IL-17 axis is a significant driver of skin inflammation in Card14 mutant pityriasis rubra pilaris model mice

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Abstract

Pityriasis rubra pilaris (PRP) is a rare inflammatory keratinization disorder with perifollicular erythema, and most autosomal dominant familial cases of atypical juvenile (type V) PRP are caused by gain-of-function mutations in CARD14, which encodes caspase recruitment domain-containing protein 14 (CARD14). We report the first mouse model of PRP to carry a homozygous knock-in mutation, c.380G>C (p.Cys127Ser) corresponding to a PRP-causative human mutation, in CARD14. The Card14C127S/C127S knock-in mice recapitulate key aspects of human PRP, including hair follicle dilatation, follicular plugs, and palmoplantar hyperkeratosis, and show skin barrier dysfunction, the hyperactivation of innate immunity via the IL-36 signaling and inflammasome pathways, and the excessive activation of the IL-17 axis in the outer root sheath and interfollicular epidermis. Administering anti-IL-17A neutralizing antibody significantly attenuates the skin symptoms in mutant mice. Thus, this knock-in mouse is a valid model for further evaluating early events in the PRP pathogenesis and for developing PRP therapies.

Introduction

Several of the genetic causes or predisposing factors for inflammatory keratinization disorders are associated with autoinflammatory mechanisms. We have proposed that inflammatory keratinization disorders whose pathogenic mechanisms are autoinflammatory can be regarded as autoinflammatory keratinization diseases (AiKDs)

CARD14, which encodes caspase recruitment domain-containing protein 14 is one of the causative genes of AiKDs

A number of CARD14 gain-of-function variants/mutations have been reported as predisposing factors for psoriasis vulgaris (PV)

generalized pustular psoriasis (GPP) with PV

and atypical juvenile (type V) pityriasis rubra pilaris (PRP)

CARD14 is expressed and localized mainly in the skin, especially in keratinocytes

Psoriasis-causative CARD14 variants hyperactivate nuclear factor κB (NFκB) and upregulate the expression of a subset of psoriasis-associated genes in keratinocytes

CARD14 mutants expressed in the keratinocytes are thought to be responsible for the disease pathology and clinical manifestations of those inflammatory keratinization diseases that result from CARD14 mutations. Subsequent reports have also implicated CARD14 variants in various autoinflammatory skin disorders, although the pathogenic relevance in each disease has not been well determined.

Pityriasis rubra pilaris (PRP), a clinical entity distinct from psoriasis, is an inflammatory erythematous keratinization disorder that shows perifollicular erythema, often with confluent configurations, follicular plugging, pityriasis capitis, and palmoplantar hyperkeratosis. Although most PRP cases are regarded as sporadic, some are familial, particularly type V PRP, which is one of six subtypes. Notably, the skin eruptions in patients with type V PRP first appear in infancy or early childhood and tend to run a chronic course with no sustained clearance of the skin lesions. In 2012, gain-of-function mutations in CARD14 were identified in some autosomal dominant familial cases of PRP

In 2017, we reported that, among 22 patients with PRP, all 3 patients with type V PRP were found to have CARD14 mutations

To date, 8 heterozygous mutations in CARD14 have been reported in patients with type V PRP

However, the
detailed contribution of CARD14 gain-of-function to PRP pathogenesis remains unclarified because type V PRP is rare and there have been no reports of PRP model mice. Therefore, we sought to determine the functional impact of the CARD14 mutation c.380G > C (p.Cys127Ser) in vivo, a mutation that was identified in PRP patients\(^4\). Using a knock-in (KI) mouse model generated by CRISPR-Cas9 editing, we present further pathogenic mechanisms and describe the therapeutic effectiveness of anti-IL-17A neutralizing antibody in type V PRP due to the CARD14 variant p.Cys127Ser.

**Results**

C57BL/6J-Card14\(^{em1Nmea}\) homozygous mice (Card14\(^{C127S/C127S}\)) show a PRP-like phenotype and defective skin barrier function.

To gain insights into the roles of CARD14 hyperactivity in the autoinflammatory pathogenesis in vivo, we used a CRISPR-Cas9 gene-targeting approach to generate Card14KI mice. The alteration and deletion of the Glu138 residue in CARD14 is associated with both GPP\(^5\) and PRP phenotypes\(^8,9,10\). In contrast, p.Cys127Ser has been previously reported in a patient with type V PRP\(^4\) and in one kindred with CARD14-associated papulosquamous eruptions with PRP-like skin features, classic islands of sparing and palmoplantar keratoderma\(^11\). We reported that skin lesions caused by p.Cys127Ser are severe and that the patient showed diffuse erythema with fine scaling on the trunk, the extremities and the face, and had a large number of brown macules and patches on the trunk\(^4\) (Supplementary Fig. 1). In this context, we generated Card14KI PRP model mice carrying the substitution mutation c.380G > C, which corresponds to the previously reported CARD14 mutation p.Cys127Ser in a patient with type V PRP\(^4\). p.Cys127Ser lies between the CARD domain and the coiled-coil domain of the CARD14 protein, close to residues that correspond to the pathogenic mutations in GPP (p.Glu138Ala\(^5\)) and in type V PRP (p.Leu124Pro\(^4\), p.Gln136Leu\(^4\), p.Glu138Lys\(^9,10\), and p.Glu138del\(^8\)) (Supplementary Fig. 2). Frequencies of Card14KI genotypes in pups derived from intercrosses between heterozygous Card14KI mice showed that there were few or no embryonic lethal homozygous Card14KI mice (Supplementary Table 1).

**Skin phenotypes of the Card14KI PRP model mice**

Card14\(^{C127S/C127S}\) mice were produced at the expected frequencies (Supplementary Table 1) and were indistinguishable from their wild-type (WT) littermates at birth. At postnatal days 4–6 (P4–P6), the Card14\(^{C127S/C127S}\) mice started to show abnormal scaling of the skin over the whole body, including the trunk, limbs, paws, tail, and face (Fig. 1b). Histological analysis of skin biopsied from the back, tail, and hind paw of the Card14\(^{C127S/C127S}\) mice at P5 revealed severe hyperkeratosis, parakeratosis, acanthosis, and infiltration of lymphocytes in the dermis (Fig. 1e). Importantly, many follicular plugs, a typical feature that distinguishes PRP from psoriasis, were present in the Card14\(^{C127S/C127S}\) mice (Fig. 1e). Transepidermal water loss (TEWL) and a toluidine blue exclusion assay indicated severe defects in both the “inside-out” and “outside-in” skin permeability barriers in the Card14\(^{C127S/C127S}\) mice (Fig. 1f, g). Moreover, their weight gain markedly slowed after P5 (Fig. 1a) and most of them died by P11, although...
about 19% (8/42) of the $\text{Card14}^{C127S/C127S}$ mice lived as long as the WT and C57BL/6J-Card14$^{em1Nmea}$ heterozygous mice ($\text{Card14}^{C127S/+}$). These data suggest that the postnatal lethality in the $\text{Card14}^{C127S/C127S}$ mice was caused by dehydration as the result of the epidermal barrier defect that developed after birth. In the adult $\text{Card14}^{C127S/C127S}$ survivors, the ear thickness was significantly greater than in WT mice (Fig. 1d). The mean weight of the surviving adult $\text{Card14}^{C127S/C127S}$ mice at the age of 2–12 months was 25.8g (n = 7) (data not shown). Many of these abnormal phenotypic features of the $\text{Card14}^{C127S/C127S}$ survivors continued throughout life, except the skin scaling on the front and hind paws, which improved spontaneously over time. In contrast, heterologous $\text{Card14}^{C127S/+}$ mice showed mild hyperkeratosis and acanthosis in the back, tail, and fore and hind paws, and the dilatation of hair pores and some keratinous plugs at P5 (Fig. 1e). At 2–3 weeks of age, they displayed hyperkeratosis and scaling on the tail (Fig. 1c). The scaling gradually increased until 4–5 weeks, and then spontaneously disappeared by 6–7 weeks (Fig. 1c). Other phenotypes seen in homologous $\text{Card14}^{C127S/C127S}$ mice, such as permeability barrier dysfunction, weight loss, postnatal lethality, ear thickness, and infiltration of lymphocytes, were not significant in the $\text{Card14}^{C127S/+}$ mice than in their WT littermates (Fig. 1a–e).

Trans-epidermal Water Loss (Tewl)

The Tewl of the $\text{Card14}^{C127S/C127S}$ mouse skin at P7 was approximately 6 times as high as those of the WT and $\text{Card14}^{C127S/+}$ mice (Fig. 1f). In contrast, there was no difference in Tewl between the WT and the $\text{Card14}^{C127S/+}$ mice (Fig. 1f). These findings indicate that the $\text{Card14}^{C127S/C127S}$ mouse skin has severe water permeability barrier dysfunction. In addition, a toluidine blue exclusion assay showed that more dye remained in some parts of the skin of the $\text{Card14}^{C127S/C127S}$ mice than in those of the WT and $\text{Card14}^{C127S/+}$ mice at P7 (Fig. 1g), suggesting that the $\text{Card14}^{C127S/C127S}$ mice also has a defect in the outside-to-inside barrier function in the skin.

Gene expression profiling in the skin of $\text{Card14}^{C127S/C127S}$ mice reveals the hyperactivation of IL-17, and the down-regulation of filaggrin family genes and $S100a7a$

To define the impact of the mutation in the $\text{Card14}$ gene on early transcriptional events associated with pathogenesis, DNA microarray analysis was performed on the WT and $\text{Card14}^{C127S/C127S}$ mice at P0 and P5. By the criteria of a ≥ 2-fold expression change, we found that 760 genes were upregulated and 459 genes were downregulated in the $\text{Card14}^{C127S/C127S}$ skin at P5 (Fig. 2a). In contrast, only a small number of differentially expressed genes (DEGs) were identified between the two genotypes at P0 (Fig. 2a). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using the DAVID bioinformatics resources indicated the “cytokine–cytokine receptor interaction” and the “IL-17 signaling pathway” to be the top enriched functional clusters in the upregulated DEGs of the $\text{Card14}^{C127S/C127S}$ mice at P5 (Fig. 2b, Supplementary Table 2), suggesting a close link between PRP and Th17 cell responses. Other enriched clusters that are potentially associated with skin diseases include the NOD-like receptor signaling pathway, Th17 cell differentiation, and the TNF signaling pathway (Fig. 2b,
Supplementary Table 2). Both microarray and qRT-PCR analyses confirmed the elevated expression of inflammatory cytokines (Il1b, Il1f6, Il6, Il12b, Il17a, Il17f, Il23a, Tnfa) and key genes in the IL-17 signaling pathway (Fig. 2c, d). These IL-17 target genes include chemokines (Cxcl1, Cxcl2, Cxcl5, Cxcl10, Ccl2, Ccl7, Ccl20), hyperproliferative keratins (Krt6a, Krt6b, Krt16), small proline-rich proteins (Sprr1b, Sprr2b, Sprr2d, Sprr2e, Sprr2g), antimicrobial peptides (Defb1, Defb3, Defb14), S100 proteins (S100a8, S100a9), late cornified envelope proteins (Lce3a, 3b, 3c, 3d), serine protease inhibitors (Sepinb3a, 3b, 3c, 3d), caspases (Casp1, Casp4), and lipocalin-2 (Lcn2) (Fig. 2c, d).

S100a7a, which is generally upregulated in the skin lesions of psoriasis, was slightly downregulated in these PRP model mice at P5 (Fig. 2c, d). Supporting this observation, the immunohistochemical staining of skin from the Card14C127S/C127S mice at P5 indicated that S100a9 was expressed at high levels, whereas S100a7 was significantly decreased in all epidermal layers (Fig. 2e). The expression of the hyperproliferation keratin genes Krt6a, Krt6b, and Krt16 was significantly higher in the skin of the Card14C127S/C127S mice, although the expression of Krt17, which is another hyperproliferative keratin gene, was not significantly upregulated compared with that expression in the WT controls at P5 (Fig. 2c, d). The cytosolic phospholipase A2 genes Pla2g4d and Pla2g4e, which were reported to be highly expressed in the epidermis in PRP and psoriasis human patients13, were highly expressed in the skin of the Card14C127S/C127S mice at P5 (Fig. 2d). The expression of Flg and Flg2 was decreased in the skin of the Card14C127S/C127S mice at P5 (Fig. 2c, d). The expression of Stat3 encoding signal transducer and activator of transcription 3 (STAT3) was slightly upregulated, but the expression of Jak1 or Jak2 was not upregulated in the skin of Card14C127S/C127S mice at P5 (Supplementary Table 3).

Single-cell transcriptional profiling of skin from the WT and Card14C127S/C127S mice revealed the hyperactivation of IL-17 target genes (Krt16, chemokines), IL-1 family cytokine genes, and NFκB family genes in granular layer cells and cells in the outer roof sheath (ORS). S100a7a was down-regulated in granular layer cells, and Pla2g2f and Il17ra were upregulated in the ORS.

Although most of the Card14C127S/C127S mice died before weaning, we performed single-cell RNA sequencing (scRNA-seq) to reveal the population heterogeneity of the mutant mice that survived to 12 months.

8,364 single cells from WT and Card14C127S/C127S mice are visualized in uniform manifold approximation and projection (UMAP) plots in Fig. 2f. Based on the expression levels of well-known markers to identify cell populations (Supplementary Fig. 3a), we defined 8 main clusters of cell types (Epi_Whole, Imm_Whole, Mes_Whole, End_Whole, Neu, Ery, Death_1, and Death_2), as visualized in the UMAP plots (Fig. 2g). Of these 8 clusters, Epi_Whole, Mes_Whole, End_Whole and Imm_Whole, which were expected to be significantly involved in inflammation and/or keratinization, were subdivided into 20 subclusters: Epi_Basal-1, Epi_Basal-2, Epi_Spinous-1, Epi_Spinous-2, Epi_Granular, Epi_SG, Epi_ORS, Mes_Fibro, Mes_Per/DS/DP/SM, EndVEC, EndLEC, Imm_Cd4, Imm_Cd8, Imm_Treg, Imm_B, Imm_DC-1, Imm_DC-2, Imm_LC, Imm_Mast/Baso, and Imm_Mp (Fig. 2g).
Differentially expressed genes in epidermal cells, mesenchymal cells, and endothelial cells are shown in Supplementary Tables 5 to 7 (epidermal cells: Supplementary Table 5; mesenchymal cells: Supplementary Table 6; endothelial cells: Supplementary Table 7). *Il23a* was intensively expressed in epidermal cells (Epi_Whole, Epi_Granular), and JAK/STAT signaling-associated genes, such as *Jak1* and *Stat3*, were highly expressed in epidermal cells (Epi_Whole, Epi_Granular). In addition, tumor necrosis factor (TNF) signaling-associated genes, including *Tnf*, *Tnfrsf1a* (*Tnfr1*), and *Tnfaip3*, were highly expressed in epidermal cells (Epi_Whole, Epi_Granular). Some cytokine genes in the IL-1 family (*Il33, Il1f5, Il1f6, Il1f9* and *Il1f10*), some IL-1 receptor family genes (*Il1rn, Il1r2, Il1rl2* and *Il1rap*), and the IL-1 signaling adaptor molecule gene *Myd88* were highly expressed in various epidermal cells (Epi_Whole, Epi_Granular, Epi_Basal-1, Epi_Basal-2, Epi_ORS). In addition, some inammasome-related genes (*Casp1, Casp4*, and *Il18* were highly expressed in various epidermal cells (Epi_Whole, Epi_Granular, Epi_Basal-1, Epi_Basal-2, Epi_ORS). *Il6* and *Il18* were highly expressed in mesenchymal cells (Mes_Whole, Mes_Fibro). Epi_Spinous-2, Epi_SG, Mes_Per/DS/DP/SM, and End_LEC showed no remarkable changes in the expression of genes involved in proinflammation. The Nod-like receptors family genes *Nod1* and/or *Nod2* were highly expressed in epidermal cells and in mesenchymal cells (Epi_Whole, Epi_Basal2, Epi_ORS, Mes_Fibro). *Card10* and some genes in the NFκB family, such as *Nfkb2, Rela, Relb*, and *Rel*, were highly expressed mainly in epidermal cells (Epi_Whole, Epi_Granular, Epi_ORS), although *Rel* was also highly expressed in mesenchymal cells (Mes_Fibro). Some chemokine genes, such as *Cxcl1, Cxcl2, Cxcl10, Cxcl13, Cxcl16, Ccl2, Ccl7*, and *Ccl20*, were highly expressed in epidermal cells, mesenchymal cells, and endothelial cells (Epi_Whole, Epi_Granular, Epi_Basal-1, Epi_ORS, Mes_Fibro, End_VEC). The upregulated expression of *Cxcl1* in fibroblasts in the present single-cell analysis was consistent with the results of immunohistochemical staining for CXCL1 in fibroblasts in the skin lesions. (See the following section, “Neutralization of the T helper type 17-polarizing cytokine IL-17 alleviates disease symptoms.”) Some S100 protein genes, including *S100a8* and *S100a9*, were highly expressed in epidermal cells (Epi_Whole, Epi_Granular, Epi_spinous-1, and Epi_ORS). Notably, the expression of *Flg* and *S100a7a* was significantly down-regulated in Epi_Granular. Consistently, the protein expression of filaggrin was reduced in the granular layers of the epidermis in the *Card14*<sup>C127S/C127S</sup> mice (Fig. 2i). *Krt16*, a hyperproliferative keratin, was significantly upregulated in Epi_whole, Epi_Granular, Epi_Spinous-1, and Epi_ORS, whereas *Krt17*, which is another hyperproliferative keratin, was not significantly upregulated in any cluster. *Pla2g2f*, a secretory phospholipase A2, was upregulated in Epi_whole and Epi_ORS. The expression of *Il17ra* encoding IL-17 receptor A, *Il23r* encoding IL-23 receptor, and *Il6ra* encoding IL-6 receptor subunit α were upregulated in Epi_ORS. Immunohistochemical staining with the anti-IL-17 receptor A (IL-17RA) antibody showed stronger IL-17RA staining in the ORS of the *Card14*<sup>C127S/C127S</sup> mice than in that of the WT mice (Fig. 2j). Some genes with significantly different expression are shown in Supplementary Fig. 4.

scRNA-seq found large changes in gene expression in epidermis cells and mesenchymal cells, especially in granular layer cells, cells in the ORS, and fibroblasts. Interestingly, *Krt16* and *Pla2g2f*, which are genes related to hyperkeratosis, and IL-17RA proteins were highly expressed in the ORS cells of the *Card14*<sup>C127S/C127S</sup> mice.
Neutralization of IL-17 ameliorates disease symptoms in Card14C127S/C127S mice.

To assess whether CARD14-induced PRP-like disease symptoms can be attenuated in vivo by the targeted disruption of the IL-17A/Th17 axis, we intraperitoneally injected the Card14C127S/C127S mice with a monoclonal antibody that selectively neutralizes murine IL-17A. This treatment produced a significant reduction in ear thickness in the mutant mice after 2 days of therapy (Day 2) and a significantly decreased PRP-like phenotype for the ears and tail after Day 5 compared with those seen before treatment and those seen in the IgG control animals. The phenotypic improvements and reduced ear thickness continued until Day 8 (Fig. 3a, b), although there was no apparent improvement in TEWL for the ear skin of the Card14C127S/C127S mice (data not shown).

We histologically and immunohistochemically evaluated the skin tissue before treatment (Day 0) and at Day 8. Skin biopsy specimens from the ear showed that scales and crusts had decreased, and inflammatory cell infiltration of the entire dermis also had decreased in the samples obtained at Day 8, although the acanthosis did not improve much (Fig. 3c). The entire ear became thinner and the deformation was greatly reduced at Day 8 (Fig. 3a, c). A skin biopsy specimen from the back at Day 8 showed that the entire epidermis, especially the stratum corneum, was markedly thinned, and the basket-weave pattern in the stratum corneum was recovered. In addition, lymphocytes in the entire dermis were also reduced (Fig. 3c).

Immunohistochemical staining of the back skin showed lower staining levels in the cytoplasm of keratinocytes with anti-IL-1β, IL-36γ (IL-1F9), IL-17C, IL-23, TNFα, MYD88, CCL20, CXCL1, and IL-10 antibodies at Day 8 than at Day 0 (Fig. 3d and Supplementary Fig. 5). Similarly, NF-κB (p65/RELA), JAK1, and STAT3 signals in the nuclei of keratinocytes were markedly decreased after treatment with anti-IL17A antibodies (Fig. 3d). The intense CXCL1 staining in the keratinocytes and fibroblasts was also profoundly reduced after treatment (Supplementary Fig. 5). These data strongly support the idea that blocking IL17 receptors could improve the inflammatory profile of the skin in the PRP model animals via suppression of the NF-κB pathway (Fig. 4).

Discussion

Activation of the IL-17/Th17 axis in immune cells and of the IL-1/IL-36 axis in granular layer cells and ORS cells are important in the formation of PRP lesions.

PRP, a disease related to psoriasis, is a rare inflammatory keratinization disorder, and the details of its triggers and pathogenic mechanisms remain unknown. Recently, it has become clear that the causes of type V PRP are CARD14 mutations\(^4\), while psoriasis vulgaris, psoriatic arthritis and pustular psoriasis with CARD14 variants/mutations have also been reported\(^2,14\). Why CARD14 variants/mutations produce the varied phenotypes is currently unclear. CARD14 mutant mice have been reported, including those with alteration\(^15,16,17\) and the deletion\(^15,16,17,18\) of p.Glu138, those with the accidentally obtained deletion of Gln136\(^15\), and those with a Gly117Ser\(^16\) missense mutation. Model mice with an amino acid alteration at
Card14Glu138 and the deletion of Card14Gln136 showed psoriatic phenotypes\textsuperscript{15}, and model mice carrying Card14Gly117Ser were clinically and histologically normal\textsuperscript{16}. Regarding therapeutic experiment, Millett et al.\textsuperscript{18} reported that the efficacy of the anti-IL-23p19 neutralizing antibody for ameliorating psoriatic skin showing the transcriptional landscape typical of human PV in model mice with the deletion of Card14Glu138 (Card14Δ138E). Manils et al. 2020\textsuperscript{17} reported the therapeutic efficacy of anti-TNF and anti-IL-17 neutralizing antibodies in Card14Glu138Ala mice.

In humans, an amino acid alteration and the deletion of p.Glu138 in CARD14 were reported in patients with PRP and patients with psoriasis, including GPP patients\textsuperscript{5,8,9,10}. There have been no reports of Gln136 deletion in CARD14 in humans. In contrast, the CARD14 missense mutation p.Cys127Ser was reported in patients who showed PRP or PRP-like skin lesions\textsuperscript{4,11}. The present study is the first report of PRP model mice carrying the CARD14 missense mutation p.Cys127Ser and is the first report on the single-cell analysis of skin from Card14KI model mice, as far as we know. We established Card14\textsuperscript{C127S/C127S} mice that carry a Card14 mutation that specifically causes PRP. To clarify the pathogenic pathways that work to produce the PRP phenotype, we analyzed the patterns of gene expression by the cells in the PRP skin lesions of the Card14\textsuperscript{C127S/C127S} mice and performed treatment experiments with anti-IL-17A antibodies.

Microarray analysis of the whole skin from PRP lesions in the Card14\textsuperscript{C127S/C127S} mice at P5 showed Il17a, Il17f, and Il23a to be predominantly upregulated (Fig. 2c, d). The mRNA expression of Pla2g4d and Pla2g4e was significantly higher in the Card14\textsuperscript{C127S/C127S} mice (Fig. 2d). PLA2G2F and PLA2G4D/PLA2G4E are highly expressed in the epidermis in PRP and psoriasis, and the combination of TNF and IL-17A is a strong inducer of PLA2G2F, PLA2G4D, and PLA2G4E expression\textsuperscript{13}. PLA2G4D leads to the production of IL-22 and IL-17A\textsuperscript{19}. The upregulation of the cytokines Pla2g4d and Pla2g4e strongly suggests that CARD14 contributes to driving the pathogenic IL-23/IL-17 axis \textit{in vivo}, and enrichment analyses of microarray data also showed the importance of the IL-17 signaling pathway (Fig. 2b).

Moreover, numerous IL-1 family cytokine genes (Il33, Il1f5, Il1f6, Il1f9 and Il1f10), IL-1 receptor genes (Il1r1m, Il1r2, Il1r1l2 and Il1rap), an IL-1 signaling adapter molecule gene (Myd88), and inflammasome-related genes (Casp1, Casp4 and Il18) were upregulated in the epidermis, especially in the granular cell cluster and ORS cluster (Supplementary Table 5). These data indicate that, in addition to the IL-17 axis being important for the pathogenesis in Card14\textsuperscript{C127S/C127S} mice, the activation of the IL1/IL36 axis and the inflammasome pathways in granular cells and in cells in the ORS are also important for this pathogenesis.

**Stimulation By Il17a To Ors Cells Leads To Follicular Plugging**

In Card14\textsuperscript{C127S/C127S} mice at the age of 12 months, Epi_ORS expressed not only the genes of cytokines and chemokines, but also genes related to cytokine receptors, such as Il17ra, Il6ra, and Il23r. The upregulation of Il6ra, Il23r, and Il17ra in Epi_ORS (Supplementary Table 5) and intense
immunohistochemical staining of IL-17RA in the ORS in Card14C127S/C127S mice (Fig. j) indicate that IL-17/Th17 axis signaling is likely to stimulate ORS cells and cause the abnormal keratinization of hair follicle epithelia by promoting the proliferation and differentiation of keratinocytes, leading to follicular plugging.

Indeed, Krt16 and Pla2g2f, which are genes related to hyperkeratosis, were upregulated in cells in the ORS. Pla2g2f in a transgenic mouse model led to the development of chronic epidermal hyperplasia and hyperkeratosis, similar to the pathology of psoriasis. The present study suggests that the activation of IL-17/Th17 axis not only in the interfollicular epidermis and the immune cells but also in the ORS is important in the formation of PRP lesions. As shown by multilayer analysis of genome/transcriptome/ligand-receptor and pathological investigation in psoriatic skin, we speculate that, also in the development of PRP skin lesions, different cell populations might interact in a complex manner and might be involved (Fig. 4).

S100A7A and keratin 17 might be useful for distinguishing PRP from psoriasis.

Microarray analyses of RNA samples from the whole skin of P5 mice showed that the expression of S100a8 and S100a9 was upregulated (Fig. 2c, d, Supplementary Table 3). S100a8 and S100a9 are usually co-expressed and are assumed to be associated with many autoinflammatory diseases. S100A8 and S100A9 alter neither the differentiation status nor the inflammatory response pattern of keratinocytes in vitro. However, S100A8 and S100A9 represent additional inflammatory factors during IL-17-mediated dermal inflammation, and IL-17A and IL-17F promote the expression of S100A8 and S100A9 during the inflammatory response of keratinocytes. The present Card14KI mice and all previously reported Card14KI mice showed higher gene/protein expression of S100a8 and/or S100a9. S100A7 is produced by epidermal keratinocytes and peripheral leukocytes and is significantly overexpressed in patients with psoriasis. It was also reported that the serum levels of S100a7a were elevated in patients with moderate to severe psoriasis. However, S100a7a expression was down-regulated in Epi_whole and Epi_Granular of the present Card14C127S/C127S mice (Supplementary Fig. 4, Supplementary Table 5). In contrast, S100a7a expression was upregulated in previously reported psoriasis patients and in Card14ΔE138 mice. Thus, down-regulated or not upregulated expression of S100a7a might be useful for distinguishing PRP from psoriasis. Indeed, the vitamin D analog calcipotriol suppresses Th17 cytokine-induced proinflammatory S100A7 in psoriasis. Treatment with the vitamin D analog calcipotriol may be ineffective for patients whose PRP is caused by the p.C127S mutation in CARD14.

Keratins 6, 16, and 17 are key alarmins during skin wounding and/or psoriasis, and these keratins are generally considered to be psoriasis biomarkers and potential therapeutic targets for psoriasis. However, in the present PRP model, Krt17 was not significantly upregulated in qRT-PCR for the skin of neonatal mice (Fig. 2d) nor in scRNA-seq for the skin of adult mice (Supplementary Table 5). Therefore, the absence of elevated expression of keratin 17 might be a hallmark of PRP, and keratin 17 may not be a therapeutic target for patients whose PRP is caused by the p.C127S mutation in CARD14.
Card14 C127S/C127S mice showed skin barrier dysfunction.

The expression of Flg and Flg2 was down-regulated in Card14 C127S/C127S mouse skin at P5 (Supplementary Table 3), and filaggrin protein expression was also low in mutant mouse skin (Fig. 2i). In addition, the expression of Casp14, the gene encoding caspase-14, which mediates profilaggrin processing to produce filaggrin, was not increased in Card14 C127S/C127S mice skin tissue at P5 (Supplementary Table 3), although Casp14 expression increased in previously reported Card14ΔE138 mice skin tissue at 8 weeks of age in RNA sequencing analysis. Card14ΔE138 mice showed significantly high expression of Flg and no skin barrier dysfunction. In contrast, in the present Card14 C127S/C127S mice, Flg expression was down-regulated in the whole skin with qPCR analysis, and this down-regulation was particularly notable in the granular layer cells (Epi_Granular) with scRNA-seq analysis (Supplementary Table 5). Decreased Flg expression is associated with epidermal barrier dysfunction, which enables microbes to invade the epidermis, as occurs in atopic dermatitis. In the present Card14 C127S/C127S mice, skin barrier dysfunction was shown by TEWL measurements and toluidine blue exclusion assay (Fig. 1f, g), and it was suggested that the abnormal skin barrier function is secondary to the development of skin inflammation due to the Card14 variant.

Patients with PRP caused by the Card14 mutation p.Cys127Ser might respond to anti-IL-17A neutralizing antibodies.

The neutralization of IL-17A suppressed the expression of various types of pro-inflammatory molecules in the epidermis. We examined the therapeutic efficacy of the anti-IL-17A neutralizing antibody for PRP lesions in the Card14 C127S/C127S mice. IL-17A inhibition led to clear improvements in macroscopically and histopathologically abnormal phenotypic features, although TEWL from the ear did not improve after the administration of the anti-IL-17A neutralizing antibody. The present scRNA-seq analysis revealed that the skin tissue in adult Card14 C127S/C127S mice without any treatment showed the significantly upregulated expression of Jak1 and Stat3 in Epi_Whole (Supplementary Table 5), although the present microarray analysis of RNA samples suggested that the involvement of JAK/STAT signaling might be insignificant in the early stages of PRP lesion development (Supplementary Table 3). Immunohistochemically, the protein expression of IL-23, IL-17C, IL-1β, IL-36γ (IL1F9), MYD88, JAK1, STAT3, TNFα, CCL20, and CXCL1 was decreased in the skin tissue treated with anti-IL-17A antibody (Fig. 3d, Supplementary Fig. 5). IL-1β, IL-17, and TNF-α induce the expression of CCL20, and CCL20 suppresses the differentiation of Treg cells and promotes the differentiation of Th17 cells. IL-23, IL-1β, and TNFα also promote Th17 cell differentiation. The neutralization of IL-17A suppressed the abnormal enhancement of the IL-17/Th17 signaling axis, resulting in the down-regulated expression of genes related to the IL-23 axis, inflammasomes, JAK/STAT signaling, and innate immunity. In addition, not only might the IL-23 that is secreted by dendritic cells and the CXCL1 that is secreted by keratinocytes be involved in the pathogenesis of the PRP phenotype, but also might be the IL-23 that is secreted by keratinocytes and the CXCL1 that is secreted by fibroblasts (Supplementary Fig. 5, Supplementary Table 5, 6).
The present IL-17A inhibition experiments further confirmed that the development of the PRP phenotype due to CARD14 gain of function is mediated by the activation of the IL-17 signaling axis and that the disruption of the IL-17/Th17 immune signaling axis is sufficient to reverse the aberrant epidermal signaling networks in Card14C127S/C127S mice. Thus, targeting IL-17A is a rapid and effective therapeutic option for PRP, at least in this mouse model. The therapeutic efficacy of the anti-IL-17A receptor antibody\textsuperscript{36} and the anti-IL-17A antibody\textsuperscript{37, 38} were recently reported for patients with PRP. Strunck \textit{et al.}\textsuperscript{37} reported that there were responders and non-responders to ixekizumab in PRP patients who had no Card14 mutations. The non-responders showed the sustained elevation of mRNA expression of IL-17C and CCL20 in lesional skin tissue after the administration of the monoclonal anti-IL-17A antibody ixekizumab, although the mRNA expression of IL-17C and CCL20 was decreased in the skin lesions after administration in the responders. An immunohistochemically apparent decrease of the protein expression of IL-17C and CCL20 in lesional skin after treatment with anti-IL-17A neutralizing antibody suggest that patients whose PRP is caused by the CARD14 mutation p.Cys127Ser are responders to anti-IL-17A neutralizing antibody.

\textbf{Conclusion}

We assumed the pathways and processes of inflammatory response in PRP from the results of microarray and scRNA-seq analyses (Fig. 4).

This study suggests that the Card14C127S/C127S mouse model might be an invaluable tool for elucidating the complex signaling networks that are associated with skin lesions in PRP. For example, in the present model mice, the absence of upregulated expression of S100A7A and keratin 17 was suggested to be a sign for distinguishing PRP from psoriasis. This model also promises to be beneficial for the preclinical assessment of therapeutics that specifically target molecular drivers of PRP.

Skin barrier dysfunction, the hyperactivation of innate immunity via IL-36 signaling and inammasome pathways, and the excessive activation of the IL-17 axis in the ORS and the interfollicular epidermis might be important for development of PRP phenotypes, and the inhibition of IL-36 signaling and the IL-17 axis might be effective for AiKDs that are associated with \textit{CARD14} variants/mutations, including type V PRP.

\textbf{Materials And Methods}

\textit{Generation of the Card14 knock-in mice}

C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were fed a commercial CE-2 diet (CLEA Japan) and had ad libitum access to water. The mice were bred in a pathogen-free facility at the Institute for Laboratory Animal Research, Graduate School of Medicine, Nagoya University, and were maintained at a controlled temperature of 23 ± 1°C, a humidity of 55% ± 10%, and a light cycle of 12h light (from 09:00 to 21:00)/12h dark (from 21:00 to 09:00). All animal care and experimental procedures were approved by the Animal Care and Use Committee of the Graduate School of Medicine,
Nagoya University, and were conducted in accordance with the Nagoya University Regulations on Animal Care and Use in Research.

Targeted editing of the Card14 gene on a C57BL/6J background was carried out by using the CRISPR/Cas9 method\(^39\) (Wang et al., Cell 2013). We used the cloning-free CRISPR/Cas9 method, as described previously by Aida et al.\(^40\) (Aida et al., Genome Biol 2015). The CRISPR RNA (crRNA, 5'- ATC CAA GCT GAC CGA GTG TC-3') that targets exon 6 was designed using the CRISPOR website\(^41\) (Haeussler et al., Genome Biol 2016). The designed crRNA and trans-activating crRNA (tracrRNA) (Genome CraftType CT, FASMAC, Kanagawa, Japan) and Cas9 protein (New England Biolabs, Tokyo, Japan) were mixed and incubated at 37°C for 20 min to form a ribonucleoprotein (RNP) complex. The single-stranded oligodeoxynucleotide (ssODN, 5'-atc tgt gca gGT CxTC ATG GAG ACA TCC AAG CTG ACC GAG TCT CTG GCT GGG GCC ATC AGC AGC CTG CAG GAG GAG CTG GCC-3') was designed to target c.380G > C mutation and was obtained from FASMAC. The final concentrations of components in RNP preparation with ssODN were 8µM guide RNA (crRNA + tracrRNA), 200 ng/µl Cas9 protein, and 250 ng/µl ssODN. The mixture was electroporated into zygotes using a NEPA 21 electroporator (NEPA GENE Co., Ltd., Chiba, Japan), and the embryos were transferred into the oviductal ampulla of pseudo-pregnant ICR mice purchased from Charles River Laboratories Japan (Yokohama, Japan).

For sequencing and genotyping, genomic DNA was extracted using KAPA Express Extract (Kapa Biosystems, Woburn, MA, USA) from the pinna and tail of the offspring and was used for PCR amplification. The region targeted by the Cas9 nuclease was amplified by using a GoTaq Green Master mix (Promega, Madison, WI, USA) and a primer pair (5'-gaa ctc tcg ggg aac tca aag cag-3' and 5'-TTC TCT CTG AGT GCG TTG CTG TAG-3'). The PCR amplification conditions were as follows: 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min. Mutations in the Card14 gene in offspring were confirmed by Sanger sequencing of the PCR products using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the 3730xl DNA Analyzer (Thermo Fisher Scientific) by Eurofins Genomics K.K (Tokyo, Japan). Potential off-target cleavage sites predicted by the CRISPOR website (Supplementary Table 8, top five high-scoring regions according to the Mit off-target score) were amplified by PCR and sequenced to confirm off-target mutations, and no mutations were detected in these sites. Heterozygous Card14KI mice were obtained as founders. We crossed heterozygous Card14KI mice (founders) and C57BL/6J to make male and female heterozygous Card14KI mice. We obtained homozygous Card14KI mice by crossing the male and female heterozygous Card14KI mice.

**Skin Permeability Assays**

TEWL was measured using a VAPO SCAN AS-VT100RS (Asahi Techno Lab., Ltd., Yokohama, Japan). Measurements were performed after calibration of the device at room temperature with minimized influence of air turbulence, and the results were recorded when the TEWL values stabilized 30–45 s after probe placement. Toluidine blue staining of mice was described previously\(^42\) (Radner FP et al., J Biol Chem 2010). In brief, P7 mice were anesthetized, dehydrated in methanol, washed in PBS and stained for
30 min in 0.1% (w/v) toluidine blue/PBS\(^4\) (Hirabayashi et al., Nat Commun 2017). After being washed in PBS, the pups were photographed.

**Immunohistochemistry**

Immunohistochemical analyses of skin samples from the mice were performed as described previously, with slight modifications. Thin sections (3 µm) were cut from samples embedded in paraffin blocks. The sections were soaked for 20 min at room temperature in 0.3% \(\text{H}_2\text{O}_2\)/methanol to block endogenous peroxidase activity. After being washed in PBS with 0.01% Triton X-100, the sections were incubated for 30 min in PBS with 4% BSA, followed by overnight incubation with the primary antibodies in PBS containing 1% BSA according to the manufacturer's instructions. After being washed in PBS, the thin sections were stained with the corresponding secondary antibodies for 1 hour at room temperature and were washed in PBS. The Vectastain Elite ABC-PO kit (Vector Laboratories, Burlingame, CA) was used for staining. The following polyclonal antibodies were purchased from commercial sources: anti-S100A9 (NB110-89726; Novus Biologicals, Colorado, USA), S100A7A (bs-6575R; BIOSS, Boston, USA), anti-FILAGGRIN (NBP1-87528; Novus Biologicals), anti-IL-17RA (bs-2606R; BIOSS), anti-IL-1β (ab9722; Abcam, Cambridge, UK), anti-IL-1F9 (PA5-99822; Thermo Fisher Scientific, Massachusetts, USA), anti-IL23 (ab45420; Abcam), anti-IL-17C (bs-2611R; BIOSS), anti-TNFα (bs-2081R; BIOSS), anti-MyD88 (NB100-56698; Novus Biologicals), anti-p-JAK1 (phospho-Tyr1022, anti-p-Jak1 (Tyr1022), SAB4504446; Sigma Aldrich, St. Louis, MO), anti-NFκB p65 (bs-0465R, BIOSS), anti-p-STAT3 (phospho-Tyr705, anti-p-Stat3 (Tyr705), #11045; Signalway Antibody, College Park, MD).

**Microarray**

Total RNA was extracted from the trunk skin of newborn mice at P0 and P5 with RNAiso Plus (Takara Bio), and then equal amounts of total RNA pooled from 5 mice for each genotype were purified using an RNeasy Mini Kit (QIAGEN). The quality of RNA was assessed with a 2100 Bioanalyzer (Agilent Technologies). Fluorescently labeled antisense RNA (cRNA targets) was synthesized with a Low Input QuickAmp Labeling Kit in accordance with the manufacturer's protocol (Agilent Technologies). Samples were hybridized to the Mouse Gene Expression 4\(\times\)44K v2 Microarray (G4846A, Agilent Technologies), washed, and then scanned using a SureScan Microarray Scanner (Agilent Technologies). Microarray data were analyzed with Feature Extraction software (Agilent Technologies) and then imported into GeneSpring GX software (Agilent Technologies). Signal intensities were globally normalized by shifting to the 75th percentile of each sample. Scatter plots were generated using GeneSpring GX software to compare transcript levels for genes in CARD14 KI and WT. For functional enrichment analysis, a list of upregulated DEGs was uploaded to DAVID bioinformatics resources (https://david.ncifcrf.gov/) and significantly enriched functional clusters were identified with the KEGG pathway database. The Benjamini–Hochberg false discovery rate (FDR < 0.01) was used to correct for multiple hypothesis testing.
Qrt-pcr

Total RNA was reverse transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix (Toyobo) in accordance with the manufacturer's instructions. qPCR reactions were performed on a LightCycler480-II (Roche) using the THUNDERBIRD Probe qPCR Mix (Toyobo) or the THUNDERBIRD SYBR qPCR Mix (Toyobo). The primers and probes were designed using the Universal ProbeLibrary (UPL) Assay Design Center (Roche) or NCBI primer-BLAST. The UPL probes were pre-validated short hydrolysis probes that contained locked nucleic acids, which were labeled with 6-carboxyfluorescein (6-FAM) at the 5' end and dark quencher near the 3' end. The sequences of primers and UPL probes are shown in Supplementary Table 9. Cycling conditions were as follows: 95°C for 15 min (one cycle), 95°C for 15 s and 60°C for 1 min (45 cycles). A total of 1 µl of cDNA per sample was used for quantification of the endogenous mRNA levels. Expression levels were normalized to the housekeeping gene Hprt1.

Single-cell transcriptional profiling

Tissue Isolation And The Preparation Of The Single-cell Suspension

Full-thickness skin was surgically excised from the backs from 1 WT mouse and 1 Card14C127S/C127S mouse at 12 months. Digestion for the two samples was performed using the Whole-skin Dissociation Kit for humans (Miltenyi Biotec, Germany). Enzymatic digestion was completed in 73 min, followed by mechanical dissociation using the gentleMacs Octo Dissociator with heaters (Miltenyi Biotec), running the gentle MACS mice program 37C_m_Bo_WSDK_2. Next, dead cells and debris were removed using the Dead Cell Removal Kit (Miltenyi Biotec). After the tissue was enzymatically and mechanically disrupted, a single-cell suspension (viability of WT > 80%, of homozygous > 80%) was subjected to scRNAseq.

Preparation Of The Scrnaseq Library, Sequencing, And Data Preprocessing

Preparation of the scRNAseq library, sequencing, and data preprocessing were outsourced to AZENTA Corp., Japan branch. Single-cell cDNA libraries were prepared by using 3' Gene Expression v 3.1(10X Genomics), and the constructed libraries were sequenced on DNBSEQ-G400 (MGI-Tech, China) with 150-bp paired-end runs. Raw sequencing data were processed via Cell Ranger (10X Genomics) and were analyzed using Loupe Browser ver 5.1.0 (10X Genomics). A total 8,364 single cells passed the initial quality control with a median detected unique molecular identifier (UMI) read of 5,937 and a median detected protein coding genes of 2,016.

Main Clustering And Subclustering

8,364 single cells are visualized in UMAP plots in Fig. 2f. Based on the expression levels of well-known markers for identifying cell populations (Supplementary Fig. 3a), we defined 8 main clusters of cell types.
These clusters include whole epidermal cells (Epi_Whole, 2635 cells; 32%) majorly expressing Krt1, Krt10, Krt5, Krt14, Flg, Apoc1 and Acsl5; whole immune cells (Imm_Whole, 1170 cells; 14%) majorly expressing Ptprc (Cd45), Itgam (Cd11b) and Cd3g; whole mesenchymal cells (Mes_Whole, 3804 cells; 45%) majorly expressing Pdgfrb, Lum, Col1a1 and Acta2; whole endothelial cells (End_Whole, 227 cells; 3%) majorly expressing Cdh5, Pecam1 and Lyve1; cells derived from the neural crest (Neu) majorly expressing Sox10 and Mlana (12 cells; <1%); erythrocytes (Ery) majorly expressing Hba-a2 and Hbb-bt (297 cells; 3%); and the two death cell clusters, Death_1 (177 Cells; 2%) and Death_2 (50 Cells; <1%), majorly expressing mitochondrial genes.

Of these 8 clusters, Epi_Whole, Imm_Whole, Mes_Whole and End_Whole, which were expected to be significantly involved in inflammation and/or keratinization, were further divided into subclusters. We used specific markers (Supplementary Table 4) to extract total 20 subclusters from Epi_Whole, Imm_Whole, Mes_Whole, and End_Whole. Epi_Whole could be divided into 7 distinct subclusters: basal cell type-1 cells (Epi_Basal-1) majorly expressing the basal epithelium markers Krt5 and Krt14, but not Krt1 or Krt10 (311 cells; 4%); basal cell type-2 cells (Epi_Basal-2) majorly expressing Krt5, Krt14, Krt1, and Krt10, but not Grhl1 (72 cells; <1%); spinous cell type-1 cells (Epi_Spinous-1) majorly expressing epithelial differentiation markers Krt1, Krt10, and Grhl1, but not Krt5 or Krt14 (156 cells; 2%)44,45; spinous cell type-2 cells (Epi_Spinous-2) majorly expressing Krt1, Krt10, Krt5, Krt14, and Grhl1 (247 cells; 3%); granular cell-type cells (Epi_Granular) majorly expressing Flg and Flg2 (489 cells; 6%); sebaceous gland-type cells (Epi_SG) majorly expressing Abcc3, Apoc1, Acsl5, Myc, Sox9, Elov15, Fasn and Rdh11 (101 cells; 1%)46; and outer root sheath-type cells (Epi_ORS) majorly expressing Cd34, Cd200 and Lnx2 (112 cells; 1%)47. End_Whole were represented by 2 subclusters: vascular endothelial cell-type cells (End_VEC) and lymphatic endothelial cell-type cells (End_LEC). End_VEC was identified by expression of its archetypal marker Pecam1 (not coexpressing Lyve1) (69 cells; <1%), and End_LEC was identified by the expression of Lyve1 (111 cells; 1%)48,49. Mes_Whole were represented by 2 subclusters; fibroblast-type cells (Mes_Fibro) and cells including pericytes-like cells, dermal sheath cells, dermal papilla cells, and smooth muscle cells (Mes_Peri/DS/DP/SM). Mes_Fibro was identified by the expression of Col1a1, Col3a1, and Col5a1 (3249 cells; 39%). Mes_Peri/DS/DP/SM expressed Acta2 as the marker gene (381 cells; 5%). Imm_Whole is divided into 9 subclusters: Cd4-positive T cell-type cells (Imm_Cd4) (55 cells; <1%), Cd8-positive T cell-type cells (Imm_Cd8) (89 cells; 1%) and Foxp3, a marker of regulatory T cells (Treg), positive T cell-type cells (Imm_Treg) (35 cells; <1%); B cell-type cells (Imm_B) majorly expressing Ms4a1 (Cd20) and Cd79a (62 cells; <1%); dendritic cell type-1 cells (Imm_DC-1) majorly expressing Il1b, Cd209a and Cd80 (50 cells; <1%); dendritic cell type-2 cells (Imm_DC-2) majorly expressing Il1b, Cd209a and Cd86 (44 cells; <1%); Langerhans cells (Imm_LC) majorly expressing Cd207 and Cldn1 (27 cells; <1%); mast cells and basophils (Imm_Mast/Baso) majorly expressing Cd200r3 and Ms4a2 (7 cells; <1%); and Cd163- and Cd68-positive macrophages (Imm_Mp) (48 cells; <1%). We visualized these 20 subclusters in a UMAP plot (Fig. 2g). In addition, differentially expressed genes in 20 subclusters were shown with a heatmap (Supplementary Fig. 3b), and the relative expression of selected significant maker genes in 20 subclusters were visualized as a violin plot (Fig. 2h). The other cells that were difficult to divide into subclusters numbered 2,110 cells (25%) (other).
Therapeutic Approach With Anti-il-17a Neutralizing Antibody

We intraperitoneally injected the Card14^{C127S/C127S} mice with a neutralizing antibody specific to mouse Il-17a (Clone 17F3, #BE0173, Bioxcell) over the course of 5 consecutive days, and control mice were administered an IgG isotype antibody (BE0083, Bioexcell). The Card14^{C127S/C127S} mice received 50 µg of each antibody per body weight per day (50µg/g/day).

Declarations

Data availability


Study approval

This study was approved by the ethics committee of the Nagoya University Graduate School of Medicine. All studies were conducted according to the Declaration of Helsinki Principles. Animal care and all experimental procedures were approved by the Animal Experiment Committee, Graduate School of Medicine, Nagoya University, and were conducted according to the Regulations on Animal Experiments of Nagoya University.

Author contributions

Designing research studies: TY, TT, MA; conducting experiments: TY, TH, YMiyasaka, TO; acquiring data: TH, YN; YMiyasaka, TO; analysing data: TY, TH, YMurase; providing reagents: TH, YMiyasaka, TO; writing the manuscript: TY, TT, TH, YMuro, YMiyasaka, MA.

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Conlicts of interest: The authors declare that we have no conlicts of interest.

References


Figures
Figure 1

Phenotypes of the Card14$^{C127S/+}$ and Card14$^{C127S/C127S}$ mice.

(a) At birth, the Card14$^{C127S/+}$ mice and Card14$^{C127S/C127S}$ mice are normal in weight. The weight gain of the Card14$^{C127S/C127S}$ mice became significantly poor at 5 days of age (P5). * $p<0.01$, ** $p<0.001$.

(b) The Card14$^{C127S/+}$ mice show no abnormal phenotypic features until about 2 weeks after birth. In contrast, the Card14$^{C127S/C127S}$ mice at P4–P6 show hyperkeratosis and severe scaling on the entire body, including the face, forepaws, and hind paws.

(c) At 2–3 weeks after birth, the Card14$^{C127S/+}$ mice show hyperkeratosis and scales on the tail, with the scales gradually increasing until 4–5 weeks of age. The scales spontaneously disappear 6–7 weeks after birth. No macroscopic phenotypic abnormalities are seen on the ears throughout the entire life of the Card14$^{C127S/+}$ mice.

(d) The ear thickness of the adult Card14$^{C127S/+}$ mice does not differ from that of the adult WT mice, and the ear thickness of the adult Card14$^{C127S/C127S}$ mice is significantly greater than that of the adult WT mice. Each mouse is at the age of 2–12 months. * $p<0.01$.

(e) Skin samples from the back, tail, and hind paw of the Card14$^{C127S/+}$ mice at P5 show mild hyperkeratosis and acanthosis compared with those of WT mice at P5, and demonstrated dilatation of
hair pores and keratinous plugs (hematoxylin-eosin (HE), original magnification, x200). Those of \textit{Card14}\textsuperscript{C127S/C127S} mice at P5 showed severe hyperkeratosis, parakeratosis, acanthosis and the infiltration of mainly lymphocytes in the dermis compared with those of WT and \textit{Card14}\textsuperscript{C127S/+} mice (HE, original magnification, x200), and many follicular plugs (arrows) were present in \textit{Card14}\textsuperscript{C127S/C127S} mice (bottom) (HE, original magnification, x400).

(f) TEWL was measured in the back skin of WT, \textit{Card14}\textsuperscript{C127S/+}, and \textit{Card14}\textsuperscript{C127S/C127S} mice at P7. The back skin of the \textit{Card14}\textsuperscript{C127S/C127S} mice shows significantly increased TEWL, and the TEWL of the \textit{Card14}\textsuperscript{C127S/C127S} mouse skin is approximately 6 times as great as those of the WT and \textit{Card14}\textsuperscript{C127S/+} mice. *\(p< 0.01\).

(g) A toluidine blue exclusion assay on the skin of WT, \textit{Card14}\textsuperscript{C127S/+} and \textit{Card14}\textsuperscript{C127S/C127S} mice at P7 shows that more dye remains in the face and some parts of the skin of the \textit{Card14}\textsuperscript{C127S/C127S} mice than in those of the WT and \textit{Card14}\textsuperscript{C127S/+} mice (white arrows).

**Figure 2**


(a) Scatter plots of the microarray data showing gene expression profiles in \textit{Card14}\textsuperscript{C127S/C127S} mice (y-axis) and WT controls (x-axis) at P0 and P5. Each axis is on a logarithmic scale, and the diagonal lines define 2-fold changes. Differentially expressed genes (DEGs) of particular interest are labeled.

(b) A bubble plot showing the results of the KEGG pathway enrichment analysis on microarray data obtained from the skin at P5 of panel (a). The 20 most significant terms upregulated in \textit{Card14}\textsuperscript{C127S/C127S}
mice as compared to controls are shown. The rich factor indicates the ratio of upregulated DEGs to all annotated genes in the pathway. Dot color and size correspond to the enrichment FDR and the gene number enriched in the pathway, respectively.

(c) Heatmaps show the gene expression of a panel of proinflammatory cytokines, chemokines, and adhesion molecules, as well as keratinocyte proliferation and differentiation markers in Card14$^{C127S/C127S}$ mice and WT controls at P0 and P5.

(d) qRT-PCR analysis in Card14$^{C127S/C127S}$ mice at P5. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. n=4–5 animals per group.

(e) Anti-S100a9 and anti-S100a7a staining is strong in nuclei and weak in the cytoplasm of keratinocytes, respectively, in the back skin Card14$^{C127S/C127S}$ mice at P5.

(f) Visualization using UMAP of the whole skin single cells of WT and Card14$^{C127S/C127S}$ mice at the age of 12 months. Blue points represent WT mouse cells, and orange points represent Card14$^{C127S/C127S}$ mouse cells.

(g) 8 main clusters are represented as Epi_Whole, Imm_Whole, Mes_Whole, End_Whole, Neu, Ery, Death_1, and Death_2. Epi_Whole, Imm_Whole, Mes_Whole and End_Whole are further divided into 20 subclusters from the expression of specific markers.

(h) The relative expression of selected significant marker genes for 20 subclusters is visualized as violin plots. Each plot is color-coded to match the colors in Fig. 2g.

(i) Immunohistochemical staining shows stronger IL-17RA staining in the ORS (*) of the Card14$^{C127S/C127S}$ mice than in that of the WT mice.

(j) Immunohistochemical staining shows the reduction of filaggrin protein expression in the granular layers of the epidermis in Card14$^{C127S/C127S}$ mice.
Treatment with IL-17A neutralizing antibodies improves the skin lesions of \textit{Card14}^{C127S/C127S} mice.

(a) \textit{Card14}^{C127S/C127S} mice receiving anti-IL-17A neutralizing antibodies show significantly decreased PRP-like phenotypic features on the ears, face, and tail at Day 5 compared with those seen before treatment and those seen in IgG control-treated animals.

(b) \textit{Card14}^{C127S/C127S} mice receiving anti-IL-17A neutralizing antibodies show a significant reduction in ear thickness at Day 8 (P value:<0.01).

(c) The skin tissue of \textit{Card14}^{C127S/C127S} mice was histologically evaluated at Day 0 and Day 8. Top: The ear skin sample shows decreased scales and crusts, the inflammatory cell infiltration of the entire dermis is also decreased in the samples obtained at 8 days of treatment, and the entire ear has become thinner and the deformation has greatly improved at Day 8 (hematoxylin–eosin, original magnification, x200). The back skin sample at Day 8 shows the entire epidermis, especially the stratum corneum, to be markedly thinned and inflammatory cell infiltration in the entire dermis to be also reduced (HE, original magnification x400).

(d) Immunohistochemical staining using anti-IL-1β, IL-1F9, MYD88, IL-17C, IL-23, TNFα, JAK1, STAT3, and RELA antibodies was performed for skin tissue from the back at Day 0 and Day 8 (original magnification
The back skin samples show weaker staining for anti-IL-1β, IL-1F9, IL-17C, and TNFα antibodies in the cytoplasm of keratinocytes at Day 8 than at Day 0. The same skin samples show weaker RELA, JAK1, and STAT3 staining in the nuclei of keratinocytes at Day 8 than at Day 0. IL-23 staining in keratinocytes is clearly weaker after treatment.

**Fig. 4**

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*Figure 4*
Inflammatory pathways induced by CARD14 gain-of-function mutants in the skin

The pathways and processes of inflammatory responses induced by CARD14 gain-of-function variants. Mutant CARD14 hyperactivates NFκB, leading to the secretion of chemokines/cytokines, IL-23, IL-36, TNF-α, CXCL1, CXCL2, and CXCL16 from keratinocytes, and IL-33 and CCL2 from ORS cells, resulting in the activation of neutrophils, dendritic cells, fibroblasts, and vascular endothelial cells in the dermis. Fibroblasts stimulated by these chemokines/cytokines secrete IL6, IL18, CXCL1, CXCL2, CXCL10, and CXCL13, and in like manner, stimulated vascular endothelial cells secrete CCL2 and CCL7. These chemokines/cytokines from fibroblasts and vascular endothelial cells stimulate immune cells and keratinocytes, including ORS cells. In addition, Th1 and Th17 cells are induced, and Th1 cytokines and IL-17 are secreted. Then, IL-17 stimulates vascular endothelial cells and keratinocytes, including ORS cells. KRT16, a hyperproliferative keratin, is elevated in keratinocytes, including ORS cells. IL-17A promotes the expression of S100A8 and S100A9 during the inflammatory response of keratinocytes.

DC, dendritic cell; F, Fibroblast; N, neutrophil; ORS, outer roof sheath; VEC, vascular endothelial cell

Supplementary Files

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- SupplementaryinfoPDF.pdf
- SupplementaryTable3.xlsx
- SupplementaryTable5.xlsx
- SupplementaryTable6.xlsx
- SupplementaryTable7.xlsx