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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Keratin 16 regulates innate immunity in response to epidermal barrier breach

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Mutations in the type I keratin 16 (Krt16) and its partner type II keratin 6 (Krt6a, Krt6b) cause pachyonychia congenita (PC), a disorder typified by dystrophic nails, painful hyperkeratotic calluses in glabrous skin, and lesions involving other epithelial appendages. The pathophysiology of these symptoms and its relationship to settings in which Krt16 and Krt6 are induced in response to epidermal barrier stress are poorly understood. We report that hyperkeratotic calluses arising in the glabrous skin of individuals with PC and Krt16 null mice share a gene expression signature enriched in genes involved in inflammation and innate immunity, in particular damage-associated molecular patterns. Transcriptional hyper-activation of damage-associated molecular pattern genes occurs following de novo chemical or mechanical irritation to ear skin and in spontaneously arising skin lesions in Krt16 null mice. Genome-wide expression analysis of normal mouse tail skin and benign proliferative lesions reveals a tight, context-dependent coregulation of Krt16 and Krt6 with genes involved in skin barrier maintenance and innate immunity. Our results uncover a role for Krt16 in regulating epithelial inflammation that is relevant to genodermatoses, psoriasis, and cancer and suggest a avenue for the therapeutic management of PC and related disorders.

Results

Molecular Convergence Between Krt16−/− Front Paw Calluses and Human Palmoplantar Keratoderma Lesions. Skin lesions in adult Krt16−/− front paws have an impaired outside-inside epidermal barrier, correlating with loss of the stratum corneum protein filaggrin, induction of the wound healing-associated Krt17, and hyperproliferation (14). We now confirm the presence of inflammation by showing that CD4+ T-cells, monocytes, macrophages, and neutrophils accumulate in lesional Krt16−/− front-paw skin lesions (Fig. 3).

Significance

Here we report that keratin 16 (Krt16), a type I intermediate filament cytoskeletal protein, is an integral and functionally important component of a genetic network regulating danger signals, innate immunity, and barrier function in skin epidermis. Our findings help explain the pathogenesis of the conspicuous skin lesions arising in genetic skin disorders caused by mutations in Krt16, such as pachyonychia congenita and focal palmoplantar keratoderma, and in diseases in which Krt16 is induced and misregulated, such as psoriasis and cancer.


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skin (Fig. L4). CD207+ Langerhans cells (LCs), normally rare in murine glabrous skin, are also markedly increased in Krt16−/− calluses, especially in areas immediately adjacent to tissue showing hyperpidermerization (Fig. L4) (14).

Genome-wide association and gene expression profiling studies have linked Krt16 to key players in cutaneous inflammation and cancer susceptibility (15–18). We used quantitative RT-PCR (qPCR) to look at a panel of proinflammatory signature mRNAs (Table S1) relevant to these settings. At 8 wk after birth, Krt16−/− front paws feature prominent lesions whereas hind-paw pads still appear normal (Fig. S1). Using hind-paw–derived data as an internal control for each mouse, we observe prominent expression of several DAMPs and proinflammatory cytokines in Krt16−/− front-paw skin (Fig. 1B and Fig. S1). Particularly notable are the high levels of Spr2d, Sfn, and Krt6a mRNAs as they are selectively induced in keratinocytes at the wound edge (10, 15, 19), are connected to each other in cancer susceptibility networks (20), and, in the case of Spr2d, possess antioxidant properties (21, 22). Krt16−/− front-paw lesions also feature high transcript levels for Sfa1 and SerpinB3a, which are keratinocyte-specific protease inhibitors associated with proliferation, differentiation, and increased susceptibility to skin cancer (23, 24). Up-regulation of DefB3 and DefB4 further suggests an impairment of both the permeability and the antimicrobial barrier in Krt16−/− glabrous skin. By comparison, genes involved in LC trafficking (Ccr6, Ccl20), apoptosis (Casp8), the inflammasome (Nlips3), and the amplification and coordination of the adaptive immune response (IL-22) are only moderately induced in Krt16−/− front-paw lesions (Fig. 1B). Of note, the rupture or lysis of keratinocytes is not a predominant feature in Krt16−/− glabrous epidermis as confirmed by transmission electron microscopy (Fig. S1).

We next analyzed global gene expression in plantar keratoderma biopsies from human PC patients carrying mutations in KRT16, KRT6, or KRT17 and normalized the data to nonlesional glabrous skin from the same individuals. Interestingly, although expression of proinflammatory cytokines was generally low, several genes encoding for S100s, Spr proteins, and β-defensins were among the most abundant transcripts in lesional tissue (Fig. 1C). Expression of KRT6 paralogs (a and b) was also markedly elevated (Fig. 1C), consistent with their wound-inducible nature (25) and their newly proposed status as DAMPs (26). Such a DAMP-centric profile occurs independently of the disease-causing mutation and is strikingly similar to our findings in Krt16−/− front-paw calluses, suggesting that the misregulation of barrier-related genes is a general feature of PC-related palmoplantar keratoderma. These data validate the Krt16−/− mouse as a relevant model in which to study the pathogenesis of PPK.

### Keratinocytes Lacking Krt16 Hyper-Activate Alarmin Expression in Response to Chemical and Mechanical Challenges to the Epidermis.

To test the hypothesis that the absence of Krt16 alters the course of acute cutaneous inflammation, we treated ear skin of 8-wk-old WT or Krt16−/− and Krt10−/− mice twice with 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA is a well-known activator...
of protein kinase C that initiates epidermal inflammation and promotes tumor formation (27). Topical TPA application to mouse ear skin causes epidermal thickening, hyperproliferation, up-regulation of Krt16 and Krt17 proteins, and recruitment of wound-associated immune cells (10) (Fig. 2A and Fig. S2). Before TPA treatment, histology and epidermal thickness are normal in Krt16−/− ear skin (Fig. 2A). At 48 h following the last of two TPA treatments, expansion of the postmitotic suprabasal layers is modestly but significantly greater in Krt16−/− relative to control (Fig. 2A). The origin of this expansion is unknown, as the mitotic index remains the same in Krt16−/− and control TPA-treated ears (Fig. 2A). Onset of Krt16 expression precedes epidermal thickening and thus can be uncoupled from hyperproliferation in such settings (19, 28).

TPA treatment of ear skin tissue also results in the increased expression of a group of proinflammatory and barrier-related gene targets similar to Krt16−/− front-paw lesions (Fig. S2). Normalization of gene expression fold changes to the control mice suggests a similar basal state of adult Krt16−/− and control epidermis. This said, we observed a modest decrease in mRNA levels for IL-1b, Ccl2, Ccl5, and HO-1 in acetone-treated Krt16−/− ear epidermis (Fig. S2). However, these changes do not overlap with the increases in gene expression in acetone-treated ears relative to control ears.

**Fig. 2.** Chemical and mechanical irritation leads to hyper-activation of DAMPs and cytokines in Krt16−/− skin. (A) Phorbol ester (TPA) treatment of ear skin in 8-wk-old mice induces Krt17 expression in suprabasal keratinocytes and infiltration by nonresident immune cells (CD11b+). epi, epidermis; hf, hair follicle. (Scale bar, 50 μm; the first scale bar refers to the IF stainings, the second to the H&E stainings.) H&E stainings illustrate the epidermal expansion in response to TPA. (Scale bar, 25 μm.) Insets show ears treated with acetone vehicle. Note the equal epidermal thickness as well as the absence of Krt17 or immune cell staining in the interfollicular epidermis of vehicle-treated ears. Krt16−/− mice develop significantly more epidermal thickening in response to TPA than controls without a change in the mitotic index. *P < 0.05, n.s., not significant, one-way ANOVA with Bonferroni correction. (B) qPCR data from TPA-treated ear skin represented as fold changes (TPA/acetone) and normalized to control mice. Krt16−/− mice show a significant over-induction of alarmins and cytokines. Each bar represents the mean ± SD of 5–10 biological replicates. *P < 0.05, **P < 0.01, Mann–Whitney test, two-tailed. (C) Mechanical disruption of the epidermal barrier via tape stripping also leads to an over-induction of DAMP and cytokine RNA in Krt16−/− mice. Data represent the mean fold changes (stripped ear/normal ear) + SD of three biological replicates relative to control mice.
findings in hind-paw skin epidermis (Fig. S1), normal ear epidermis (Fig. S3), or newborn keratinocytes in primary culture (Fig. S3) and could be related to the very low steady-state levels of these mRNAs in normal skin. Consistent with Krt17’s proposed role as a proinflammatory immunomodulator (11) and in contrast to our findings in Krt16−/− skin, DAMP mRNA levels are essentially unchanged in Krt17−/− ear skin after TPA treatment (Fig. S2). An over-reaction to external stimuli is a wide-ranging characteristic of Krt16−/− embryos because tape stripping of ear skin—a superficial mechanical insult that removes the stratum corneum and induces Krt16 mRNA and protein expression (33)—also consistently leads to exaggerated expression of several alarmins, IL-1β, SPR2D, HO-1, and Krt6δ in Krt16−/− ear skin (Fig. 2C).

Unbalanced DAMP expression in response to trauma has long-term implications for an organism. Patients with atopic dermatitis (AD) overreact to injury with increased production and secretion of DAMPs (34). Likewise, chronically elevated levels of thymic stromal lymphopoietin (TSLP) in mouse epidermis trigger the formation of AD-like lesions (35), and overexpression of S100A7A leads to an immunological overreaction to mechanical stress (36). In addition to their PPK-like paw lesions, older Krt16−/− mice also develop spontaneous chronic dermatitis, which is fully penetrant yet variable in onset, severity, and location (Fig. S4). Such lesions appear in areas where Krt16 is not normally expressed and show markedly elevated levels of Krt6, Krt17, TSLP, and S100A7A mRNAs and proteins (Fig. S4).

The analyses of our TPA and tape-stripping experiments were conducted at 48 h posttreatment, which allows for the arrival of systemic immune cells at the site of inflammation (Fig. 2A and Fig. S2). These immune cells likely make a contribution to the elevated mRNA levels for several genes, including S100A8, S100A9, and IL-1β (6, 37). Induction of targets such as the epithelial-specific Krt6a and SPR2D, however, suggests a keratinocyte-autonomous component in this phenomenon. To test this hypothesis, newborn skin keratinocytes were seeded in primary culture, treated once with TPA, and processed for qPCR analysis. In this setting, DAMP gene expression peaks at 3–6 h posttreatment and returns to baseline within 24 h (Fig. 3A). Relative to controls, Krt16−/− but not Krt17−/− keratinocytes overexpress several DAMPs after TPA exposure (Fig. 3B). Before TPA treatment, Krt16−/− and control keratinocytes express similar levels of all mRNAs tested (Fig. S3). Other keratins do not appear to compensate for the loss of Krt16 in the primary culture setting (Fig. S3). At this time, we cannot comment on whether the absence of Krt16 alters the secretion of DAMPs from keratinocytes. The induction of alarmin transcription 3 h after TPA addition is largely mediated by the Erk1/2 arm of MAPK signaling and does not appear to require IL-1β-dependent amplification (Fig. 3C and Fig. S3). The ex vivo findings strongly suggest that the specific induction of Krt16 in skin keratinocytes subject to cellular stress is critical for the proper transcriptional regulation of innate danger signals.

**Systems Genetics Analysis Independently Links Krt16 to Alarmin and Skin Barrier Genes.** In 2009, Quigley et al. reported an unbiased systems genetics analysis yielding genome-wide expression and association networks for adult mouse skin during normal homeostasis and different stages of carcinogenesis (20). Here, we reanalyzed this data set for genes correlated with Krt16. In normal tail epidermis, Krt16 expression is constitutive (similar to glabrous epidermis) and strongly positively correlated with several DAMPs and other regulators of skin barrier function (Fig. 4A, top 30 hits shown). Many of the top-scoring genes in this Krt16-anchored network—e.g., Krt6a, S100A8, S100A9, DefB3, SerpinB3a, and Sfia1 (Fig. 4A)—are markedly misregulated in Krt16−/− mouse skin subject to barrier challenges and in PC-related PPK (Figs. 1–3). Krt16 expression in benign papillomas sampled from back skin in the same set of mice is notably higher and more uniform relative to normal tail skin (Fig. 4B). A striking proportion of the top-scoring genes lose their correlation with Krt16 in papillomas (Fig. 4A), suggesting that the interrelationships between Krt16 and barrier-related genes may differ in settings of chronic inflammation. These findings significantly extend the notion that Krt16 is an integral part of a genetic network that includes DAMP-encoding and skin barrier-associated genes.

**Discussion**

We show here that when the epidermal barrier is experimentally challenged by acute proinflammatory and mechanical stimuli, keratinocytes lacking Krt16 fail to properly regulate the production of innate danger signals and overactivate the expression of DAMPs, cytokines, and other regulators of skin barrier function. Our results imply a role for Krt16 in this form of innate immunity, provide an innovative framework to understand the complex pathogenesis of several chronic inflammatory skin diseases, and, finally, may have direct implications for the treatment of the painful and debilitating palmoplantar keratodermas associated with PC and related genodermatoses. Early activation of Krt16 expression after various types of insults to the skin is therefore functionally relevant to the progression of cutaneous inflammation. We infer that loss of Krt16 eliminates an important inflammatory checkpoint, leaving the organism vulnerable to inappropriate immune responses, and we further speculate that loss of Krt16 function, whether complete or partial, impairs the resolution of PPK-like calluses in glabrous skin.

![Fig. 3. Misregulation of innate danger signals is specific to Krt16−/− keratinocytes.](image-url)

(A) DAMP expression peaks at 3–6 h post-TPA treatments in newborn keratinocytes in primary culture and returns to baseline by 24 h. (B) Cultured Krt16−/− primary keratinocytes retain the ability to hyper-activate alarmins 3 h after TPA treatment. Krt17−/− cells do not show a difference compared with controls. Data represent the mean ± SD of three to eight biological replicates. *P < 0.05, Mann–Whitney test, two-tailed. (C) DAMP transcription in Krt16−/− keratinocytes in response to TPA is mediated by the Erk arm of MAPK signaling. Data represent the mean ± SD of four biological replicates. *P < 0.05, Student t test, two-tailed.
Krt16 mouse lines (C57BL/6 background) (10, 14) were maintained under allele-specific skin. Phorbol esters heighten keratinocyte in mice dose-dependently keratinocytes in culture. However, in is also a direct target for signaling mediated by the Nrf2 fi Krt16 skin skin and human PPK lesions. In our treated hypersensitivity to proinflammation, which interacts with hemidesmosomal integrins, causes epidermal during wound healing, chronic in induction in keratinocytes. For example, intermediate S100A8, and S100A9 secretion from keratinocytes (30). in the differentiating layers of the epidermis, is essential for con- tier maintenance. Krt1, a type II keratin constitutively expressed (44), which are altered in epidermal keratinocytes null with MAPK signaling to activate and amplify keratinocyte pro- (EGFR) activation via Erk1/2 signaling, resulting in the increased production of IL-1a (42). IL-1a is known to interact functionally with MAPK signaling to activate and amplify keratinocyte prolif- eration and epidermal inflammation, creating an autoimmune feedback loop (37, 43). Integrons, the IL-1 receptor, and the EGF receptor are all located in focal adhesion complexes at the plasma membrane (44), which are altered in epidermal keratinocytes null for Krt6, the type II keratin partner for Krt16 (8). In addition, focal adhesion kinase links mechanical stress to Erk1/2 signaling and cytokine production in dermal fibroblasts (45). Possibly, Krt16 may be involved in regulating a pathway that is activated when the skin experiences increased or altered mechanical forces, e.g., in normal glabrous skin or at the wound edge.

DAMP and cytokine transcription in cultured keratinocytes depends on the MAPK signaling cascade, a major switchboard for relaying and amplifying stress signals (46 and this study). 

Krt16 is a direct target for EGFR and Erk1/2-mediated signaling (47–50), and its overexpression in mice dose-dependently enhances EGFR activity (51). Following stress, Krt16 could conceivably impact MAPK and/or EGF signaling to modulate the total level of DAMPs in a keratinocyte-autonomous fashion. Krt16 is also a direct target for signaling mediated by the Nrf2 transcription factor, a master regulator of ROS levels and the oxidative stress response in skin (50, 52, 53). In adult mice, misregulation of Nrf2 levels raises the risk for tumorigenesis (54, 55) and promotes cutaneous inflammation secondary to stratum corneum abnormalities (22). In utero, Nrf2 stimulates epidermal barrier repair via the up-regulation of Spr22d and Spr22h (56). We observe high levels of Spr22d as well as HO-1 in Krt16TT- TPA-treated ears, suggesting the activation of the Nrf2-mediated oxidative stress response and raising the possibility that Krt16 may play a role in this cellular defense mechanism.

Autoantibodies to KRT16 have been tied to an exaggerated activation of innate immunity signaling pathways in psoriatic lesions (57, 58). Furthermore, IL-1a treatment of human primary keratinocytes elicits a transcriptional profile enriched in antimicrobial peptides and genes from the epidermal differentiation complex (59) that is strikingly similar to challenged Krt16TT- skin and to Krt16’s association with skin barrier-related factors as revealed by computational analysis. In the absence of Krt16, improper control of the IL-1a-signaling pathway and/or its proinflammatory feedback loop could explain the phenotypes we observe in both Krt16TT- skin and human PPK lesions. In our hands, inhibiting IRAK1/4 did not alter DAMP transcription in response to TPA in Krt16TT- keratinocytes in culture. However, in vivo cellular architecture and feedback from other cell types, e.g., fibroblasts as well as resident and infiltrating immune cells, play a major role in IL-1a-mediated autoimmune feedback (37). The lack of an intact tissue microenvironment could thus account for the modest induction of DAMP expression occurring in newborn skin keratinocyte cultures compared with adult whole ear tissue.

Various strategies have been applied toward the therapeutic management of PC (60–62) or for palmoplantar keratoderma of various etiologies (63, 64) with mixed results and, in the end, limited relief for the patient. This includes topical treatments (e.g., with corticosteroids or retinoids) designed to antagonize inflammation in a broad and rather nonspecific fashion (64–66). A recent trial involving the use of a mutant KRT6a allele-specific siRNA in plantar skin led to the recession of calluses and associated pain in one patient, but the extreme pain associated with the direct injection is problematic considering the large areas covered by PPK (61). The development of therapies designed to attenuate the alarmin response in skin, especially when combined with keratin mutant al- late-specific interventions, could prove beneficial for PC patients.

Materials and Methods

Procedures for the collection, processing, and analysis of patient plantar biopsies, gene expression correlation and regression, epidermal barrier challenges 

Krt16 and Krt17 null mice, qPCR, cell culture, histology, reagents, and the system genetics analysis are described in the SI Materials and Methods. Animal experiments involving mice were approved by The Johns Hopkins University Institutional Animal Care and Use Committee. Krt16TT- and Krt17TT- mouse lines (C57BL/6 background) (10, 14) were maintained under specific pathogen-free conditions and fed chow and water ad libitum. De-identified plantar human skin samples were obtained, with informed con- sent, from one affected and one unaffected site of five nonrelated, adult PC patients harboring a KRT16 R127C, KRT6A N171K, KRT6B E472K, or KRT17 N925 single nucleotide mutation (patients #1009, #1015, #10, #66, and #394 from the International Pachyonychia Congenita Research Registry).
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