisms such as mechanical stress caused by stratum corneum detachment could also induce skin inflammation (Wood *et al.*, 1992; Nickoloff and Naidu, 1994). Indeed, tape stripping induces IL1 β expression and secretion by keratinocytes (Nickoloff and Naidu, 1994). IL1 β , which is strongly expressed in the skin of *Spink5*^{−/−} mice (Briot *et al.*, 2009), could then be processed and activated by unrestricted KLK7 (Nylander-Lundqvist and Egelrud, 1997) or caspase 1 (Hosomi *et al.*, 2008), and participated in the recruitment of innate immune cells.

Although IL1 β is not considered as a Th2 cytokine, it is not excluded that residual production of TSLP and TNF α in the DKO epidermis could be sufficient to switch inflammation toward an AD-like phenotype. Independently of the PAR2 pathway, residual TSLP expression could be due to other unidentified mechanisms or secondary to IL1 β and TNF α production, which have been reported to induce its expression (Lee and Ziegler, 2007). In our grafted skin model, the recruitment of polynuclear eosinophilic cells and mast cells could be first a consequence of IL1 β production and residual TNF α and TSLP. Together, these molecules are known to induce the secretion of Th2 cytokines by activated mast cells (Allakhverdi *et al.*, 2007).

Interestingly, these innate immune cells could arise not only from the donor skin but also from host nude mice bearing the functional PAR2 receptor. Indeed, our skin graft model is somehow different from *Par2*^{−/−} mice, in which *Par2* is inactivated in the epidermal, dermal, and immune compartments. *Par2*^{−/−} mice exhibit significant reduction of immune cell infiltration after experimental induced-allergic dermatitis compared with WT mice (Kawagoe *et al.*, 2002; Seeliger *et al.*, 2003). Thus, with time, phenotype aggravation

of the DKO-grafted skin could be due to PAR2 activation at the surface of cells from the host, such as mast cells and eosinophilic cells (Shpacovitch *et al.*, 2008).

Six weeks after grafting, we observed an aggravated cutaneous inflammation probably resulting from the “vicious circle” promoted and maintained by hyperactivated proteases, mechanical stress, skin barrier impairment, and activation of innate immune cells, which also secrete cytokines and proteases. In an immune competent model of DKO, one can argue that the secretion of the proallergic chemokines, macrophage-derived chemokine and thymus and activation-regulated chemokine, overexpressed in NS patient keratinocytes and the *Spink5*^{−/−} epidermis independently of the KLK5-PAR2 pathway (Briot *et al.*, 2009), together with residual expression of TSLP could still promote a Th2 response in the adult skin.

To date, there is no evident point demonstrating that either intrinsic or extrinsic signals are promoting cutaneous allergic-type inflammation in AD. The fact that only 37–50% of patients with *Ichthyosis vulgaris* develop atopic manifestations (Kuokkanen, 1969; Smith *et al.*, 2006), supports the notion that in NS in which all patients develop allergic manifestations, additional mechanisms to primary skin barrier defect have a key role in immune response polarization toward a Th2 response. This study highlights the fact that the KLK5-PAR2-TSLP pathway, already activated *in utero* in *Spink5*^{−/−} mice, is an early inducer of proinflammatory and proallergic signals seen in our models (Briot *et al.*, 2009). This observation could be relevant to the development of “intrinsic atopy” before sensitization to environmental allergens. However, skin integrity is essential to protect our body against allergens that could trigger “extrinsic atopy” when the epidermal barrier is defective.

This study also provides strong evidence that several mechanisms trigger skin inflammation in NS models *in vivo*. These include skin barrier defect and keratinocyte mechanical stress induced by stratum corneum detachment as well as protease-induced proinflammatory and proallergic pathways, including KLK5-PAR2-mediated overexpression of TSLP (Briot *et al.*, 2009) and the consequences of other hyperactivated proteases (Hansson *et al.*, 2002; Descargues *et al.*, 2005; Bonnart *et al.*, 2010). Taken together, unregulated biological cascades lead to a severe clinical phenotype in NS. These specific pathophysiological mechanisms triggering atopic manifestation in NS may serve as a model for more complex unknown biological cascades involved in the more common disease, AD.

Therapeutic developments for NS should consider multiple sides of the disease. Reducing inflammation through TSLP repression together with restoring skin barrier function through inhibition of unrestricted protease activities would probably be a promising approach to significantly improve the clinical phenotype.

MATERIALS AND METHODS

Mouse housing conditions and skin grafting

This study 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 conducted

in accordance with the relevant guidelines and regulations. Mice embryos were delivered by Caesarean section at E19.5.

Total dorsal skin from E19.5 embryos was transplanted onto athymic immunodeficient nude mice using the skin-flap technique (Barrandon *et al.*, 1988) and analyzed after 6 weeks.

Gestate, newborn, and host grafted mice were maintained under specific pathogen free housing conditions during every experiment.

Histological and immunohistochemical analysis

Mouse skin samples from E19.5 embryos or skin grafts were fixed in 10% formalin and embedded in paraffin. Sagittal sections of 4µm were stained with hematoxylin-eosin for histological examination or toluidin blue for mast cell counting. Luna staining was performed to reveal polynuclear eosinophilic cells. For immunohistochemistry experiments, antigen retrieval of paraffin-embedded cross-sections were performed in citrate buffer (1 mM, pH6) boiled for 20 minutes. TSLP and ICAM (R&D Systems, Minneapolis, MN) primary antibodies were used and immunodetection was performed using appropriate Horseradish peroxidase (diaminobenzidine)-conjugated polymers (EnVision System, Dakocytomation, Glostrup, Denmark).

RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA were extracted from the E19.5 skin using RNeasy mini kit (Qiagen, Courtaboeuf, France) and cDNA synthesis from 2 to 3 µg of total RNA was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was realized on ABI prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA) using the qPCR MasterMix Plus SYBR Green I kit (Eurogentec, Angers, France). Results were normalized with the *Hprt* gene and analyzed using Sequence Detection System version 1.2 (Applied Biosystems). The following primers were used: *Tslp* forward 5'-CGACAGCATGGTCTTCTCA-3' and reverse 5'-CGATTGCTCGAACTTAGCC-3'; *Icam* forward 5'-AGGGCTGGCATTGTTCTCTA-3' and reverse 5'-CTTCAGAGGCAGGAAACAGG-3'; *Hprt* forward 5'-CTGGTTAAGCAGTACAGCCCCA-3' and reverse 5'-CGAGAGGTCCTTTTACCAGC-3'. *P*-values were calculated using the Student-Fischer test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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