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The Inflammatory Caspases-1 and -11 Mediate the Pathogenesis of Dermatitis in Sharpin-Deficient Mice

Todd Douglas,* Claudia Champagne,† Alexandre Morizot,‡ Jean-Martin Lapointe,§ and Maya Saleh*†‡§

Chronic proliferative dermatitis in mice (cpdm) is a spontaneous multiorgan inflammatory disorder with pathological hallmarks similar to atopic dermatitis and psoriasis in humans. Cpdm mice lack expression of SHANK-associated RH domain–interacting protein, an adaptor of the linear ubiquitin assembly complex, which acts in the NF-kB pathway to promote inflammation and protect from apoptosis and necroptosis. Although skin inflammation in cpdm mice is driven by TNF- and RIPK1-induced cell death, the contribution of initiating innate immunity sensors and additional inflammatory pathways remains poorly characterized. In this article, we show that inflammasome signaling, including the expression and activation of the inflammatory caspase-1 and -11 and IL-1 family cytokines, was highly upregulated in the skin of cpdm mice prior to overt disease onset. Genetic ablation of caspase-1 and -11 from cpdm mice significantly reduced skin inflammation and delayed disease onset, whereas systemic immunological disease persisted. Loss of Nlrp3 also attenuated skin disease, albeit more variably. Strikingly, induction of apoptosis and necroptosis effectors was sharply decreased in the absence of caspase-1 and -11. These results position the inflammasome as an important initiating signal in skin disease pathogenesis and provide novel insights about inflammasome and cell death effector cross-talk in the context of inflammatory diseases. The Journal of Immunology, 2015, 195: 000–000.

The inflammasome is a pattern recognition complex that acts to coordinate immediate innate immune responses to invading microorganisms, perturbations, and tissue damage. Direct or indirect sensing of microbial- or danger-associated molecular patterns triggers the assembly of an inflammasome, resulting in cell death and autoactivation of the effector protease caspase-1. Active caspase-1 cleaves a number of substrates, including the cytokines pro–IL-1β and pro–IL-18, to initiate the inflammatory cascade and, in some contexts, a rapid form of caspase-1 and -11 from cpdm mice is driven by TNF- and RIPK1-induced cell death, the contribution of initiating innate immunity sensors and additional inflammatory pathways remains poorly characterized. In this article, we show that inflammasome signaling, including the expression and activation of the inflammatory caspase-1 and -11 and IL-1 family cytokines, was highly upregulated in the skin of cpdm mice prior to overt disease onset. Genetic ablation of caspase-1 and -11 from cpdm mice significantly reduced skin inflammation and delayed disease onset, whereas systemic immunological disease persisted. Loss of Nlrp3 also attenuated skin disease, albeit more variably. Strikingly, induction of apoptosis and necroptosis effectors was sharply decreased in the absence of caspase-1 and -11. These results position the inflammasome as an important initiating signal in skin disease pathogenesis and provide novel insights about inflammasome and cell death effector cross-talk in the context of inflammatory diseases.

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Abbreviations used in this article: AD, atopic dermatitis; cpdm, chronic proliferative dermatitis in mice; LUBAC, linear ubiquitin assembly complex; Sharpin, SHANK-associated RH domain–interacting protein.

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The linear ubiquitin assembly complex (LUBAC) plays a critical role downstream of various cytokine receptors and pattern recognition receptors by modifying RIPK1 and IKKγ (NEMO) (NEMO) with linear polyubiquitin chains to promote inflammation and cell survival. LUBAC consists of two catalytic subunits, HOIP and HOIL-1, and a regulatory subunit: SHANK-associated RH domain–interacting protein (Sharpin). Interestingly, mice deficient in the different LUBAC components display radically different phenotypes; Hoil1<−/−> mice are viable and phenotypically normal (28), Hoip<−/−> mice die during embryogenesis at midgestation (29), and a homozygous inactivating mutation in Sharpin triggers development of severe multiorgan inflammation, with the most prominent feature being chronic proliferative dermatitis (cpdm) (30–33). Sharpin<+/−> mice display skin lesions with traits similar to AD and psoriasis in humans, such as hyperkeratosis, acanthosis, dermal spongiosis, and keratinocyte apoptosis (19, 34). Along with dermatitis, cpdm mice develop systemic inflammation and adaptive immune defects, such as splenomegaly, disrupted secondary lymphoid organ development, and inflammatory infiltrates throughout the body. Skin disease in cpdm mice was initially considered a model of human AD, given the similar histopathological findings, the prominent Th2 cytokine profile in the skin and spleen, and the associated eosinophilia (35). However, the observations that neither depletion of eosinophils nor neutralizing anti–IL-5 Ab administration nor genetic ablation of IL-4R alleviated skin inflammation suggested that type 2 immune responses could be manifestations of alternate mechanisms driving skin disease. This is unlike AD, in which neutralizing the IL-4R common α subunit proved to be of high therapeutic value in human patients (38). TNF-mediated cell death pathways play a critical role in cpdm disease pathogenesis, because genetic loss of Tnf (31) or transgenic expression of a kinase-dead Ripk1 (39), which is capable of inducing apoptosis but not necroptosis, rescued cpdm skin disease. Loss of IL-1RAP, a common adaptor of IL-1R and IL-33R, also was shown to attenuate skin inflammation, implicating these inflammatory pathways in disease pathogenesis (40).

Although these studies provide important genetic insights into the role of cell death in inflammatory disease, it remains unclear what pathways are engaged by Sharpin deficiency upstream of, or in parallel with, TNF-induced cell death. In this article, we demonstrate that the inflammasome pathway is an important mediator of skin disease in Sharpin<+/−> mice and may act upstream of apoptotic and necrototic cell death.

Materials and Methods

Animal strains

All mice were bred and maintained at McGill University. C57BL/KaLaw-Rij-Sharpin<+/−> (RijSun) (Sharpin<+/−> mice) (30) were obtained from J.P. Sundberg (The Jackson Laboratory, Bar Harbor, ME). All breeding animals were heterozygous for cpdm. In all experiments, unaffected wild-type or heterozygous littermates were used as controls. Cpdm, Iice−/−, and cpdm; Nlrp3<−/−> mice were generated by intercrossing Sharpin<+/−> heterozygotes with B6N.129S2-Csp1<tm1HFlj/Ice−/−> mice (41) or B6N.129-Nlrp3<tm3HFlj/Ice−/−> (Nlrp3<−/−>) mice (42) to obtain Sharpin<+/−>; Iice−/− or Sharpin<+/−>; Nlrp3<−/−> breeding parents, respectively. Mice were monitored biweekly for skin disease. Both male and female mice were used in all experiments. All animal experiments were approved by the McGill University Animal Care and Ethics Committee, in accordance with the guidelines of the Canadian Council on Animal Care.

Tissue processing and Western blotting

Sections (1.5 × 1.5 cm) of shaved dorsal skin were excised and flash frozen for later use. Skin was homogenized in 1.2 ml ice-cold lysis buffer (50 mM HEPES-NaOH [pH 7.5], 100 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% CHAPS) supplemented with 10 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, and complete protease inhibitors (Roche), using a GLH homogenizer. After 30 min on ice, homogenized samples were subjected to four freeze-thaw cycles, followed by sonication (3 × 15 s at 80% amplitude). Following three rounds of centrifugation (13,000 g for 20 min) to pellet debris and remove lipids, proteins in the supernatant were quantified using the Bradford assay, and samples were diluted to 2 μg/ml with lysis buffer. A total of 20–40 μg lystate was separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk in 0.1% TBS–Tween 20 for 1 h at room temperature followed by overnight incubation with primary Ab at 4˚C with the following Abs: anti–capsase-1 p20 (Genentech; 1:1000), anti-cleaved caspase-3 (Cell Signaling; cat. no. 9661; 1:500), anti-cleaved caspase-8 (Cell Signaling; cat. no. 9429: 1:1000), anti–capsase-11 (Sigma; cat. no. C7611; 1:1000), anti-IL-18 (BioVision; cat. no. 5180R; 1:2000), anti–IL-18R<α1> (Prosci; cat. no. 2233; 1:1000), anti-Ripk1 (BD; cat. no. 610459; 1:1000), anti-Milki (Abcam; cat. no. 172686; 1:1000), anti-Sharpin (Protechtech; cat. no. 14626-1-AP; 1:2000), and anti-GAPDH (Santa Cruz; cat. no. sc-25778; 1:2000). Signal was detected following incubation with HRP-conjugated secondary Abs for 1 h at room temperature using ECL (Perkin Elmer; cat. no. NE104001EA or Thermo Scientific; cat. no. 1856189).

Quantitative real-time PCR

Total RNA was extracted from 1.5 × 1.5-cm shaved skin sections by homogenization in TRIzol reagent, followed by chloroform extraction and isopropanol precipitation. A total of 1.0 μg RNA was reverse transcribed into cDNA using random hexamers and MMLV Reverse Transcriptase in a total volume of 20 μL, according to the manufacturer’s protocol. Quantitative PCR was performed using iTaq SYBR Green SuperMix, following normalization to the housekeeping gene Hprt1 or Gapdh, fold induction over wild-type was calculated using the 2−ΔΔCt method. Primer sequences are listed in Supplemental Table I.

Skin explants culture and ELISA

Sections (1.5 × 1.5 cm) of dorsal skin were excised, weighed, washed in PBS with antibiotics/antimycotics (Life Technologies; cat. no. 15240-062), and cultured for 24 h in 1 ml KGM (Lonza; cat. no. CC-3108) at 37˚C, 5% CO2. Following centrifugation to pellet debris, supernatant was used for ELISA. The following ELISAs were performed on the supernatant: IL-1β (R&D Systems; cat. no. DY401), IL-18 (MBL International; cat. no. 1625), IL-1α (R&D Systems; cat. no. DY403), IL-33 (R&D Systems; cat. no. DY3626), TSLP (R&D Systems; cat. no. DY555), and TNF (R&D Systems; cat. no. DY410). All values were normalized to skin weight.

Histology

Skin, spleen, and liver sections were fixed in 10% buffered formalin overnight and embedded in paraffin, and 4-μm-thick sections were cut onto glass slides and processed for H&E staining. H&E sections were scanned using a ScanScope XT digital scanner (Aperio Technologies). Histopathological analysis was performed using H&E-stained slides by a trained veterinary pathologist (J.-M.L.) blinded to genotype. The changes observed (hyperkeratosis, parakeratosis, intracorneal pustules, acanthosis, apoptotic keratinocytes, spongiosis, erosion and ulceration, dermal–epidermal interface edema and disarray, pigmentary incontinence, fibrosis and granulation, and inflammatory infiltrates in the epidermis, dermis, and subcutis) were graded using a 0 to 4 scale, where 0 is normal/absent and 4 is extreme/severe, culminating in a maximum dermatitis score of 56. Epidermal thickness was measured from at least five randomly selected fields/mouse using ImageScope (Aperio Technologies). To identify mast cells, paraffin-embedded skin sections were deparaffinized, rehydrated with distilled water, stained with a toluidine blue/sodium chloride solution (pH 2.3) for 3 min, and dehydrated with 95 and 100% ethanol, followed by clearing in xylene. Slides were scanned using a ScanScope XT digital scanner (Aperio Technologies).

Immunofluorescence staining and quantification

Following deparaffinization and Ag retrieval, slides were permeabilized with 0.25% Trition X-100 in PBS for 20 min at room temperature. Click-iT TUNEL Alexa Fluor 647 (Invitrogen; cat. no. C10247) staining was performed, according to the manufacture’s instructions. Slides were blocked (10% FBS, 3% BSA) for 30 min at 37˚C, followed by a 30-min incubation with Alexa Fluor 594 (Invitrogen)-coupled secondary Abs. The slides were rinsed with PBS and stained with Hoechst 33342 (Invitrogen; cat. no. H3570). Tissues were mounted with cover slips and analyzed on...
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The severity of dermatitis was scored by evaluating the regions affected (head, neck, and/or back; front limbs and/or ventral area; hindlimbs and/or rump), with each affected region scoring 1 point, with the exception of the head (2 points), for a maximum of 5 points. The severity of lesions was evaluated and graded from 0 to 3, as follows: 0 = no lesion, 1 = mild scaling and hair loss, 2 = erosion and mild ulceration, and 3 = coalescent ulcerations (>5 mm). The score for the number of regions affected was added to the score for the severity of the lesions for a maximum score of 8. Clinical end point was considered a score ≥ 5 or the presence of corneal ulceration.

Flow cytometry staining

Splenocytes were stained with viability dye (LIVE/DEAD Fixable Aqua Dead cell stain kit; Molecular Probes) and the following Abs in PBS containing 2% FBS for 20 min: B220-allophycocyanin (clone RA3-6B2; eBioscience), NK1.1-PE (clone 1H7; BioLegend), CD11c-FITC (HL3; BD Biosciences), CD11b-eFluor 450 (M1/70; eBioscience), Ly6C-PE (clone HK1.4; eBioscience), Ly6G-PE-Cy7 (1A8; BioLegend), and TCRβ-PerCP-Cy5.5 (H57-597; eBioscience). Cells were washed in PBS containing 2% FBS and fixed with PBS containing 2% formaldehyde. Samples were analyzed on a FACSCanto II (BD Biosciences), and data were analyzed using FlowJo vX0.7.

Primary bone marrow–derived macrophage culture and stimulation

Bone marrow–derived macrophages were isolated, cultured, and stimulated as previously described (43).

Statistical analysis

Data are shown as mean ± SE. The two-tailed Student t test was used for evaluating statistical significance between two groups. One-way ANOVA followed by the Bonferroni post hoc comparison test was used to evaluate statistical significance among three or more groups.

Results

Early activation of inflammasome signaling in the skin of cpdm mice

To uncouple causative and consequential processes that occur in cpdm mice, we sought to examine the inflammatory milieu of the skin prior to overt disease onset. At weaning, 3–4 wk-old cpdm mice were indistinguishable in appearance from their Sharpin+/+ and Sharpin−/− littermates (Fig. 1A), although mild epidermal hyperplasia and inflammatory cell recruitment in cpdm mice were already apparent by H&E staining (Fig. 1B, 1C). The absence of splenomegaly in 3–4 wk-old cpdm mice suggested that systemic immunological disease had not fully developed at this early time point (Fig. 1D). Despite the lack of overt lesions, skin explants from cpdm mice contained higher levels of IL-1 family cytokines (i.e., IL-1β, IL-18, and the alarmin IL-33) compared with Sharpin+/+ littermates (Fig. 1E), with no significant differences in other cytokines known to initiate type II cytokine–driven diseases, such as TSLP (data not shown). At this time point, no significant differences in gene expression in the skin of these cytokines or other type I or II cytokines were found (Fig. 1F). Because IL-1β and IL-18 are regulated by the inflammasome and inflammatory caspases-1 and -11 at the posttranslational level, and because their increased levels in cpdm skin could not be explained by enhanced gene expression, we posited that the inflammasome pathway might be engaged in the skin of these animals. To test this hypothesis, we performed immunoblot analysis to determine the expression and activation levels of various inflammasome pathway components. Fig. 1G shows increased expression and processing of inflammatory caspase-1 and -11 and enhanced processing of IL-18 in the skin, indicative of inflammasome activation. Because LUBAC regulates cell death downstream of TNF signaling, which was implicated in cpdm skin disease pathogenesis (39, 44, 45), we examined, in parallel, the levels of cell death effectors in the skin of young cpdm mice. The kinases Ripk1 and Ripk3, which are involved in both apoptosis and necroptosis, also were induced in cpdm skin (Fig. 1G). Consistently, the proapoptotic effector Mlkl was slightly induced, and proapoptotic caspase-3 and -8 were more notably activated and cleaved in the skin of young cpdm animals (Fig. 1G). Collectively, these results indicate that activation of cell death and inflammasome pathways are early events that occur prior to overt skin disease manifestations and may be important drivers of pathology.

Inflammatory caspase-1 and -11 mediate cpdm skin pathology

To examine the role of the inflammatory caspases in cpdm pathology, we crossed cpdm mice with Ice−/− mice, which are deficient in both caspase-1 and -11 (46). Strikingly, at 8 wk of age, when cpdm mice are typically nearing clinical end point in our facility and require euthanasia, age-matched cpdm; Ice−/− compound mutant mice were completely free of skin lesions (Fig. 2A). H&E staining revealed the absence of epidermal hyperplasia (Fig. 2B, 2C), and histological analysis identified an almost complete lack of microscopic disease traits typically observed in the skin of cpdm mice, such as hyperkeratosis, parakeratosis, acanthosis, dermal–epidermal interface edema, and dermal fibrosis (Fig. 2D). Qualitative examination of inflammatory infiltrates in the dermis of cpdm and cpdm; Ice−/− mice revealed the most striking difference to be a paucity of macrophages in the skin of the compound mutant mice compared with cpdm mice (Fig. 2E). This was supported by decreased mRNA expression of Emr1 (F4/80) (Supplemental Fig. 1). Similar to monocytes and macrophages, staining skin sections with toluidine blue revealed that mast cells numbers also were decreased (Fig. 2F). Consistent with inflammasome activation in cpdm skin, skin organ cultures from diseased cpdm mice contained significantly higher levels of IL-1β and IL-18 compared with wild-type mice, which were sharply decreased in age-matched cpdm; Ice−/− compound mutants (Fig. 2G). Interestingly, the danger-associated molecular pattern IL-1α, although not a direct substrate of the inflammatory caspases, also was slightly decreased in the compound mutants, whereas caspase-1 and -11 deficiency had varying effects on IL-33 levels (Fig. 2G). The elevated expression of type II cytokines documented in cpdm mice also was reversed to wild-type levels with the loss of caspase-1 and -11, whereas that of type I cytokines, with the exception of Ifnβ, was largely unaffected (Supplemental Fig. 1). In addition to dampening cytokine production, inflammatory caspase deficiency rescued the excessive keratinocyte proliferation seen in cpdm mice, as evidenced in cpdm; Ice−/− mice by a relative decrease in mRNA expression of keratin-14 (Supplemental Fig. 1). Together, these
results indicate a critical role for the inflammatory caspases in mediating dermatitis in cpdm mice. Despite the reduction in inflammasome-dependent cytokines in the circulation (Fig. 2H), and in striking contrast to the impact of the inflammatory caspases on the skin phenotype, systemic disease was largely unaffected in cpdm; Ice<sup>−/−</sup> mice, as noted by elevated serum KC levels (Fig. 2I), splenomegaly (Fig. 2J), and aberrant secondary lymphoid architecture (Supplemental Fig. 2A). Flow cytometric analysis of the spleen revealed an expansion of myeloid cells with prominent eosinophilia and a relative decrease in B and T cell frequencies in cpdm mice. Notably, the lack of caspase-1 and -11 in cpdm; Ice<sup>−/−</sup> compound mutant mice had no effect on the examined splenic cell composition (Supplemental Fig. 2C, 2D).

Interestingly, histological analysis of liver sections from cpdm mice revealed prominent perivascular inflammatory infiltrates that were notably less abundant and reduced in size in cpdm; Ice<sup>−/−</sup> mice (Supplemental Fig. 2B). These data suggest that caspase-1 and -11 primarily control skin disease progression, with intermediate tissue-specific effects on systemic disease.

**Inflammasome activation lies upstream of cell death signaling in cpdm dermatitis**

Given our previous observations that both the inflammasome and cell death effectors were activated early in cpdm mice prior to overt skin disease manifestation, we sought to investigate whether loss of caspase-1 and -11 modulated the expression and activation of apoptosis and necroptosis effectors and, consequently, cell death induction in the skin. Strikingly, the upregulation of Mlkl, Ripk1, and Ripk3 in the skin of cpdm mice was strongly reduced in cpdm; Ice<sup>−/−</sup> compound mutant mice (Fig. 3A). This was accompanied by diminished activation and cleavage of caspase-8 and -3 (Fig. 3A). Concordantly, immunofluorescence staining revealed a significant reduction in cleaved caspase-3+, TUNEL+, and double-positive staining cells in the epidermis (Fig. 3B, 3C). Overall, these data suggest that inflammasome signaling contributes to the inflammatory milieu via cytokine production, as well as to the induction of various cell death pathways in the context of cpdm skin pathology.
FIGURE 2. Genetic ablation of caspase-1 and -11 attenuates cpdm skin disease. (A) Photographs of 8-wk-old cpdm and 7-wk-old cpdm; Ice−/− mice. (B) H&E staining of skin cross-sections from mice of the indicated genotype. Scale bars, 100 μm. (C) Thickness of the epidermis measured using H&E-stained skin sections (n ≥ 4). (D) Cumulative histopathological dermatitis score of cpdm and age-matched cpdm; Ice−/− mice. (E) Qualitative analysis of inflammatory infiltrate composition in the skin of cpdm and cpdm; Ice−/− mice using H&E-stained skin sections. (F) Toluidine blue–stained skin sections from cpdm and cpdm; Ice−/− mice. Arrowheads indicate mast cells. Scale bars, 100 μm. (G) Cytokine levels in cell-free supernatants from skin explants cultured overnight at 37˚C, as measured by ELISA. IL-18 (H) and KC (I) levels were measured by ELISA using serum from 7–12-wk-old wild-type (WT), cpdm, and cpdm; Ice−/− mice. (J) Spleen weight of mice from the indicated genotypes at 7–12 wk of age. Data represent the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, followed by the Bonferroni post hoc test. ns, not significant.
Ablation of inflammasome signaling delays \( cpdm \) skin disease and attenuates its severity

To determine whether skin disease eventually manifests in \( cpdm \); \( \text{Ice}^{-/-} \) compound mutant mice over time, we aged a cohort of mice beyond 8 wk. In contrast to \( cpdm \) mice, which exhibited severe skin disease by 10 wk of age, \( cpdm; \text{Ice}^{-/-} \) mice showed only mild signs of disease (Fig. 4A, 4B), with focal lesions beginning to appear in the thoracic dorsal region. Although the severity of disease slowly progressed over time, lesions were largely confined to the same region by 14 wk of age. At this time, induration of the region, scaling, and concomitant mild ulceration developed. Severe dermatitis developed much later in \( cpdm; \text{Ice}^{-/-} \) mice compared with \( cpdm \) mice (Fig. 4B), although this analysis was complicated by the development of corneal ulcerations in several compound mutant mice that necessitated euthanasia prior to severe disease onset. Interestingly, although alopecia typically

**FIGURE 3.** Loss of caspase-1 and -11 prevents the activation of apoptosis and necroptosis effectors and the induction of cell death. (A) Western blot analysis of skin homogenates for different cell death and inflammasome-associated effector proteins. (B) Representative epifluorescence images of skin sections from \( cpdm \) and \( cpdm; \text{Ice}^{-/-} \) mice stained with Abs against active caspase-3 and TUNEL. Insets correspond to boxed regions (original magnification \( \times3 \)). (C) Quantification of active caspase-3-, TUNEL-, and caspase-3 and TUNEL double-positive stained cells in the epidermis of \( cpdm \) and \( cpdm; \text{Ice}^{-/-} \) mice (n = 3). \( *p < 0.05, **p < 0.01 \), Student two-tailed \( t \) test.

**FIGURE 4.** Skin disease is delayed in \( cpdm; \text{Ice}^{-/-} \) and \( cpdm; \text{Nlrp3}^{-/-} \) mice. (A) Representative photographs of mice at 8–10 and 12–14 wk of age. Dashed lines highlight affected areas. (B) Age at the onset of severe dermatitis (score \( \geq 5/8 \)). Four \( cpdm \); \( \text{Ice}^{-/-} \) and two \( cpdm; \text{Nlrp3}^{-/-} \) mice developed ulcerated corneas necessitating euthanasia prior to the appearance of severe dermatitis and, thus, were excluded from analysis. Each symbol represents an individual mouse. \( *p < 0.05, **p < 0.001 \), Student \( t \) test.
manifests as a precursor of inflammation in cpdm mice, hair loss in cpdm; Ice−/− mice occurred uniquely as a consequence of inflammatory lesion formation and was not observed in noninflamed regions. This finding is supported by a previous report implicating caspase-1 activation in androgenetic alopecia in humans (47).

Next, to define which inflammasome sensor contributed to the activation of the inflammatory caspases in the skin, we generated cpdm mice deficient in the inflammasome scaffolding receptor Nlrc4, a well-described sensor of both microbial and endogenous danger signals. Unlike cpdm; Ice−/− mice, certain cpdm; Nlrc4−/− mice began showing mild skin disease by 8 wk of age (Fig. 4A). Nevertheless, disease progression was notably blunted compared with cpdm mice, with some mice developing severe focal lesions with similar anatomical confinement as seen with cpdm; Ice−/− mice by 12 wk of age. By 16 wk of age, one of five examined cpdm; Nlrc4−/− mice, although significantly runted, had not developed any visible skin lesions. The onset of severe dermatitis also was delayed significantly compared with cpdm mice (Fig. 4B). Overall, our results indicate that the absence of inflammasome signaling delays skin disease onset and attenuates disease progression.

**Attenuated inflammasome activation in cpdm macrophages**

Because we noted a relative loss of macrophage infiltration in the dermis of cpdm; Ice−/− mice and a corresponding decrease in IL-1β and IL-18 secretion, we hypothesized that infiltrating macrophages might be responsible for the enhanced inflammasome response observed in the diseased skin of cpdm mice. To test this hypothesis, we set out to determine whether LUBAC impairment directly impacted the inflammasome pathway in isolated primary macrophages. Treatment of bone marrow–derived macrophages from cpdm mice with a panel of inflammasome agonists induced significantly blunted IL-1β secretion downstream of Nlrc4, Nlrc3, and Aim2 inflammasome activation (Supplemental Fig. 3A). This was likely due to a defect in priming, as reflected by decreased pro–IL-1β expression following LPS treatment (Supplemental Fig. 3B). In contrast to IL-1β, we observed a small, but consistent, increase in IL-18 secretion downstream of Nlrc4 or Aim2 stimulation in cpdm macrophages (Supplemental Fig. 3C). No differences in pyroptosis were observed (Supplemental Fig. 3D). These results are in line with recent studies demonstrating a role for LUBAC in controlling the priming step of the NLRP3 inflammasome in ex vivo macrophages (48). Further, they suggest that alternative priming mechanisms, independent of LUBAC function in the NF-κB pathway, are engaged in vivo, allowing full inflammasome activation or that macrophages are not the primary pathogenic cell type in cpdm skin.

**Discussion**

Inflammatory skin disease is multifactorial and is likely driven by several inflammatory and cell death processes occurring in parallel. It was reported recently that dermatitis of cpdm mice is initiated through TNFR1-dependent cell death, because genetic ablation of Tnfr1 from cpdm mice prevented skin disease manifestation up to 35 wk of age (45). However, although it is agreed that TNF-induced cell death is pathogenic in this context, there is a discrepancy about the primary cell death modality that drives cpdm skin pathology (39, 44). Because both apoptosis and necroptosis are induced by TNF in cpdm keratinocytes ex vivo (31), it is conceivable that they are both detrimental. By examining young cpdm mice prior to the onset of overt disease, we showed that upregulation of inflammasome signaling is not a consequence of an established inflammatory milieu but, rather, is an important initiating signal in disease pathogenesis. However, it is worth noting that the increased IL-18 detected in skin organ culture supernatants may be attributable, in part, to the moderate keratinocyte expansion observed in these young mice and cross-reactivity to the proform of IL-18 (Fig. 1B, 1C), although other epithelial-derived cytokines, such as TSLP, were not significantly elevated (data not shown). The function of inflammasome activation as an initiating signal is supported by the significant attenuation of dermatitis and inflammatory skin lesions in cpdm; Ice−/− compound mutant mice.

Hyperactivation of the inflammatory caspases in the skin of cpdm mice may be a result of cell-intrinsic or cell-extrinsic interactions between Sharpin and the inflammasome. Genetic deficiency of Sharpin may sensitize mice to aberrant reactions to environmental factors that subsequently trigger inflammasome activation. Likewise, predisposition of LUBAC-deficient cells to TNFR1-mediated cell death, in particular necroptosis, could release danger-associated molecular patterns known to trigger inflammasome activation (49). We observed an upregulation of both cell death and inflammasome effectors in cpdm skin before the appearance of outward signs of disease (Fig. 1G), supporting the possibility that very early inflammatory cell death triggers inflammasome activation, initiating a local inflammatory environment. However, if caspase-1 and -11 were activated solely downstream of cell death, their loss should not influence cell death execution. Interestingly, our observation that the induction of both apoptotic and necroptotic effectors was impaired in cpdm; Ice−/− mice, despite genetic susceptibility, places the inflammasome upstream of TNF-driven cell death. Indeed, recent evidence suggests that caspase-1 can act upstream of UVB-induced apoptosis in human keratinocytes (50). Given the intimate association between these pathways and the recent reports highlighting cross-regulatory roles of cell death effectors in inflammasome activation (51–56), our findings may provide insight into how this cross-regulation contributes to inflammatory disease in vivo.

Alternatively, Sharpin could impose a direct negative regulation on caspase activity. Although our data and that of other investigators (48) do not support such a function in macrophages, it is plausible that LUBAC might differentially modulate inflammasome activation in other cell types, such as keratinocytes. Humans with mutations in HOIL1 develop waves of systemic inflammation followed by bouts of immune deficiency, leading to hypersusceptibility to viral and bacterial infections (57). Interestingly, these paradoxical pro- and anti-inflammatory states were attributed to cell type–specific roles for LUBAC, in which LUBAC deficiency led to hyperinflammation in monocytes but abrogated inflammation in fibroblasts. Cpdm bone marrow does not transfer disease to wild-type mice (44, 58), and cpdm skin grafts transplanted onto nude mice retain their diseased state (59), two findings that highlight the potential pathogenic role of skin-resident cells in this context.

Incomplete rescue by loss of Nlrc3 may suggest the involvement of other inflammasome sensors upstream of caspase activation or noninflammasome functions of caspase-11; however, we cannot ignore the possibility that the different genetic backgrounds of the compound mutants and cpdm mice account for some of the observed variability. Further, it remains unclear whether cytokine production, pyroptosis, or an undescribed pathway downstream of caspase-1 and -11 activation contributes to cpdm skin disease. Because genetic ablation of either Il1r1 (44) or Il1rap (39) was shown to attenuate skin disease and delay its onset, IL-1α/β signaling clearly contributes to pathology. Notably, transgenic overexpression of caspase-1 in keratinocytes under the keratin 14 promoter was sufficient to induce severe dermatitis that is reminiscent of eczema in humans (17). As in cpdm mice, these mice displayed severe pruritus, excessive keratinocyte apoptosis, and...
mast cell expansion. Further, despite the lack of keratin 14 expression in splenocytes, skin-specific caspase-1 overexpression skewed splenic T cell polarization toward a Th2 phenotype. In this model, IL-18 plays a dominant role, because its genetic loss fully prevented disease, whereas the absence of IL-1β only delayed its onset (18). Although IL-1β is known to initiate a Th1 response, IL-18–mediated control over Th1/Th2 skewing is dependent on the local cytokine milieu. Indeed, IL-18 was shown to contribute to detrimental Th2 responses in both infectious disease (60) and sterile inflammatory contexts (61), and it was suggested as a useful biomarker to assess AD severity in humans (62, 63); all of these data support the notion that IL-18 may also contribute to biomarker to assess AD severity in humans (62, 63); all of these data support the notion that IL-18 may also contribute to skin disease in cpdm mice. Although loss of caspase-1/11 or Nlrp3 attenuated skin disease, it did not completely prevent it, highlighting the complex nature of this disease. This is further supported by the dichotomy in pathways contributing to skin inflammation versus systemic disease. For example, although we demonstrated that deficiency in caspase-1 and -11 imposes drastic effects on skin disease manifestation but has minimal effects on myeloid cell expansion in the spleen or splenic architecture, other investigators recently showed that genetic ablation of Mki67 from cpdm mice attenuated hepatic and splenic inflammation, whereas dermatis persisted (44). Even diseases that are monogenic in nature can be the product of multiple interacting pathways, complicating therapeutic intervention. Thus, it is important to tease apart cause from consequence to differentiate between pathogenic processes and secondary symptoms. In this article, we provide evidence that inflammasome activation is an important pathogenic process in the initiation of skin disease in cpdm mice. In the future, it will be critical to dissect which other upstream pathways mediate caspase activation in the skin and what endogenous, environmental, or microbial ligands stimulate the inflammasome.

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Disclosures
The authors have no financial conflicts of interest.

References

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Supplemental Figure 1. Gene expression profile in WT, cpdm and cpdm; ice⁻/⁻ mice. Quantitative real-time PCR analysis of skin cDNA obtained from 7-8 weeks old WT, cpdm and cpdm; ice⁻/⁻ mice. Cₚ values were normalized to the housekeeping gene Tbp and fold expression over mean of wild-type levels was calculated. Data points represent individual mice ± SEM. Student's t-test was used for statistical analysis. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001.
Supplemental Figure 2. Systemic disease is largely unaffected by loss of caspase-1/-11. (A) H&E-stained spleen sections demonstrating disrupted splenic architecture in \textit{cpdm} and \textit{cpdm; Ice}^- mice compared to wild-type mice. Scale bar = 800 µm. (B) H&E-stained liver sections demonstrating minimal perivascular inflammatory infiltrates in \textit{cpdm; Ice}^- mice compared to \textit{cpdm}. Scale bar = 100 µm. (C) Flow cytometry analysis of spleen from WT (n=4), \textit{cpdm} (n=6) and \textit{cpdm; Ice}^- (n=4) mice. Eosinophils were defined as ViD^-B220^-NK1.1^-CD11c^-CD11b^-SSC$^{\text{high}}$Ly6C$^{\text{int}}$, Monocytes ViD^-B220^-NK1.1^-CD11c^-CD11b^-SSC$^{\text{low}}$Ly6C$, Macrophages ViD^-B220^-NK1.1^-CD11c^-CD11b^-SSC$^{\text{low}}$Ly6C$^{\text{high}}$, Neutrophils ViD^-B220^-NK1.1^-CD11c^-CD11b^+Ly6G^+^, NK cells ViD^-B220^-NK1.1^+, B cells ViD^-B220^+ and T cells as TCRβ^+. Frequency of total splenocytes with SEM is represented. (D) Total numbers of Eosinophils, Monocytes, Macrophages, Neutrophils, NK cells, B cells and T cells in WT (n=4), \textit{cpdm} (n=6) and \textit{cpdm; Ice}^- (n=4) spleens. Student’s t-test was used for statistical analysis between two genotypes. *p < 0.05, **p < 0.01, ***p < 0.001
Supplemental Figure 3. *Cpdm* bone marrow-derived macrophages (BMDMs) secrete less IL-1β following inflammasome activation. (A) WT and *cpdm* BMDMs were primed with 1µg ultra-pure LPS for 4 hours followed by stimulation with 5µM nigericin (45 min), 5mM ATP (45 min), 500µg/mL Alum (5 hrs), 2µg/mL DOTAP-transfected flagellin (5 hrs), 1.5µg/mL lipofectamine-transfected poly(dA:dT) or OPTI-MEM alone. IL-1β (A) and IL-18 (C) were measured in cell-free supernatants by ELISA. (B) Cell lysates from (A) were harvested and immunoblot analysis was performed to probe for pro-IL-1β induction following LPS stimulation. (D) LDH activity in the supernatant was measured to assess cell death and expressed as a percentage of total LDH (0.1% Triton-lysed cells). Data points represent individual mice ± SEM, n≥4. Data in (D) represent the mean ± SEM. Student's t-test was used for statistical analysis between two genotypes. **p < 0.01, ***p < 0.001.
### Supplemental Table 1. qPCR primer sequences used in this manuscript

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