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Conditional PDK1 ablation promotes epidermal and T cell-mediated dysfunctions leading to inflammatory skin disease

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Abstract

Phosphoinositide dependent kinase-1 (PDK1) is a key signaling molecule downstream of the phosphatidylinositol 3-kinase (PI-3 kinase) pathway and is a master regulator of multiple kinases in cells of epithelial and hematopoietic lineages. The physiological role of PDK1 in regulating skin and immune homeostasis is not known. Here we developed a mouse model in which PDK1 is conditionally ablated in activated CD4 T cells, regulatory T cells and mature keratinocytes, through OX40-Cre recombinase expression. The resultant mice (PDK1-CKO) spontaneously developed severe dermatitis, skin fibrosis and systemic Th2 immunity, succumbing by 11 weeks of age. Through a series of T cell transfers, bone marrow reconstitutions and crossing to lymphocyte-deficient backgrounds, we demonstrate that ablation of PDK1 in keratinocytes is the major driver of disease pathogenesis. PDK1-deficient keratinocytes exhibit intrinsic defects in expression of key structural proteins including cytokeratin-10 and loricrin, resulting in increased keratinocyte turnover, which in turn, triggers inflammation, T cell recruitment and immune-mediated destruction. Our results reveal PDK1 as a central regulator of keratinocyte homeostasis which prevents skin immune infiltration and inflammation.

INTRODUCTION

Inflammatory skin diseases such as atopic dermatitis (AD) and psoriasis involve immune-mediated and skin-intrinsic defects with each disease having specific immune signatures and skin pathology (Bergboer *et al.*, 2012; Boguniewicz and Leung, 2011; Guttman-Yassky *et*

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CONFLICT OF INTEREST

The authors have no conflict of interest.

al., 2011; Quaranta *et al.*, 2014). Genome-wide association studies (GWAS) have identified genes involved in skin barrier integrity and immune regulation (Ellinghaus *et al.*, 2013; Tsoi *et al.*, 2012) to be associated with inflammatory skin diseases; however, the respective role of skin-intrinsic versus immune-mediated factors in disease induction are not known. Mouse models targeting keratinocyte signaling can lead to development of skin pathology and immune activation with features of human inflammatory skin diseases (Sano *et al.*, 2005; Zenz *et al.*, 2005). Identifying new targets by which keratinocytes interface with the immune system is important for developing new therapies for these complex diseases which have no cure.

Phosphoinositide dependent kinase-1 (PDK1) is a key signaling regulator downstream of the phosphatidylinositol 3-kinase (PI-3 kinase) pathway, and signals upstream of multiple protein kinases including PKA, Akt, PKC, and p70S6 kinase (Mora *et al.*, 2004; Toker and Newton, 2000). PDK1 is broadly expressed in many cell types including epithelial and hematopoietic lineages, and is important for embryonic development, cell growth, survival and metabolism (Chen *et al.*, 2013; Hashimoto *et al.*, 2006; Mora *et al.*, 2003). The PI3 kinase/Akt/mTOR pathway promotes proliferation of epidermal precursors (Murayama *et al.*, 2007) and is implicated in skin tumorigenesis (Segrelles *et al.*, 2002; Suzuki *et al.*, 2003), although the physiological role of PDK1 in skin is not defined. PDK1 is known to play central roles in immune cells, as conditional PDK1 ablation in developing T cells results in impaired function through the abrogation of downstream PKC θ phosphorylation and NF- κ B activation (Park *et al.*, 2009).

In this study, we identify PDK1 as a molecular regulator of keratinocyte homeostasis and of the skin-immune interface, that when disrupted *in vivo* triggers severe skin pathology, systemic inflammation and morbidity. We generated a mouse model with conditional ablation of PDK1 by OX40-directed Cre expression resulting in simultaneous PDK1 deletion in subsets of activated and regulatory CD4 T cells and mature keratinocytes. The resultant PDK1-CKO mice are born healthy but gradually develop severe inflammatory skin disease, with systemic Th2-mediated inflammation, skin thickening and fibrosis. We dissected the relative contribution of PDK1-deficient T cells and –keratinocytes to disease pathogenesis, and demonstrate a dominant role for PDK1-deficient keratinocytes in driving disease through dysregulation of keratinocyte differentiation and turnover. Our results reveal that PDK1-signaling as a central regulatory pathway for keratinocyte homeostasis which prevents pathological immune infiltration and skin inflammation.

RESULTS

Spontaneous dermatitis and skin fibrosis in PDK1 conditional knockout mice

We constructed a mouse model with conditional ablation of PDK1 in activated CD4 T cells by crossing three mouse strains (Figure S1a): **1.** PDK1 flox/flox mice (Mora *et al.*, 2003), to **2.** ROSA26-yellow fluorescent protein reporter mice (R26-YFP) (Srinivas *et al.*, 2001), to **3.** OX40-Cre mice (Klinger *et al.*, 2009) which restrict Cre expression to activated CD4 T cells and a subset of regulatory T cells (Tregs) (Redmond *et al.*, 2009). The resultant OX40^{+/Cre} PDK1^{F/F} R26-YFP mice, designated PDK1-CKO, express YFP in all PDK1-ablated CD4 T cells, while the control strain (PDK1-CHET) is heterozygous at the floxed PDK1 locus

(OX40^{+/Cre} PDK1^{F/+}R26-YFP) and maintains PDK1 expression in YFP⁺ cells (Figure S1b). PDK1-CKO mice were born healthy; however, starting at 5 weeks of age, they developed severe, systemic dermatitis accompanied by hair loss and skin thickening (Figure 1a). PDK1-CKO mice further developed peripheral lymphadenopathy, an enlarged spleen (Figure 1a) and wasting syndrome, succumbing to disease by 11 weeks of age, whereas PDK1-CHET mice maintained normal health (Figure 1a,b).

The skin of PDK1-CKO mice contained multiple alterations by histological analysis, including epidermal scales, hyperplasia, hyperkeratosis, loss of hair follicles and hypodermal fat, and increased dermal fibrosis, while the skin of PDK1-CHET mice remained healthy (Figure 1c and Table S1). The skin of PDK1-CKO mice with advanced disease contained lesions with epidermal damage, resulting in loss of skin barrier integrity, as shown by dye penetration (Figure S1c). This skin barrier defect was observed in mice with severe disease at 7-8wks of age and not in infant mice (Figure S1c). We did not observe inflammation in other organs (lung, liver, kidney, gut) (Figure S1c), indicating that the tissue target of disease pathology in PDK1-CKO mice was limited to skin.

PDK1-CKO mice develop spontaneous Th2 responses and Treg deficiency

OX40-directed PDK1 ablation resulted in alterations in effector and regulatory T cell frequencies and function in PDK1-CKO mice. CD4 T cells isolated from diseased PDK1-CKO mice exhibited an activated phenotype and higher proportion of YFP⁺ cells compared to PDK1-CHET mice (Figure 1d). Functionally, a significant fraction of YFP⁺ CD4 T cells from PDK1-CKO mice, but not PDK1-CHET mice produced IL-4 (but not IFN- γ) following stimulation with anti-CD3/anti-CD28 antibodies (Figures 1e and S2a). GATA3 staining confirmed that YFP⁺ T cells were Th2 T cells, suggesting spontaneous differentiation of Th2 effector cells in PDK1-CKO mice (Figure S2b). A small fraction of YFP⁺ CD4 T cells from PDK1-CKO mice but not PDK1-CHET mice also produced IL-17A after stimulation (Figure S2c).

In diseased PDK1-CKO mice, the frequency of Foxp3⁺ CD25⁺Tregs was significantly decreased with a corresponding increase in a Foxp3⁻CD25⁺ effector T cells (Figure S3a). To assess the role of Tregs in the disease process, we transferred CD4⁺CD25⁺ Tregs from wild type (WT) mice into PDK1-CKO recipients (<4 weeks of age). While PDK1-CKO recipients of WT Tregs exhibited improved survival and reduced skin symptoms, morbidity and disease score were only slightly reduced in Treg recipients which continued to exhibit skin pathology (Figure S3c,d), despite reconstitution of Foxp3⁺ Tregs (Figure S3e). Therefore, the reduced Treg frequency in PDK1-CKO mice is not a primary disease trigger.

T cell-intrinsic immune impairments in PDK1-CKO mice cause mild skin disease

We assessed the role of aberrantly activated T cells from PDK1-CKO mice in promoting disease development by transfer of purified T cells from PDK1-CKO or -CHET mice into lymphocyte-deficient RAG2^{-/-} recipients, to generate RAG2+CKO and RAG2+CHET recipient mice. RAG2+CKO mice gradually developed skin lesions and hair loss in a limited region, while RAG2+CHET mice remained healthy (Figure 2a). Notably, the overall disease score of RAG2+CKO mice was much lower compared to intact PDK1-CKO donor mice

(Figure 2b), with 8 of 9 mice surviving beyond 12 weeks post transfer. Skin histology revealed skin scale formation, hyperkeratosis, epidermal hyperplasia and fibrosis (Figure 2c and Table S1). Moreover, YFP⁺ CD4 T cells infiltrated into the dermal layer of RAG2+CKO but not RAG2+CHET mice (Figure 2d). These results indicate that pathogenic skin-homing T cells develop in diseased PDK1-CKO mice, yet these T cells do not appear to be broadly autoreactive and do not promote severe systemic morbidity manifested in PDK1-CKO mice.

Hematopoietic lineage cells from PDK1-CKO mice are not sufficient to trigger skin pathology

We asked whether hematopoietic lineage cells in PDK1-CKO mice contributed to disease pathogenesis using bone marrow (BM) reconstitutions. Lethally irradiated RAG2^{-/-} mice were reconstituted with T cell-depleted BM from PDK1-CKO or -CHET mice resulting in CKO-BMT and CHET-BMT mice, respectively. CKO-BMT mice did not exhibit mortality, but gradually developed weight loss and morbidity 12 weeks post-BMT coincident with reduced Treg levels while CHET-BMT mice did not develop disease and had normal Treg levels (Figure S4a). Notably, skin symptoms in CKO-BMT mice were only sporadically observed with no overt hair loss, dermatitis, or epidermal hyperplasia, nor was there infiltration of YFP⁺ CD4⁺ T cells into the skin (Figure S4c and Table S1). These BMT results indicate that the T cell-intrinsic impairments were not responsible for the severe skin disease and development of pathogenic skin-homing T cells observed in parent PDK1-CKO mice, and suggest that a non-hematopoietic cell may promote skin disease pathogenesis.

OX40-Cre mediated PDK1 ablation targets mature keratinocytes

OX40-directed gene ablation has been associated with potential manifestations in skin (Cornish *et al.*, 2012). During our analyses, we observed YFP expression not only in CD4⁺ T cells, but also in the epidermis of both PDK1-CKO and -CHET mice (Figure 3a). Notably, sorted YFP⁺ cells residing in the epidermis of PDK1-CKO mice exhibited greatly reduced PDK1 transcript expression compared to YFP⁺ cells in PDK1-CHET skin (Figure 3a, right). These results also suggested OX40 is expressed in YFP⁺ skin cells, which we confirmed by OX40-qPCR of sorted YFP⁺ epidermal cells from PDK1-CKO and -CHET mice, but not in YFP⁺ cells from homozygous OX40^{Cre/Cre} ROSA-YFP mice which lack OX40 expression due to Cre insertion (Klinger *et al.*, 2009) (Figure 3b). OX40 expression was mostly localized to basal layer keratinocytes (defined by Keratin14 expression) by immunofluorescence (Figure S5a). Together, these results demonstrate specific ablation of PDK1 in basal keratinocytes of PDK1-CKO mice through OX40-dependent Cre expression.

We investigated whether PDK1 ablation in epidermal cells affected its turnover. The basal epidermal layer of PDK1-CKO mice exhibited Ki67 staining compared with PDK1-CHET mice (Figure 3c), indicative of hyperproliferation. Because disease manifestations in PDK1-CKO mice occurred only at 5-7 weeks of age, we hypothesized that the timing of YFP expression/PDK1 ablation may occur in mature keratinocytes and not in the developing mouse skin. Interestingly, YFP was only sporadically expressed in the epidermis of mice at 10 days and 3 weeks of age, and was highly expressed only in adult mice at 6 weeks of age when significant CD4⁺YFP⁺ T cells had also infiltrated the skin (Figure 3d).

Correspondingly, mild epidermal hyperplasia and microabscess was observed in mice at 3 weeks of age but not at 10 days (Figure S5b), showing that PDK1 ablation in keratinocytes is coincident with disease development.

Lymphocyte-deficient PDK1-CKO mice develop skin pathology

To dissect the contribution of the skin-intrinsic defects in PDK1-CKO mice, we generated lymphocyte-deficient CKO×RAG2^{-/-} (and control CKO×RAG2^{+/-}) mice by interbreeding. These mice also exhibited skin-specific YFP expression and PDK1 ablation which were both largely confined to α6-integrin⁺ basal keratinocytes (Figure S6). Compared to control CKO×RAG2^{+/-} which exhibited morbidity and skin disease similar to the parent PDK1-CKO line, CKO×RAG2^{-/-} mice developed only a mild skin dermatitis with reduced morbidity (Figure 4a,b). The mice survived longer than parent PDK-CKO mice but eventually succumbed to disease at 12-16 weeks (Figure 4c). There was inflammation in the skin of CKO×RAG2^{-/-} mice, but reduced epidermal hyperplasia and fibrosis compared with CKO×RAG2^{+/-} skin (Figure 4a). However, extensive Ki67 expression was still observed in the basal layer of CKO×RAG2^{-/-} skin (Figure 4d), indicating that epidermal hyperplasia observed in the parent PDK1-CKO strain is due to a skin-intrinsic defect. This increased turnover of PDK1-deficient keratinocytes was not associated with overt cell death, based on lack of cleaved Caspase3 staining (Figure S7). Together, these results reveal a role for PDK1 in keratinocyte function and integrity, and that PDK1-deficient keratinocytes can initiate disease development.

PDK1 ablation in keratinocytes impairs differentiation and promotes inflammation

We investigated whether PDK1 ablation in epidermis affected its function, integrity and/or was associated with structural defects observed in AD and psoriasis (Boguniewicz and Leung, 2011; Roberson and Bowcock, 2010). Expression of important structural proteins including keratin-10 (krt10), loricrin and keratin-14 (krt14) was significantly impaired in the skin of PDK1-CKO compared to -CHET mice, as assessed by immunohistochemistry and/or quantitative RT-PCR (Figure 5a). By contrast, other structural proteins including keratin-14 (krt14), keratin-1 (krt1), filaggrin and involucrin were comparably expressed in PDK1-CKO and -CHET skin (Figure S8). Krt10 and loricrin expression were also reduced in CKO×RAG2^{-/-} skin (Figure 5b), indicating that PDK1 ablation in keratinocytes directly impacts keratinocyte differentiation. Interestingly, expression of thymic stromal lymphopoietin (TSLP), an inflammatory mediator, was specifically upregulated in the epidermis of PDK1-CKO mice (but not in control PDK1-CHET skin) by immunofluorescence (Figure 5c). TSLP was specifically expressed by sorted YFP⁺ keratinocytes of PDK-CKO (but not -CHET) mice, and also by YFP⁺α6⁺ keratinocytes of CKO×RAG2^{-/-} (but not CHET×RAG2^{-/-}) mice (Figure 5d). Significant levels of TSLP were also detected in the serum of both PDK1-CKO and CKO×RAG2^{-/-}, but not PDK1-CHET mice (Figure 5e). Together, these results establish that PDK1-ablated keratinocytes produce TSLP.

PDK1 ablation in keratinocytes is sufficient for inducing skin infiltration and Th2 activation

In order to assess the role of PDK1^{-/-} keratinocytes in promoting disease in the context of a normal T cell response, we transferred T cells from PDK1-CHET mice into CKO×RAG2^{-/-}

mice (with PDK1-ablated keratinocytes) or CHET×RAG2^{-/-} mice with intact keratinocytes, and assessed disease outcome. Transfer of (CHET) T cells into CKO×RAG2^{-/-} hosts significantly exacerbated disease including a higher overall disease score and severe skin pathology, including focal abscesses and ulcers (Figure 6a,b), while T cell transfers into CHET×RAG2^{-/-} hosts caused no disease. Moreover, CHET-derived CD4⁺YFP⁺ T cells infiltrated into the skin of CKO×RAG2^{-/-} mice but not CHET×RAG2^{-/-} mice (Figure 6c). Importantly, CHET T cells recovered from CKO×RAG2^{-/-} hosts with the aberrant skin phenotype produced IL-4 after stimulation while those recovered from CHET×RAG2^{-/-} recipients with intact skin produced negligible IL-4 (Figure 6d). Together, these results indicate that the altered skin environment induced by PDK1 ablation in keratinocytes is the primary driver of altered Th2 differentiation and skin-specific T cell homing that exacerbates and perpetuates disease.

DISCUSSION

We present here a model of inflammatory skin disease with systemic inflammation and morbidity in mice with conditional ablation of the PDK1 kinase (PDK1-CKO) in activated/regulatory T cell subsets and in mature keratinocytes. Through a series of T cell transfers, bone marrow reconstitutions and crossing to lymphocyte-deficient backgrounds, we demonstrate that ablation of PDK1 in keratinocytes initiates disease pathogenesis, that is further exacerbated by T cell-mediated immune responses. We therefore define a role for PDK1 in keratinocytes to maintain their homeostasis and prevent skin inflammation, and a model for studying complex pathogenesis of inflammatory skin diseases.

Inflammatory skin disease is caused by the interplay of skin barrier disruption and immune dysregulation. PDK1-CKO mice had triple defects in conventional effector/memory CD4 T cells, Tregs, and keratinocytes. We used a series of T cell transfers, bone marrow reconstitutions and crossings to lymphocyte-deficient backgrounds to identify the respective roles of PDK1 ablation in each cell type. PDK1 ablation in PDK1-CKO mice resulted in reduced Tregs, which is consistent with previous findings that PDK1 is required for Treg function (Park *et al.*, 2010). However, PDK1 ablated CD4 T cells (both conventional effector/memory CD4 T cells and Tregs) in the context of healthy skin (in BMT) caused mild morbidity, few skin symptoms and no T cell infiltration into the skin (Figure S4). Conversely, transferring wild type Tregs into PDK1-CKO mice did not prevent disease induction, which indicates that impaired Tregs were not the primary initiating factor of disease. Thus, immune-mediated defects appear secondary to disease initiation.

We demonstrate that PDK1-ablation in keratinocytes was sufficient to trigger skin pathology and drive inflammatory disease. PDK1 ablation in keratinocytes in the absence of lymphocytes (CKO×RAG2^{-/-}) exhibited similar defects in expression of keratinocyte structural proteins, epidermal hyperplasia and skin pathology as the parent PDK1-CKO mice (Table S1). Moreover, PDK1 deficient keratinocytes in the context of normal T cells developed severe dermatitis similar to the PDK1-CKO mice (Figure 6, Table S1). These results indicate that PDK1 ablated keratinocytes can initiate disease in the context of a normal immune system. Importantly, PDK1-deficient keratinocytes produced the proinflammatory cytokine-TSLP (Figure 5), known to promote Th2 responses. PDK1

ablation in keratinocytes therefore disrupts structural integrity of the skin which in turn promotes inflammation, Th2 differentiation and infiltration, setting up a cascade of tissue damage, inflammation-induced acanthosis (51, 52) and fibrosis.

Our results reveals a role for PDK1 in maintaining keratinocyte function and integrity. Skin specific gene ablation in PDK1-CKO mice occurs due to OX40-mediated Cre expression in keratinocytes also observed in a previous study (Cornish *et al.*, 2012). Based on PDK1 function in other types of cells, there are multiple pathways upstream of PDK1 which could impact keratinocyte differentiation including Akt/mTOR, and/or the canonical NF- κ B pathway (Kang *et al.*, 2013; Park *et al.*, 2010; Tanaka *et al.*, 2005). Akt signaling in keratinocytes has been shown to induce their differentiation and survival (O'Shaughnessy *et al.*, 2009; Thrash *et al.*, 2006), and PDK1 effects on keratinocyte turnover could be mediated through Akt. Keratinocyte-specific ablation of the I κ B kinase, IKK2, which is required for NF- κ B activation (Li *et al.*, 2000; Li *et al.*, 1999) resulted in psoriasis-like dermatitis, impaired expression of loricrin and filaggrin, upregulated expression of TNF- α , and triggered similar but milder skin symptoms compared to PDK1-deficient keratinocytes (Cornish *et al.*, 2012; Pasparakis *et al.*, 2002; Stratis *et al.*, 2006). We propose that the severe skin phenotypes induced by PDK1 ablation in keratinocytes may be due to dysregulation of multiple pathways (e.g. Akt, NF κ B) for which PDK1 serves as a central upstream regulator.

The skin manifestations due to PDK1 ablation mimic different features of human skin diseases, with epidermal hyperplasia, reduced Krt10 and loricrin expression, barrier defects and skewed Th2 responses similar to AD, parakeratosis and thickening observed in psoriasis, and skin fibrosis and Th2 responses seen in scleroderma (Chizzolini *et al.*, 2011; Guttman-Yassky *et al.*, 2011). The models presented here which separate skin-intrinsic and immune-mediated bases of complex skin phenotypes, as well as their combined effects, can be of important clinical relevance for designing targeted therapies for treating inflammatory skin syndromes.

MATERIAL AND METHODS

Mice

PDK1^{F/F} mice (Mora *et al.*, 2003) were crossed to R26 ROSA YFP mice (Jackson laboratories, Bar Harbor, ME) to generate PDK1^{F/F} ROSA26 YFP mice, which was further crossed to OX40-Cre mice (Jackson Laboratories, Bar Harbor, ME) to generate PDK1-CKO mice (OX40^{+cre}PDK1^{F/F}ROSA26 YFP) and PDK1-CHET mice (OX40^{+cre}PDK1^{F/+}ROSA26 YFP) mice, respectively. PDK1-CKO \times RAG2^{+/-} and PDK1-CHET \times RAG2^{-/-} were generated by crossing to RAG2^{-/-} mice (Taconic Farms, Germantown, NY). All mice were housed and bred in specific pathogen-free conditions in the Animal Barrier Facility at the Columbia University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Columbia University.

Flow cytometry

Fluorochrome-conjugated antibodies specific for CD4, -CD8, -CD25, CD44 -IFN- γ , and -GATA3, CD62L, IL-2, IL-4, and Foxp3 were purchased from BD Biosciences (San Jose, CA) and eBiosciences (San Diego, CA). For nuclear staining, cells were fixed and permeabilized by FoxP3 fixation/permeabilization buffer (eBioscience) and stained with anti-Foxp3 or GATA3 antibody. Cells were analyzed using the FACSCantoII or LSRII flow cytometer (BD Biosciences) and data were analyzed using Flowjo software (Treestar, Ashland, OR).

Histology

All H&E and masson trichome staining were performed by the Pathology Core of Columbia University.

Disease scoring

Disease was scored based on 4 aspects: activity, weight, hair loss and skin condition. For activity, 0=normal; 1=hunched position at rest; 2= hunched, mild resistance when handled; 3=severely hunched, no resistance when handled. For weight, 0=normal; 1=<15% weight loss; 2=15-30% weight loss; 3= >30% weight loss. For hair loss, 0=normal; 1=<10% hair loss; 2=10-30% hair loss; 3= >30% hair loss. For skin condition, 0=normal; 1= red, no scales or crust; 2=light skin scales and crusty appearance; 3=moderate skin scales and crusty appearance; 4=severe skin scales and crusty appearance, loss of skin elasticity. The total disease score was the sum of 4 individual attributes, with the maximum combined disease score =13.

Immunofluorescence

Skin samples were fixed in 4% paraformaldehyde, dehydrated in 30% sucrose, embedded in OCT and frozen. Skin cryostat sections (5mm) were stained with antibodies specific for YFP (life technologies, Grand Island, NY), OX40 (BD Biosciences), filaggrin (Santacruz, Dallas, TX), involucrin (Covance, Princeton, NJ), loricrin, cytokeratin14, cytokeratin15, cytokeratin10 (Genetex, Irvine, CA) Ki67, CD4 (eBioscience), cleaved caspase 3 (Cellsignaling), and TSLP (R&D Systems, Minneapolis, MN), then stained with fluorescently-coupled secondary anti-rabbit or -rat IgG (Cellsignaling Technologies, Danvers, MA), or streptavidin-conjugated Cy5 (Biolegend). Images were obtained using LAS AF 6.2 software on a motorized Leica DMI 6000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

T cell transfer and bone marrow reconstitution

T cells were purified from spleen and peripheral lymph nodes of PDK1-CKO and -CHET mice by anti CD3 ϵ microbeads (Miltenyi Biotec). T cells (5×10^6 /mouse) were transferred to RAG2 recipients intravenously (i.v.) by tail vein injection. For bone marrow reconstitution experiments, bone marrow mononuclear cells isolated from the femurs and tibia of CD45.2 PDK1-CKO or -CHET mice were depleted of CD3⁺ T cells using anti-CD3 ϵ microbeads (Miltenyi), and 5×10^6 cells were transferred i.v. into CD45.1 RAG2^{-/-} mice irradiated with 9Gy by RS2000 irradiator (Rad Source Tech, Suwanee, GA) one day previously.

T cell stimulation and intracellular cytokine staining

CD4 T cells purified by negative selection (Stem cell Tech), were activated with anti-CD3/CD28 antibodies (5µg/ml/2.5µg/ml, BD Biosciences) in the presence of 0.66µl/ml GolgiStop (BD Biosciences) for 5hrs, and cytokine production was assessed by intracellular cytokine staining.

ELISA

Serum TSLP level were measured using mouse TSLP ELISA Ready-Set-Go reagent kit (eBiosciences) according to the manufacturer's instructions.

Real time RT-PCR

Total RNA was harvested by RNAeasy isolation kit (Qiagen, Valencia, CA). Total RNA quality was determined by Bioanalyzer (Agilent, Cold Spring, NY). RT-PCR was done using SYBR green mastermix (Agilent). All primer sequences are in supplementary Table S2.

Statistical analysis

For the two group comparisons, statistical differences were determined by unpaired two-tailed t-test. Multiple sample comparisons were calculated by one way ANOVA, with $p < 0.05$ indicating significance. All values were calculated with Excel (Microsoft), Sigmaplot (Systat Software, Inc. San Jose, CA) and Prism software (GraphPad, La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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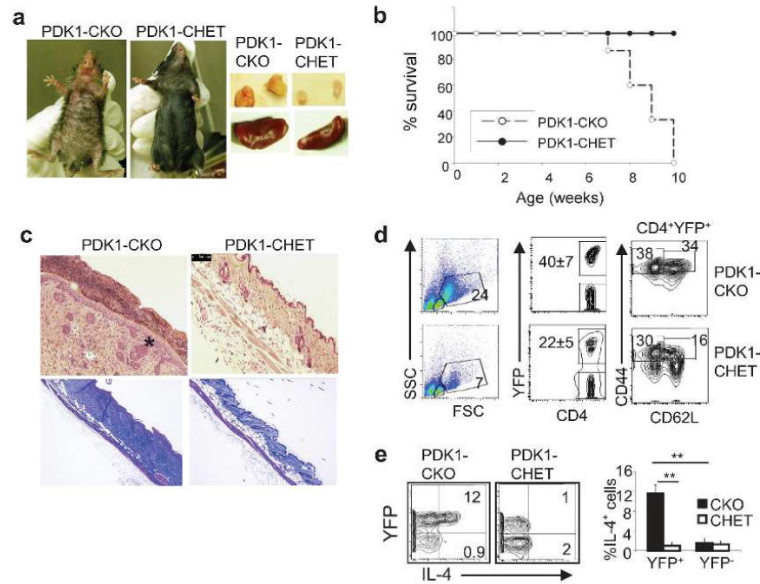


Figure 1. Conditional ablation of PDK1 in OX40-expressing cells results in diffuse dermatitis, fibrosis and Th2 polarization

(a) Macroscopic view of PDK1-CKO and PDK1-CHET mice (left), cervical lymph nodes (upper right) and spleen (lower right) from each strain. (b) Survival curve of PDK1-CHET (n=20) and PDK1-CKO (n=15) mice. (c) Skin sections from PDK1-CKO and PDK1-CHET mice showing H&E staining (upper row) and Masson Trichrome staining (lower row). Scale bars:100µm. (* indicates epidermal hyperplasia). (d) Flow cytometric analysis of CD4 T cells from peripheral lymph nodes of PDK-CKO and PDK1-CHET mice (7-8wks old). Left: forward scatter versus side scatter; middle: YFP expression by CD4 T cells (shown as mean ±SEM, n=6), right: CD44 versus CD62L expression of CD4⁺YFP⁺ cells. (e) IL-4 production from PDK1-CKO CD4 T cells expressed as percent YFP⁺IL-4⁺ in each quadrant drawn based on control unstimulated T cells.

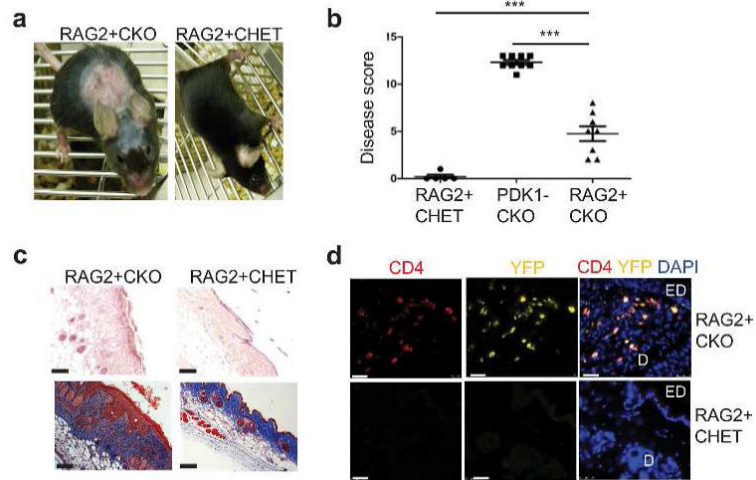


Figure 2. PDK1-deficient T cells promote skin pathology and mild disease in RAG2

(a) Macroscopic view of RAG2^{-/-} recipients 13 weeks post-transfer of 5×10^6 CD3⁺ T cells from PDK1-CKO (“RAG2+CKO”, left) or PDK1-CHET (“RAG2+CHET”, right) mice. (b) Disease score (see methods) of RAG2+CHET (n=5) or RAG2+CKO (n=8) recipients of T cells 12 weeks post-transfer compared to the disease score of 8 week-old PDK1-CKO parental mice. (c) Skin sections of RAG2+CKO and RAG2+CHET recipient mice stained with H&E (upper) or Masson Trichrome (lower). Scale bars: 100 μ m. (d) Immunofluorescence of CD4 (first column), YFP (second column) expression together with DAPI nuclear staining (third column) in skin sections from RAG2+CKO (upper row) and RAG2+CHET (lower row) mice. Scale bars: 25 μ m. D=Dermis; ED=epidermis.

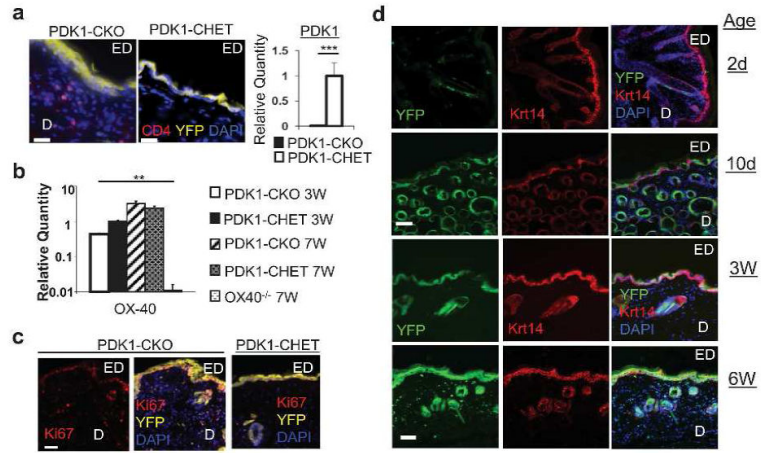


Figure 3. OX40-Cre mediated PDK1 ablation occurs in epidermal cells of PDK1-CKO mice
 (a) Left, Immunofluorescence of CD4 and YFP expression with DAPI nuclear stain in the skin of PDK1-CKO mice and PDK1-CHET mice, from 6 experiments. Scale bars: 25µm. D=Dermis; ED=epidermis. Right, PDK1 transcript expression (mean ± SEM, n=3) in YFP⁺ epidermal cells measured by qPCR, calculated relative to levels in -CHET/YFP⁺ normalized to 1. (b) OX40 expression by qPCR in YFP⁺ cells sorted from PDK1-CKO, -CHET and OX40^{-/-}/ROSA^{YFP} mice at 3 and/or 7 weeks of age, expressed relative to levels in -CHET cells (normalized to 1.0). (c) Ki67 expression in the skin of PDK1-CKO and PDK1-CHET mice (7-8wks). Scale bars: 50 µm (d) Immunofluorescence of YFP (green) and cytokeratin 14 (red,) and DAPI (third column) in the skin of PDK1-CKO mice at the indicated ages.

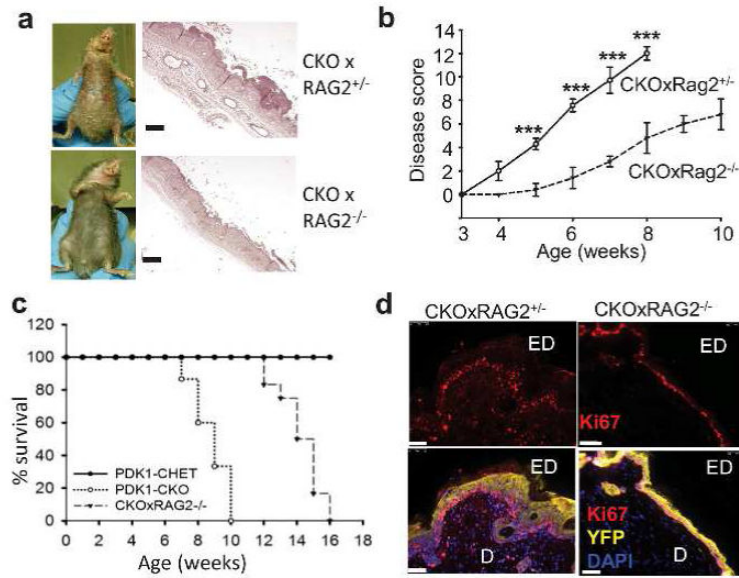


Figure 4. Lymphocyte-deficient PDK1-CKO mice spontaneously develop skin pathology and disease symptoms

(a) Macroscopic view of PDK1-CKO×RAG2^{+/-} (CKO×RAG2^{+/-}) and PDK1-CKO×RAG2^{-/-} (CKO×RAG2^{-/-}) mice, with corresponding H&E-stained skin sections obtained at 8 weeks of age. Arrows indicate location of skin lesions. Scale bars: 100µm. (b) Disease score of CKO×RAG2^{+/-} (n=6) and CKO×RAG2^{-/-} mice (n=8). (c) Survival curve of CKO×RAG2^{-/-} (n=12) mice superimposed on survival curve of PDK1-CHET (n=20), PDK1-CKO (n=15) mice. (d) Skin sections stained with Ki67 (red), YFP (yellow) and DAPI (blue) as indicated. Scale bars: 50µm.

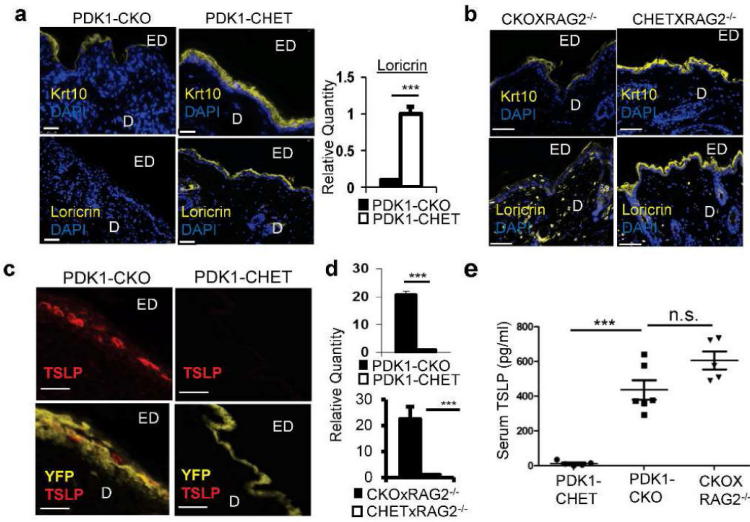


Figure 5. PDK1 ablation in skin impairs keratinocyte differentiation and increases inflammation

(a) Left, Immunofluorescence staining of Keratin 10 (upper) and Loricrin (lower) in skin sections from PDK1-CKO and PDK1-CHET mice. Scale bars, 25 μ m. Right, qPCR of loricrin expression (average of triplicates) in sorted YFP⁺ epidermal cells. (b) Immunofluorescence of Keratin 10 (upper) and Loricrin (lower) staining in skin sections from CKO \times RAG2^{-/-} and CHET \times RAG2^{-/-} mice. Scale bars: 50 μ m. (c) Left, Immunofluorescence of YFP and TSLP expression in the skin of PDK1-CKO mice and PDK1-CHET mice (7wks old). Scale bar: 25 μ m. (d) TSLP transcript expression level (qPCR, average of triplicates) in sorted YFP⁺ epidermal cells of PDK1-CKO and -CHET mice (upper) and YFP⁺ α 6-integrin⁺ cells of CKO \times RAG2^{-/-} and CHET \times RAG2^{-/-} skin (lower). (e) Serum TSLP level from 6-8 weeks old mice as indicated. *** represents p<0.001. ; n.s.:not significant.

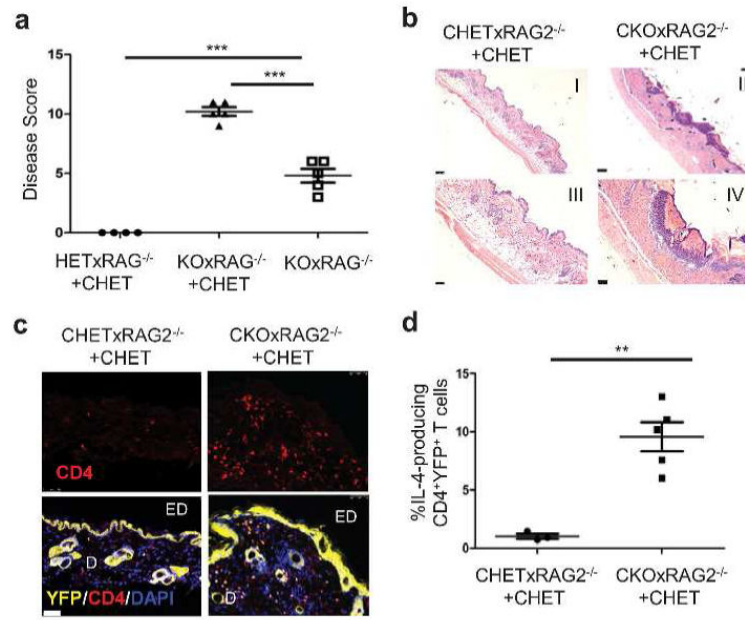


Figure 6. PDK1 ablation in keratinocytes is sufficient for inducing skin infiltration and Th2 activation.

T cells from PDK1-CHET mice were transferred to CHET×RAG2^{-/-} or CKO×RAG2^{-/-} recipients (see methods), resulting in CHET×RAG2^{-/-}+CHET or CKO×RAG2^{-/-}+CHET mice, respectively. (a) Disease score of recipient strains 2 weeks post-transfer for CHET×RAG2^{-/-} (n=4) and CKO×RAG2^{-/-} (n=5) recipient mice, compared to Disease score of age-matched CKO×RAG2^{-/-} mice (n=5) without transferred T cells. (b) Skin sections (H&E stained) from CHET×RAG2^{-/-}+CHET and CKO×RAG2^{-/-}+CHET mice two weeks post-transfer. Scale bars: I-II, 100μm; III-IV, 50μm. (c) Immunofluorescence of CD4 and YFP expression in the skin of the indicated strains. Scale bars:50μm. (d) IL-4 production by T cells isolated from CHET×RAG2^{-/-}+CHET or CKO×RAG2^{-/-}+CHET mice expressed as mean frequency±SEM of CD4⁺YFP⁺ IL-4⁺ cells after 5hrs stimulation.