NLRP3 Inhibition Ameliorates Severe Cutaneous Autoimmune Manifestations in a Mouse Model of Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy—Like Disease

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Patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy show diverse endocrine and nonendocrine manifestations initiated by self-reactive T cells because of AIRE mutation—induced defective central tolerance. A large number of American patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy suffer from early-onset cutaneous inflammatory lesions accompanied by an infiltration of T cells and myeloid cells. The role of myeloid cells in this setting remains to be fully investigated. In this study, we characterize the autoinflammatory phenotypes in the skin of both autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy—like kinase-dead Ilkka knockin mice and patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. We found a marked infiltration of autoreactive CD4 T cells, macrophages, and neutrophils; elevated uric acid; and increased NLRP3, a major inflammasome component. Depleting autoreactive CD4 T cells or ablating Ccl2/Cxcr2 genes significantly attenuated the inflammasome activity, inflammation, and skin phenotypes in kinase-dead Ilkka knockin mice. Importantly, treatment with an NLRP3 inhibitor reduced skin phenotypes and decreased infiltration of CD4 T cells, macrophages, and neutrophils. These results suggest that increased myeloid cell infiltration contributes to autoreactive CD4 T cell—mediated skin autoinflammation. Thus, our findings reveal that the combined infiltration of macrophages and neutrophils is required for autoreactive CD4 T cell—mediated skin disease pathogenesis and that the NLRP3-dependent inflammasome is a potential therapeutic target for the cutaneous manifestations of autoimmune diseases.


INTRODUCTION

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), or autoimmune polyglandular syndrome type 1, is a T cell—driven systemic autoimmune disease with diverse endocrine manifestations caused by mutations in the AIRE gene locus (Manley et al., 2011; Mathis and Benoist, 2009; Rautemaa et al., 2007; Sonal et al., 2012). Nonendocrine skin and nail phenotypes, including urticarial eruption, alopecia, and nail dystrophy, are associated with increased T-cell and neutrophil infiltration and have recently been reported in many American patients with APECED (Ferre et al., 2016). To develop effective treatments for the skin manifestations of the patients, we attempt to elucidate the mechanism underlying the pathogenic roles of skin-infiltrating immune cells.

Central tolerance is established in the thymus, where autoreactive T cells are deleted by interacting with medullary thymic epithelial cells that express tissue-restricted antigens (Manley et al., 2011). The transcriptional factor AIRE regulates the expression of tissue-restricted antigens and other molecules that facilitate the selection of proper T-cell repertoire (Akiyama et al., 2005; Mathis and Benoist, 2009). AIRE mutations impair the development of medullary thymic
epithelial cells (Manley et al., 2011; Mathis and Benoist, 2009). When a defect occurs during central tolerance development, autoreactive T cells can evade negative selection, enter the periphery, and incite tissue injury and systemic autoinflammation. Although autoreactive T cells are the main drivers of APECED, it is not clear how they interact with myeloid cells and keratinocytes (KCs) to promote skin manifestations.

IKKα, a subunit of the IKK complex, is essential for canonical and noncanonical NF-κB activation (Ghosh and Karin, 2002; Zhu and Hu, 2018). IKKα regulates the development of medullary thymic epithelial cells and the expression of AIRE and other molecules important for the negative selection of T cells in the thymus via the NF-κB pathways (Zhu et al., 2017). Kinase-dead ikka knockin (KA/KA) mice recapitulate APECED symptoms with systemic autoinflammation and increased fungal infection in the oral cavity and esophagus but not in the skin (Xiao et al., 2013; Zhu et al., 2017). Meanwhile, IKKα is required for maintaining skin homeostasis independent of its kinase activity and NF-κB pathways (Cao et al., 2001; Hu et al., 2001; Liu et al., 2008; Zhu and Hu, 2018). Therefore, in this study, we aimed to elucidate the role of IKKα in the development of T cell–driven autoreactive skin disorders.

NLRP3 plays a critical role in inflammasome activity (Goldbach-Mansky et al., 2006; He et al., 2016). Activating or gain-of-function mutations in the CIAS1 locus, which encodes NLRP3, have been reported in a spectrum of dominantly inherited autoinflammatory diseases in humans (Goldbach-Mansky et al., 2006). Elevated NLRP3 levels lead to increased IL-1β and IL-18 expression in response to a broad range of pathogen-associated molecular patterns; damage-associated molecular patterns; and insults, including uric acid released from dead cells (Braga et al., 2017; Gurung and Kanneganti, 2016; He et al., 2016; Martin et al., 2014; Masters et al., 2016).

In this study, we identified severe skin manifestations associated with increased autoreactive CD4 T-cell and macrophage and neutrophil numbers and increased inflammasome NLRP3 levels in both patients with APECED and KA/KA mice. We investigated the causal relationship among these immune cells and KCs in the pathogenesis of skin lesions by using genetic mouse models and an NLRP3 inhibitor treatment. Our findings show that myeloid cell infiltration contributes to autoreactive CD4 T cell–mediated autoinflammatory skin disorders and that a NLRP3 inhibitor can block the inflammatory cell infiltration, which provides new therapeutic approaches for autoimmune skin diseases.

RESULTS

Severe skin lesions are associated with upregulated NLRP3 expression and increased macrophage and neutrophil recruitments in both KA/KA mice and patients with APECED

We found that the skin of all C57BL/6 KA/KA mice looked normal from birth until three months of age. However, after 3 months, a large proportion of KA/KA mice developed rough fur, hair loss, skin rashes, or broken skin associated with epidermal hyperplasia and inflammation compared with wild-type (WT) mice (Figure 1a and b, Supplementary Figure S1a–c). KA/KA mice developed a T cell–driven APECED-like disease associated with increased fungal infection in the oral cavity and esophagus. We did not detect increased fungal colonization in the skin of KA/KA mice (Zhu et al., 2017), suggesting that the skin phenotype in KA/KA mice is relevant to the defective medullary thymic epithelial cells. Indeed, the urticaria-like lesions in KA/KA mice, in which infiltrating leukocytes surrounded the blood vessels in the dermal area, were similar to the urticarial manifestations in the dermis of patients with APECED (Figure 1c and d, Supplementary Figure S1d). In addition, cell proliferation and multiple types of cells in the epidermis were associated with the infiltrating inflammation from the dermis (Figure 1d, bottom and top panels and Supplementary Figure 1d, bottom). Thus, both patients with APECED and KA/KA mice show an autoinflammatory cell–induced skin phenotype from the dermis to the epidermis.

To determine the mechanism underlying the severe skin lesions, we analyzed gene expression profiles in the skin of WT and KA/KA mice at 4–5 months of age. All of the following genes were highly expressed in KA/KA skin compared with WT skin (Figure 1e): NLRP3 and IL-1β, which are related to inflammasome activation; CCL2, CXCL1, and CXCR2, which account for macrophage and neutrophil recruitment; and IL-1β, IL-6, CXCL5, CXCL2, CCL7, CCL9, MMP9, and TGFβ, which are inflammatory cytokines and chemokines. Because NLRP3 was highly expressed in KA/KA skin, we hypothesized that the KA/KA skin with tissue injury or damage releases uric acid (Braga et al., 2017) to activate inflammasome activity, which contributes to the skin lesions in a feedforward circuit. Indeed, the uric acid levels were much higher in KA/KA skin than in WT skin (Figure 1f).

We previously reported that the IKKα KA/KA protein is not stable and gradually degrades over time (Xiao et al., 2013; Zhu et al., 2017). The severity of the KA/KA skin phenotype was correlated with IKKα reduction, increased EGFR and ERK activity, and cell proliferation in the skin of KA/KA mice (Figure 1b, Supplementary Figure S1e, f), suggesting that a physiological trigger because of advancing age contributes to the pathogenesis of the severe skin phenotypes.

It has been reported that the skin phenotypes of patients with APECED are associated with increased CD4 T cells (Ferre et al., 2016). We examined NLRP3 expression and the patterns of recruited macrophages (CD163) and neutrophils (myeloperoxidase) in the skin of patients with APECED. Using immunofluorescence and immunohistochemistry staining, we detected increased NLRP3 expression and infiltration of macrophages and neutrophils in the abnormal skin of patients with APECED compared with normal human skin (Figure 1g–i). The epidermal KCs are able to express a variety of cytokines and chemokines, which recruit macrophages and neutrophils, in response to autoreactive CD4 T-cell stimulation (Ho and Kupper, 2019; Jaigirdar and MacLeod, 2015). These results suggest not only that the molecular and cellular mechanisms are shared between patients with APECED and KA/KA mice but also that KA/KA mice develop an APECED-like syndrome with skin manifestations.
Expansion of autoreactive TCR V\textsuperscript{b}5.1\textsuperscript{+} CD4 T cells and increased macrophage and neutrophil numbers in the skin of KA/KA mice

TCR V\textsuperscript{b}5.1\textsuperscript{+}CD4 T cells, which have been reported in patients with APECED (Kogawa et al., 2002), are generated in the thymi and amplified in the esophagi of KA/KA mice (Kogawa et al., 2002; Zhu et al., 2017). To demonstrate whether the skin disease in KA/KA mice is relevant to autoreactive T cells, we used flow cytometric analyses and found significantly increased TCR V\textsuperscript{b}5.1\textsuperscript{+} CD4 T-cell numbers (15–17%) in KA/KA skin compared with WT skin (3–4%) (Figure 2a and b). Most KA/KA TCR V\textsuperscript{b}5.1\textsuperscript{+}CD4 T cells were CD44-positive, indicating that these cells were activated and autoreactive (Figure 2c). In addition, the proportion of V\textsuperscript{b}5.1\textsuperscript{+}CD4 T cells in KA/KA mice was much higher in the skin than in the thymus and spleen (Figure 2b, d, and e, and Supplementary Figure S2a and b), suggesting that the significant expansion of V\textsuperscript{b}5.1\textsuperscript{+}CD4 T cells may contribute to the skin phenotypes in KA/KA mice.
KA/KA skin compared with WT (Figure 2f), and the total number of Vβ5.1⁺ CD8 T cells was very low compared with Vβ5.1⁺ CD4 T cells (data not shown). These results suggest that CD4 T cells may play a critical role in the onset of skin lesions in KA/KA mice. Thus, we focused on studying the function of CD4 T cells in the skin disease.

Similar to the findings in the skin specimens of patients with APECED, flow cytometry analyses showed significantly
increased macrophage and neutrophil numbers in the skin of KA/KA mice compared with WT mice (Figure 2g). Thus, we attempted to further understand the relationship between autoreactive CD4 T cells and these myeloid cells during the pathogenesis of skin lesions.

**Autoreactive CD4 T cells initiate skin lesions associated with increased macrophage and neutrophil infiltration**

We found that CD4 T-cell infiltration was correlated with the severity of KA/KA skin phenotypes (Supplementary Figure S3a). To examine the pathogenic role of autoreactive T cells in the skin lesions, we generated Rag1−/−;KA/KA mice that lack T cells and B cells. Histological examination showed that all organs, including the skin, were normal in Rag1−/−;KA/KA mice compared with KA/KA mice (Supplementary Figure S3b and c). We then performed CD4 T-cell adoptive transfer experiments in Rag1−/− and KA/KA;Rag1−/− mice. Neither WT nor KA/KA CD4 T-cell injection elicited skin inflammation and hyperplasia in Rag1−/− mice. Meanwhile, KA/KA CD4 T-cell (or total T-cell) injection elicited epidermal hyperplasia and infiltration of T cells, macrophages, and neutrophils in all five Rag1−/−;KA/KA mice, but WT CD4 T-cell injection did not induce any phenotypes in Rag1−/−;KA/KA mice (Figure 3a), suggesting that autoreactive CD4 T cells are required for pathogenesis of the skin disease. WT and KA/KA B-cell injection did not incite autoimmune skin phenotypes in KA/KA;Rag1−/− mice (data not shown). Thus, KA/KA CD4 T cells are pathogenic for skin disease development in KA/KA mice. Because KA/KA T-cell injection incited increased T-cell infiltration and skin phenotypes in KA/KA;Rag1−/− mice but not Rag1−/− mice, these data suggest that increased autoreactive T-cell recruitment to the skin requires additional conditions.

To verify whether the autoreactive CD4 T cells or KA/KA thymus contribute to skin lesion development in KA/KA mice, we generated KA/KA;Cd4−/− mice. CD4 T-cell deletion decreased the skin phenotypes, although minor inflammation phenotypes remained in other organs (Figure 3b and c, Supplementary Figure S3d). Thus, KA/KA CD4 T cells play a critical role for initiating skin phenotypes in KA/KA mice. To determine the effect of impaired central tolerance on KA/KA skin phenotypes, we performed thymectomy experiments to remove the thymus in newborn KA/KA (KA/KAThympo) mice. The skin phenotypes of KA/KAThympo mice significantly decreased (Figure 3b, Supplementary Figure S3e and f), suggesting that T-cell development in the thymus contributes to skin homeostasis.

Defective AIRE also impairs regulatory T cell (Treg) development (Anderson et al., 2005; Kekäläinen et al., 2007). Foxp3−/−CD25+Treg numbers were lower in KA/KA thymi and spleens than in WT thymi and spleens (Supplementary Figure S4a). To determine the effect of Treg on the autoinflammatory phenotypes in KA/KA mice, we intravenously injected WT GFP-Foxp3 cells (5 × 10^6 per mouse) into KA/KA mice at 5–6 weeks of age. The injection significantly delayed the onset of skin phenotypes associated with decreased macrophage, neutrophil, and CD4 T-cell numbers compared with untreated KA/KA mice (Supplementary Figure 4b and c). However, KA/KA GFP-Foxp3 cells did not suppress skin manifestations (data not shown). These data indicate that impaired Tregs also contribute to skin inflammatory responses in KA/KA mice.

**Ablation of Ccl2 and Cxcr2 genes in KA/KA mice reduces macrophage and neutrophil recruitment and attenuates skin lesions**

CXCL1, CXCL2, and CXCL5 are the ligands of CXCR2 (Jablonska et al., 2014). These chemokines, which are highly expressed in KA/KA skin compared with WT (Figure 1e), recruit neutrophils. In addition, CCL2, which binds CCR2 and is also highly expressed in KA/KA skin compared with WT, is a major chemokine for recruitment of monocytes in sites of inflammation (Takahashi et al., 2009). To determine the impact of elevated CCL2 and CXCR2 expression on macrophage and neutrophil recruitment and skin lesions, we generated KA/KA;Ccl2−/− and KA/KA;Ccl2−/−;Cxcr2−/− mice. Ccl2 deletion reduced epidermal thickness and macrophage and neutrophil numbers in the skin of KA/KA;Ccl2−/− mice compared with KA/KA mice, whereas double Ccl2 and Cxcr2 deletion further improved skin phenotypes (Figure 4a and b). Deletion of Ccl2 or Ccl2 and Cxcr2 also reduced T-cell infiltration (Figure 4c). The skin of KA/KA;Ccl2−/−;Cxcr2−/− mice aged one year or older looked normal as determined by histological examination and flow cytometric analyses. These results suggest that increased autoreactive T-cell infiltration requires macrophage and neutrophil recruitment to mediate skin lesions.

We also evaluated the role of macrophages in the skin lesions of KA/KA mice by injecting clodronate-loaded liposomes that induce macrophage death (Xiao et al., 2013). Treatment with clodronate-loaded liposomes significantly reduced macrophage numbers and inhibited skin phenotypes in all five KA/KA mice tested (Figure 4d), although the treated skins retained some infiltrating cells. Thus, increased macrophages play a pathological role for an autoreactive T cell–mediated skin disorder. In addition, depleting macrophages also reduced the number of infiltrating neutrophils in treated KA/KA skin (Figure 4b), suggesting that increased macrophage numbers contribute to neutrophil recruitment.

**KA/KA epidermal cells highly express CCL2, which contributes to skin phenotypes**

To determine which cell types highly express CCL2 and CXCL1 in the skin, we used immunofluorescence staining and found elevated CCL2 and CXCL1 expression in keratin 5 (K5)–positive KCs of KA/KA skin compared with WT KCs (Supplementary Figure S5a, left and right). Because Ccl2 ablation significantly affected skin phenotypes, we further examined how CCL2 is regulated in KCs using PCR. KA/KA;Ccl2−/− skins expressed decreased CCL2 compared with KA/KA skins (Figure 5a). WT and Ccl2−/− skins were used as controls. Because IKKz reduction in the skin is correlated with skin phenotypes (Supplementary Figure S1c and e), we then determined the effect of IKKz on CCL2 expression in KCs. To do so, we generated KA/KA;K5.IKKz and KA/KA;Lori.IKKz mice (Liu et al., 2006; Xia et al., 2010), in which the transgenic IKKz cDNA is controlled by a K5 promoter and by a loricrin promoter, respectively. Reintroducing IKKz into KCs significantly reduced CCL2 expression and rescued KA/KA skin phenotypes (Figure 5a...
and Supplementary Figure S5b), CCL2 expression in K5-expressing KCs with Ikka deletion was used as a positive control. Together, these results suggest that IKKα suppresses CCL2 expression. To elucidate the pathway by which IKKα regulates CCL2 expression, we first examined the effect of IKKα kinase on CCL2 expression in the skin of IkkaAA/AA (AA/AA) mice, in which two serine residues at amino acids 177 and 181 within a kinase activation loop

Figure 3. Autoreactive T cells derived from thymi mediate skin inflammation and phenotypes. (a) H&E staining and IHC analyses of CD3 T cells, macrophages (F4/80), and neutrophils (Gr1) in the skin of Rag1<sup>−/−</sup> and Rag1<sup>−/−</sup>; kinase-dead Ikka knockin (KA/KA) mice after receiving WT or KA/KA T-cell injections. Dark brown, positive staining. Bar = 50 μm. (b) H&E staining of skin from WT, KA/KA;Cd4<sup>−/−</sup>, and KA/KA<sup>Thymo</sup> (thymectomy in KA/KA mice) mice. Bar = 50 μm. (c) Flow cytometric analysis of T cells in spleens of WT, Cd4<sup>−/−</sup>, KA/KA, and KA/KA;Cd4<sup>−/−</sup> mice. IHC, immunohistochemical; WT, wild-type.
are replaced by alanine (Cao et al., 2001). AA/AA mice express normal IKK\(\alpha\) levels compared with WT (Cao et al., 2001). RT-PCR analysis showed that AA/AA skin expressed CCL2 levels similar to WT (Figure 5a). Thus, IKK\(\alpha\)'s levels but not its kinase activity regulate CCL2 expression in the skin.

Next, we investigated how IKK\(\alpha\) regulates CCL2 expression in keratinocytes. KA/KA skin expressed increased TGF\(\beta\)1 and

**Figure 4. Deletion of macrophages and neutrophils rescues skin phenotypes.** (a) H&E staining of skins of kinase-dead Ikka knockin (KA/KA), KA/KA:Ccl2\(^{-/-}\), and KA/KA:Ccl2\(^{-/-}\);Cxc2\(^{-/-}\) mice at 8 months of age. Bar = 50 μm. (b) Flow cytometric analyses of macrophages and neutrophils in the skin of WT, KA/KA, KA/KA:Ccl2\(^{-/-}\), and KA/KA:Ccl2\(^{-/-}\);Cxc2\(^{-/-}\) mice at 6 months of age. **P < 0.01; ****P < 0.0001; Student’s t-test. (c) IHC analysis of CD3 T cells in the skin of WT, KA/KA, KA/KA:Ccl2\(^{-/-}\), and KA/KA:Ccl2\(^{-/-}\);Cxc2\(^{-/-}\) mice at 6 months of age. ****P < 0.0001; Student’s t-test. (d) H&E staining and IHC analyses of CD3 T cells and macrophages (F4/80) in the skins of KA/KA mice treated with or without clodronate liposomes. Dark brown, positive staining. Bar = 50 μm. IHC, immunohistochemical; ns, not significant; WT, wild-type.
CCL2 but decreased IKKα levels (Figure 1e, Supplementary Figure S1e). Abnormal expression of TGFβ1 promotes skin inflammation (Marinari et al., 2008; Wang et al., 2006). In addition, IKKα can regulate gene expression at the transcripational level by interacting with the chromatin (Liu et al., 2008; Song et al., 2018; Xiao et al., 2013; Zhu et al., 2007). Thus, we hypothesized that TGFβ may regulate CCL2 expression through IKKα's interaction with the Ccl2 gene.
Figure 6. NLRP3 inhibition decreases inflammation and prevents skin lesions in KA/KA mice. (a) Histological examination with H&E staining of skin from WT, kinase-dead Ikkα knockin (KA/KA), and MCC-treated KA/KA mice. The numbers at the bottom of the photos represent sick mice from each group of 10. Bar = 50 μm. (b) Statistical analysis for the skin phenotypes of WT, KA/KA, and MCC-treated KA/KA mice. Data analyzed by chi-square test (n = 10 mice/group); ***P < 0.001; MCC, MCC950. (c) IHC analysis of infiltrating macrophages (F4/80), T cells (CD3), and neutrophils (Ly6G/Gr-1) in the skin of WT, KA/KA, and MCC-treated KA/KA mice. Data represent mean ± SEM (three repeats); ****P < 0.0001; Student’s t-test. (d) Uric acid assay (×100) for the skins of WT, KA/KA (KA), KA/KA;Cd4−/− (KO), and MCC-treated KA/KA mice (n = 3 samples/group). Data represent mean ± SEM (three repeats); **P < 0.01; Student’s t-test. (e) RT-PCR
promoter in KCs. We then treated KA/KA mice with an anti-TGFβ1 antibody via intraperitoneal injection. Treatment with the anti-TGFβ1 antibody reduced CCL2 expression and improved KA/KA skin phenotypes (Figure 5b–d), suggesting a functional relationship among TGFβ1, CCL2, and IKKζ. We performed a chromatin immunoprecipitation assay with an anti-IKKζ antibody on the Ccl2 promoter in KCs and found that IKKζ formed a complex with the Ccl2 promoter in WT KCs (Figure 5e–g). Treatment with TGFβ1 reduced IKKζ binding to the Ccl2 promoter in WT KCs. IKKζ-deficient KCs were used as a control. Smad3 and Smad4 bound to the Ccl2 promoter when IKKζ was absent, and TGFβ treatment also elevated the binding of Smad3 and Smad4 to the Ccl2 promoter in WT KCs (Figure 5e–g), indicating that IKKζ binding to the Ccl2 promoter suppresses CCL2 expression. In the absence of IKKζ, the increased binding of Smad3/4 to the Ccl2 promoter promotes CCL2 expression in KCs. Indeed, reintroducing IKKζ to IKKζ-null KCs suppressed CCL2 expression, whereas TGFβ promoted CCL2 expression by increasing the binding of Smad3/4 to the Ccl2 promoter (Figure 5e–h). These results suggest that IKKζ regulates CCL2 expression by modulating Ccl2 promoter activity, which antagonizes TGFβ-mediated CCL2 expression.

**NLRP3 inhibition ameliorates the skin phenotypes of KA/KA mice**

Next, we examined whether increased NLRP3 expression is biologically relevant to the pathogenesis of skin phenotypes in KA/KA mice through the increased infiltration of autoreactive CD4 T cells and myeloid cells. We intraperitoneally treated 10 KA/KA mice at 2 months of age (before the skin phenotype onset) with an NLRP3 inhibitor, MCC950 (Coll et al., 2015; Perera et al., 2018), twice weekly. We treated the mice for 3 months and then collected the results from the mice at 6 months of age. MCC950 treatment dramatically improved fur appearance and rescued skin phenotypes in KA/KA mice compared with vehicle control KA/KA mice, as determined by histological examination (Figure 6a and b). MCC950 treatment also significantly decreased the infiltration of T cells, macrophages, and neutrophils (Figure 6c, Supplementary Figure 5f). Finally, MCC950 treatment or CD4 T-cell depletion reduced uric acid levels in KA/KA skin compared with control KA/KA skin specimens (Figure 6d) and repressed the expression of NLRP3, CCL2, IL-1β, CXCL1, and TGFβ1 in KA/KA skin compared with untreated KA/KA skin specimens, as detected by RT-PCR (Figure 6e and f). WT mice were used as controls. In addition, reintroducing transgenic K5.IKKζ into KCs or depleting CCL2 decreased NLRP3 expression in KA/KA mouse skins (Figure 6g). Taken together, these results suggest that the pathogenic autoreactive CD4 T cells trigger NLRP3-dependent skin inflammatory phenotypes in KA/KA mice through interplaying with multiple immune cells and epithelial cells (Figure 6h). NLRP3, macrophages and neutrophils, and their regulators are potential therapeutic targets for some fractions of skin autoimmune diseases.

**DISCUSSION**

A large number of American patients with APECED with AIRE mutations develop skin manifestations associated with elevated infiltration of CD4 T cells and myeloid cells (Ferre et al., 2016), although American and European patients with APECED may show some discrepancies in their manifestations, which is likely because of genetic, environmental, and microbiome differences (Constantine and Lionakis, 2019). In this study, we demonstrated that the skin manifestations of patients with APECED are similar to inducible skin disorder in KA/KA mice, which is initiated by dermal inflammation and expanded to the epidermis. This disorder is associated with elevated inflammasome activity and increased infiltration of autoreactive CD4 T-cell, macrophage, and neutrophil numbers. We further showed how autoreactive CD4 T-cell-mediated skin disease development needs macrophage and neutrophil recruitment through interaction with skin cells. NLRP3 can be used as a therapeutic target for autoreactive T-cell–induced skin diseases.

Previously, we reported that impaired central tolerance results in increased Vß5.1⁺CD4 T cells in the thymus of KA/KA mice (Kogawa et al., 2002; Zhu et al., 2017). The amplified Vß5.1⁺CD4 T-cell population in the esophagus contributes to increased fungal colonization and esophageal carcinogenesis in KA/KA mice. In this study, we detected an elevated Vß5.1⁺CD4⁺CD44⁺ activated and autoreactive cell population in the skin of KA/KA mice compared with the thymus. Depleting lymphocytes or CD4 T cells in KA/KA;Rag1⁻/⁻ mice or in KA/KA;Cd4⁺/⁻ mice abolished skin phenotypes. Thus, with increased age, gradually increased autoreactive CD4 T cells irritate the skin, resulting in tissue injury or damage, inflammation, and cell growth.

Although AIRE mutations account for the pathogenesis of APECED autoimmunity disease (Jiang et al., 2005; Kuroda et al., 2005; Zhu et al., 2017), it is still unclear why Aire⁻/⁻ mice display mild symptoms compared with patients with APECED and KA/KA mice. To date, we have generated more than 100 KA/KA;Rag1⁻/⁻ mice and 100 KA/KA;Cd4⁺/⁻ mice. None of these mice have shown skin manifestations between 1 day and 1 year of age. Thus, autoreactive CD4 T cells are pathogenic for the autoimmune diseases. Of note, in addition to autoreactive CD4 T cells, patients with APECED show decreased FoxP3 Treg numbers, and their Tregs (CD25⁺/CD4⁺) lose a suppressive function compared with healthy controls (Kekäläinen et al., 2007). Consistently, here, we found that KA/KA mice developed reduced Foxp3 Treg numbers and that WT Foxp3 Tregs, but not KA/KA cells, were able to delay the onset of skin manifestations in KA/KA mice, indicating that defective Tregs enhance the severity of the
skin disease in KA/KA mice. Therefore, defective AIRE also impairs Treg numbers and functions, which contribute to the pathogenesis of the diseases. Meanwhile, Aire−/− mice develop normal Tregs (Kekäläinen et al., 2007), which may be one of the reasons for the dissimilar phenotypes in Aire−/− mice versus patients with APECED and KA/KA mice.

Furthermore, depleting lymphocytes or CD4 T cells or injecting WT Tregs decreased the infiltrating macrophage and neutrophil numbers and NLRP3 expression levels in KA/KA mice. Ablation of Ccl2 and Cxcr2, which recruit macrophages and neutrophils, attenuated the infiltration of autoreactive CD4 T cells, macrophages, neutrophils, and skin manifestations. Although depleting CCL2 or depleting macrophages significantly decreased skin phenotypes, the skin retained minor phenotypes with increased neutrophil infiltration. Deletion of both Ccl2 and Cxcr2 genes significantly decreased macrophage and neutrophil numbers and completely rescued the skin phenotypes in KA/KA mice at 1 year of age, indicating that both macrophages and neutrophils promote the skin disease. Collectively, in this study, we identified the increased macrophages and neutrophils as a main cause for autoreactive CD4 T-cell-initiated skin diseases. Excessive NLRP3, macrophages, and neutrophils were detected in skin lesions of patients with APECED. Treatment with an NLRP3 inhibitor decreased macrophage, neutrophil, and autoreactive CD4 T-cell infiltration and dampened skin phenotypes. This finding has medical significance because we can use these identified targets for therapy of skin manifestations of patients with APECED. Depleting lymphocytes or CD4 T cells not only diminished inflammatory skin phenotypes but also attenuated the phenotypes in other organs. Of note, the onset of skin symptoms in some American patients with APECED occurs earlier than other manifestations; moreover, urticarial eruption has been identified as an early symptom for European patients with APECED recently evaluated at the National Institutes of Health (Lionakis, unpublished observations) (Constantine and Lionakis, 2019), which indicates that these treatments for skin manifestation may be used to prevent other manifestations in these patients with systemic autoimmunity. In addition, hair loss and alopecia were found in KA/KA mice and some patients with APECED, respectively (Ferre et al., 2016). It has been reported that increased macrophages inhibit hair growth (Dalessandri and Kasper, 2019). Thus, elevated inflammatory cell numbers may contribute to these skin manifestations. In conclusion, our findings are important for preventing, diagnosing, and treating a fraction of human autoimmune diseases.

MATERIALS AND METHODS
Mice and human skin samples
All mice used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health. All animal experiments (Protocols 17-051 and 17-052) were approved by the Institutional Animal Care and Use Committee. The overall objective of this study was, using mouse models and pharmaceutical reagents, to define how autoreactive T cells cause autoimmune skin inflammation and skin lesions and to develop a therapeutic approach that could be applicable in treating human autoimmune disease. Mice were bred and maintained in a specific pathogen-free facility at the National Cancer Institute at Frederick. Mouse skin mRNA profiles were analyzed at the Frederick National Laboratory for Cancer Research. AA/AA mice were obtained from Michael Karin’s laboratory at University of California, San Diego (Caio et al., 2001). Cdh1−/− mice and Rag1−/− mice were obtained from The Jackson Laboratory (Sacramento, CA). Ccl2−/− mice were obtained from Joost J. Oppenheim’s laboratory at the National Cancer Institute at Frederick (Takahashi et al., 2009). Our laboratory generated KA/Ka, KA/Ka;Lor1.1Kk2, and KA/Ka;K5.1Kka mice (Liu et al., 2006; Xia et al., 2010; Zhu et al., 2007). Cxcr2−/− mice were purchased from The Jackson Laboratory (Stock No: 006848). Pharmaceutical reagents applied in this study include anti-TGFβ antagonist antibody (Clone 1D11, Giorgio Trinchieri’s laboratory at the National Cancer Institute), clodronate-loaded liposomes (Encapsula NanoSciences, Brentwood, TN), and NLRP3 inhibitor MCC950 (inh-mcc, InvivoGen, San Diego, CA). Normal human skin paraffin tissue sections were purchased from US Biomax (Derwood, MD; cat #: HuFPT136). Skin specimens of patients with APECED (enrolled in 2013–2015) were collected in accordance with a protocol approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board, and patients provided written informed consent (Ferre et al., 2016).

Data availability statement
No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: FZ, YH; Funding Acquisition: YH; Investigation: FZ, JZ, JW, ZS, XW; Methodology: FZ, JZ, JW, ZS, XW; Resources: EMNF, MSL; Supervision: YH; Writing - Original Draft Preparation: FZ, YH; Writing - Review and Editing: FZ, JW, JZ, EF, ZS, XW, MSL, YH

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.10.016.

REFERENCES
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