Modulation of autoimmune pathogenesis by T cell-triggered inflammatory cell death

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T cell-mediated autoimmunity encompasses diverse immunopathological outcomes; however, the mechanisms underlying this diversity are largely unknown. Dysfunction of the tripartite linear ubiquitin chain assembly complex (LUBAC) is associated with distinct autonomous immune-related diseases. Cpdm mice lacking Sharpin, an accessory subunit of LUBAC, have innate immune cell-predominant dermatitis triggered by death of LUBAC-compromised keratinocytes. Here we show that specific gene ablation of Sharpin in mouse Treg causes phenotypes mimicking cpdm-like inflammation. Mechanistic analyses find that multiple types of programmed cell death triggered by TNF from tissue-oriented T cells initiate proinflammatory responses to implicate innate immune-mediated pathogenesis in this T cell-mediated inflammation. Moreover, additional disruption of the Hoip locus encoding the catalytic subunit of LUBAC converts cpdm-like dermatitis to T cell-predominant autoimmune lesions; however, innate immune-mediated pathogenesis still remains. These findings show that T cell-mediated killing and sequential autoinflammation are common and crucial for pathogenic diversity during T cell-mediated autoimmune responses.

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The linear ubiquitin chain assembly complex (LUBAC) is a tripartite enzyme comprising Sharpin, HOIL-1L, and catalytic HOIP; its dysfunction is closely associated with autoimmune-inflammatory syndrome and immune deficiency in humans and mice\textsuperscript{1-3}. LUBAC conjugates linear-type ubiquitin chains on target substrates, such as NF-κB essential modulator (NEMO) and receptor-interacting serine/threonine protein kinase 1 (RIPK1), to activate NF-κB signaling in response to exogenous triggers such as TNFα, IL-1β, and T cell receptor (TCR) stimulation\textsuperscript{4-9}. Upon TNFα signaling, the ligase activity of LUBAC is requisite for protection against two types of programmed cell death, apoptosis and necroptosis; the latter is induced by generation of cytosolic death-inducible complex II comprising RIPK1, RIPK3, FAS-associated death domain protein (FADD), cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP), and Caspase-8\textsuperscript{10}. LUBAC subunits contribute differentially to the stability of the complex. Deletion of HOIP or Hoil-1 in mice, which eliminates the LUBAC complex, results in embryonic lethality due to aberrant TNFR1-mediated endothelial cell death and defective vascularization\textsuperscript{10,11}, whereas spontaneous mutant mice lacking Sharpin (called cpdm mice) are viable but develop chronic skin autoinflammation, which is triggered by death of keratinocytes\textsuperscript{12-14}.

In humans, autoinflammation is a self-directed immune disorder that manifests as chronic and recurrent inflammation. In most cases, it has a genetic etiology, leading to dysregulation of innate, but not adaptive, immune responses; this causes overproduction of proinflammatory cytokines such as IL-1β and TNFα, or exaggerated responsiveness to a steady-state level of stimulation by proinflammatory cytokines that may trigger release of other endogenous stimuli, including damage-associated molecular patterns (DAMPs), to aggravate innate immune-related inflammation\textsuperscript{15,16}. Thus, autoinflammation is defined by various forms of myeloid cell-mediated systemic inflammation, without classical autoimmune characteristics such as high-titer autoantibodies or the presence of self-reactive T cells. Other studies suggest that cpdm mice manifest additional features. Studies of cpdm Rag1\textsuperscript{-/-} mice and cpdm-derived bone marrow cell transfer experiments suggest that hematopoietic cells, including T cells and B cells, are dispensable for development of cpdm skin disease\textsuperscript{17,18}. Furthermore, Sharpin-deficient skin transplanted onto nude mice develops autonomous inflammatory responses that clearly indicate that keratinocytes showing hypomorphic LUBAC expression are susceptible to autonomous cell death mediated by FADD-caspase-8-dependent apoptosis and RIPK1-RIPK3-mixed-lineage kinase domain-like protein (MLKL)-dependent necroptosis, resulting in autoinflammation even under steady-state conditions\textsuperscript{19}.

Nevertheless, recent studies imply the presence of autoimmune aspects in LUBAC hypomorphic disease: cpdm mice show impaired development and a reduced number of Foxp3\textsuperscript{+} regulatory T cells (Treg), a critical T cell subset for immunosuppression. In addition, adaptive transfer of Sharpin-sufficient Treg into neonatal cpdm mice alleviates inflammatory responses in various tissues, but does not improve dermatitis\textsuperscript{20,21}. These reports imply that cpdm mice suffer from both autoimmune and autoinflammatory diseases, although they exhibit predominantly innate immune-mediated inflammation. Here, we examine the possibility that T cell-induced inflammation elicits an apparently innate immune-mediated pathogenesis, as observed in cpdm disease.

**Results**

**Loss of Sharpin in Treg causes cpdm-like skin inflammation.** To examine the pathogenic potency provoked by loss of Sharpin in Treg, we generated T<sub>reg</sub>-specific Sharpin-deficient mice (Sharpin<sup>fl/fl</sup>Foxp3<sup>Cre</sup>). Expression of Sharpin by CD4<sup>+</sup>Foxp3<sup>+</sup> Treg purified from the peripheral lymphoid tissues of Sharpin<sup>fl/fl</sup>Foxp3<sup>Cre</sup> mice was completely abolished, whereas that of HOIP fell, indicating a profound reduction in the amount of LUBAC complex (Fig. 1a, Supplementary Fig. 1A). However, Sharpin<sup>fl/fl</sup>Foxp3<sup>Cre</sup> mice exhibited few changes in the number and proportion of Foxp3<sup>+</sup> thymocytes and peripheral Treg (Fig. 1b, Supplementary Fig. 1B). Partial impairment of the NF-κB signaling pathway in Sharpin-deficient Treg was demonstrated by a reduction in p65 phosphorylation on Ser536 and subtle inhibition of IkBα degradation during TCR stimulation; however, the TCR-mediated ERK signaling pathway was unaffected (Fig. 1c). The cell-intrinsic roles of Sharpin in T cells were confirmed in Sharpin-KO and HOIP-KO Jurkat or murine hybridoma cells. HOIP-KO Jurkat cells lost the ability to activate NF-κB signaling in response to TCR stimulation, whereas Sharpin-KO cells still retained this signaling pathway, albeit mildly impaired (Supplementary Fig. 1C, D).

**Treg from Sharpin<sup>fl/fl</sup>Foxp3<sup>Cre</sup> mice showed normal expression of T<sub>reg</sub> functional surface markers and stimulation markers for Th1/Th2 Treg (Supplementary Fig. 1H, 1I), indicating that the trace amount of LUBAC (composed of HOIP and HOIL-1L) present in Sharpin-deficient cells is sufficient to elicit signaling for Thymic Treg development despite mild impairment of TCR signaling. However, global gene expression between Sharpin-deleted Treg and control Treg was different (63 [<two-fold] and 84 [>two-fold] out of 21,178 detected genes). Expression of genes encoding effector Treg signatures such as CD83, Pdcd1, B2m, Tnfsf9, Irf4, Egr2, Cxcl10, and Il1r1, TCR-inducing molecules such as Nrn1, Slc7a10, Tnfsf11, Cd38, Nlge5, and Plag1l, and homing receptors Ccr4, Ccr6, and Ccr9 was downregulated in Sharpin-deficient Treg (Fig. 1d).**

Furthermore, phenotypic analyses revealed that the percentage of effector Treg (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>CD69<sup>+</sup> T cells) is lower in cpdm mice than in control mice (Fig. 1e-g). In vivo co-transfer experiments using both naïve T cells and Treg revealed that Sharpin-deficient Treg failed to prevent recipient mice from developing T cell-dependent colitis and colitis-induced weight loss (Supplementary Fig. 1J). These data indicate that a sufficient amount of LUBAC is necessary to elicit signals that induce differentiation into an effector T<sub>reg</sub> phenotype and signals that regulate T<sub>reg</sub>-mediated immune responses.

**Sharpin<sup>fl/fl</sup>Foxp3<sup>Cre</sup> mice succumbed to chronic skin inflammation at around 4 weeks of age, and survived for at least 5 months (Fig. 1h).** The skin lesions displayed autoinflammatory aspects similar to those observed in cpdm mice, which is a model of autoimmune-inflammatory dermatitis that shows hyperkeratosis, parakeratosis, keratinocyte apoptosis, lamellar fibrosis, and...
dermal infiltration by granulocytes (Fig. 1i). Histological analyses also revealed limited inflammation in the lungs, but the other tissues were normal (Supplementary Fig. 1K). They had lymphadenopathy, with marked influx of CD3+ T cells, B220+ B cells, and CD11b+ myeloid cells (Supplementary Fig. 1L, M). Thus, these results demonstrate that intrinsic loss of Sharpin impairs the immunosuppressive function of Treg by inhibiting differentiation into effector Treg, resulting primarily in chronic cpdm-like skin inflammation.

T cells drive skin autoinflammation in cpdm mice. Next, we generated Sharpinfl/flLckCre mice that do not express Sharpin in most T cell lineages, including Treg and proinflammatory and cytotoxic T cells. A majority of Sharpinfl/flLckCre mice exhibited no overt symptoms and survived normally, although around 12% suffered from dermatitis with generalized lymphadenopathy (Fig. 2a, b, Supplementary Fig. 2A). Other tissues appeared normal. Peripheral T cell lineages in healthy Sharpinfl/flLckCre mice showed no significant changes, but a minor fraction acquired
activated (CD44hiCD62Lhi/lo or CD69hi) phenotypes (Fig. 2c, d, Supplementary Fig. 2B–D). To probe defects in T cell immune responses in Sharpinβ/βLckCre mice, we stimulated purified T cells in vitro. During the early phase of T cell activation, subtle inhibition of IkBα degradation was detected in Sharpin-deficient T cells, although expression of Nur77, an intermediate-early gene involved in TCR signaling, was comparable. In addition, at the late phase of activation we observed marked inhibition of CD25 and CD69 expression on the cell surface (Fig. 2g). Furthermore, an in vivo T cell cytotoxic assay revealed that Sharpin-deficient T cells induced T cell-mediated colitis and resultant weight loss (Fig. 2h, i); however, they had a lower pathogenic potency and exhibited a lower percentage of activated CD25+ T cells in the mesenteric lymph nodes (Fig. 2h, j). We also found that depletion of B cells or Gr1+ myeloid cells did not affect on the skin disease of Sharpinβ/βFoxp3Cre mice (Supplementary Fig. 2E). Considering that development of autoimmune diseases depends largely on the immunological balance between self-reactive T cells and immunosuppressive Treg, these data suggest that the cpdm-like skin inflammation observed in Sharpinβ/βFoxp3Cre mice is triggered, in some way, by T cell-mediated autoimmune pathogenesis.

Furthermore, to examine involvement of T cells in autoinflammatory skin pathogenesis in cpdm mice, we adapted a conditional expression system to express Sharpin along with EGFP (under control of the endogenous ROSA26 promoter) in a Cre recombinase-dependent manner. We then generated genetically engineered cpdm mice in which Sharpin was specifically reexpressed in Treg or the Lck+ T cell lineage (cpdm R26-Sharpin; Foxp3Cre or cpdm R26-Sharpin;LckCre, respectively) (Supplementary Fig. 3A). Recovered expression of Sharpin in these cells was detected by immunoblot or flow cytometry analysis (Supplementary Fig. 3B–D). Consistent with our findings in Sharpinβ/βFoxp3Cre and Sharpinβ/βLckCre mice, introduction of Sharpin into Treg delayed onset of dermatitis until after 3 months of age and did not induce growth disturbance (Fig. 3a, b). Histological analyses revealed suppressed epidermal hyperplasia and TUNEL-positive apoptotic cell death in the skin of cpdm R26-Sharpin;Foxp3Cre and cpdm R26-Sharpin;LckCre mice (Fig. 3c, d). The ameliorated skin inflammation led to marked suppression of autoinflammation-related cytokines such as TNFa, IL-1β, and IL-6, which were elevated in cpdm skin; similar observations were made with respect to development of immunopathological hallmarks of cpdm disease, such as spongomegaly and infiltration of multiple organs by myeloid cells (Fig. 3e–g and Supplementary Fig. 3E). In addition, the previously reported decrease in the Treg population in cpdm mice improved in these mice (Fig. 3h). These results indicate that normalization of T cell-mediated immunological functions improves skin autoinflammation in cpdm mice, and demonstrate that T cell-related mechanisms appear to play critical roles in multiple pathogeneses underlying skin autoinflammation of cpdm mice.

### Autoinflammation extrinsically downregulates Foxp3 in Treg

In contrast to Sharpinβ/βFoxp3Cre mice, cpdm mice harbored a significantly lower population of peripheral Treg (Supplementary Fig. 4A). Since inflammation reduced expression of Foxp3 in Treg, we generated TNFa−/− cpdm mice to suppress progression of dermatitis. Suppression of TNFa-mediated dermatitis led to marked rescue of Foxp3 expression in Treg, indicating that reducing the Foxp3+ T cell population in cpdm mice was a secondary effect with respect to chronic dermatitis (Supplementary Fig. 4B). Furthermore, keratinocyte-specific Sharpin-deficient mice (Sharpinβ/βK5Cre) developed cpdm-like dermatitis, suggesting that Sharpin expression by keratinocytes is requisite for tissue integrity, at least through inhibition of keratinocyte cell death (Supplementary Fig. 4C, D). Onset of the skin disease was independent on the presense of lymphocytes, but required for TNFa expression (Supplementary Fig 4I, J). Sharpinβ/βK5Cre mice also displayed liver inflammation albeit to a lesser extent; however, other tissues were normal. Although Treg from Sharpinβ/βK5Cre mice were genetically intact, they displayed reduced expression of Foxp3 and shifted to an activated and short-lived phenotype as dermatitis progressed (Supplementary Fig. 4E–G). In addition, experiments with thymic epithelial cell (TEC)-specific Sharpin-deficient mice (Sharpinβ/βR5tCre) revealed that expression of Sharpin in Keratin5+ TECs was irrelevant with respect to Treg instability and development of dermatitis (Supplementary Fig. 4H). Collectively, these data indicate that chronic autoinflammation induced by keratinocyte cell death causes extrinsic suppression of Foxp3 in Treg that possibly elicited the previously reported autoimmune aspects of cpdm mice by augmenting the functions of effector T cells.

### Similar disease outcomes in Sharpinβ/βFoxp3Cre and K5Cre

Involvement of T cells in development of cpdm-like skin disease in Sharpinβ/βFoxp3Cre mice seems contradictory to the current concept of autoinflammatory disease. To compare immunopathological changes in Sharpinβ/βFoxp3Cre with those in classical autoinflammatory disease, we used Sharpinβ/βK5Cre mice in which augmented susceptibility to TNFa-induced keratinocyte cell death underlies dermatitis. The skin lesions in Sharpinβ/βK5Cre mice resembled those in cpdm, including infiltration by F4/80+ macrophages and MPO+ granulocytes, but not lymphocytes (including T cells), which seems consistent with the context of autoinflammation (Fig. 4a). Next, we performed a detailed comparison of the immunological phenotypes of Sharpinβ/βK5Cre and Sharpinβ/βFoxp3Cre mice. Despite the T cell-mediated skin inflammation in Sharpinβ/βFoxp3Cre mice, the skin lesions were infiltrated predominantly by myeloid cells (Fig. 4a, b). Both strains suffered from eosinophilia and neutrophilia, as defined by an increase in CD11b+SiglecF+ and CD11b+Gr1hi cells, respectively, in the spleen (Fig. 4c). Moreover, expression of...
proinflammatory (IL-6, TNFα, and IL-1β) and allergy-related (TSLP) cytokines was very similar in Sharpin<sup>fl/fl</sup>Foxp<sup>3Cre</sup> and Sharpin<sup>fl/fl</sup>K5<sup>Cre</sup> mice (Fig. 4d). However, there were some differences between Sharpin<sup>fl/fl</sup>Foxp<sup>3Cre</sup> and Sharpin<sup>fl/fl</sup>K5<sup>Cre</sup> mice based on etiology. Peripheral granulocytes in Sharpin<sup>fl/fl</sup>K5<sup>Cre</sup>, but not Sharpin<sup>fl/fl</sup>Foxp<sup>3Cre</sup>, mice showed increased IL-6-mediated STAT3 phosphorylation, which is indicative of cytokine-dominant autoinflammatory traits (Fig. 4e). By contrast, Sharpin<sup>fl/fl</sup>Foxp<sup>3Cre</sup> mice exhibited increased numbers of self-reactive CD69<sup>+</sup>T cells in peripheral tissues and the presence of skin-reactive autoantibodies in serum (Fig. 4f, g). In addition, only Sharpin<sup>fl/fl</sup>Lck<sup>Cre</sup> mice showed distinct differences in progression of skin disease severity between males and females (gender-specific differences in severity are a common
Fig. 2 Sharpin predisposes mice to spontaneous T cell-dependent dermatitis. a Representative appearance of 12-week-old control, healthy, and sick Sharpin<sup>+</sup>/LckCre<sup>+</sup> (left) mice and photos of H/E-stained skin (right). Scale bar: 200 μm. b Incidence of spontaneous dermatitis. All mice were monitored over a period of 6 months. c Percentage of effector CD4<sup>+</sup>CD62L<sup>+</sup> or memory CD4<sup>+</sup>CD62L<sup>+</sup> subsets within the CD4<sup>+</sup> T cell populations in the spleen and pLNs from 12-week-old healthy Sharpin<sup>+</sup>/LckCre<sup>+</sup> mice. n = 6 biological independent animals. d Percentage of recently activated CD69<sup>+</sup> cells within the CD8<sup>+</sup> T cell population in pLNs. n = 4 biological independent animals. e MFI of IκBα in T cells at 10 min after CD3/28 antibody-based TCR stimulation. Intracellular staining was performed. n = 6 biological independent animals. f MFI of Nur77 in T cells at 3 h after CD3 or CD3/CD28 antibody-based TCR stimulation. n = 3 biological independent animals. g CD69 and CD25 expression by CD4<sup>+</sup> or CD8<sup>+</sup> T cells at the indicated times after TCR stimulation. h Experimental induction of colitis by naïve CD4<sup>+</sup>T cells. Rag2<sup>−/−</sup> mice were intravenously injected with HBSS alone or with 2.5 × 10<sup>5</sup> sorted naïve CD4<sup>+</sup> T cells (CD45R<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup>CD3<sup>+</sup>), which were obtained from pooled cells isolated from the spleen and pLNs of 4-week-old mice. Bodyweight of injected Rag2<sup>−/−</sup> mice was monitored twice or thrice weekly. Data indicate average values. i Representative photos of H/E and immunofluorescent staining against CD3<sup>+</sup> or CD69<sup>+</sup> or CD25<sup>+</sup> or CD44<sup>+</sup> or CD62L<sup>+</sup> T cells at the indicated times after TCR stimulation. j Representative appearance of 12-week-old control, healthy, and sick Sharpin<sup>+</sup>/LckCre<sup>+</sup> mice from autoimmune inflammatory skin disease. Representative skin sections from the indicated 12-week-old mice. n = 4 biologically independent animals. Small horizontal lines indicate the mean (± s.e.m.). p < 0.05; **p < 0.01; ***p < 0.001. Kruskal–Wallis test with Bonferroni correction (α value = 0.05) was used for b, e, f and j. Data are pooled from at least three independent experiments (c–g, i). Also, see Supplementary Fig. 2. Source data are provided in a Source Data file.

Fig. 3 Genetic compensation of Sharpin in T lineages rescues cpdm mice from autoimmune inflammatory skin disease. a Representative appearance of 10-week-old cpdm and cpdm R26-Sharpin;Foxp3<sup>Cre</sup> mice. b Body weight of indicated 10–12-week-old mice. n = 4 biologically independent animals. c, d Representative skin sections from the indicated 12-week-old mice. Scale bars: 200 μm for H/E (c) and 50 μm for TUNEL staining (d). TUNEL-positive epidermal cells are indicated as arrowheads. e Quantitative analyses of mRNAs encoding inflammation-related cytokines in the skin of 10–12-week-old mice. f Spleen weight from the indicated 10–12-week-old mice. n = 7 biologically independent animals. g Absolute number of eosinophils (CD11b<sup>+</sup>Gr1<sup>+</sup>SiglecF<sup>+</sup>) and neutrophils (CD11b<sup>+</sup>Gr1<sup>−</sup>) in the spleen (upper) and liver (lower). n = 6 biologically independent animals. h Absolute number and percentage of Treg, and MFI of Foxp3 in Treg, in the spleen and liver of 10–12-week-old mice. n = 5 biologically independent animals. Data are representative of two independent experiments (c, d) or pooled from three independent experiments (b, e–h). Small circles indicate individual mice. Small horizontal lines indicate the mean (± s.e.m.). p < 0.05, **p < 0.01, ***p < 0.001. Kruskal–Wallis test with Bonferroni correction (α value = 0.05) was used for b, e–h. Also, see Supplementary Fig. 3. Source data are provided in a Source Data file.
characteristic of autoimmune diseases) (Fig. 4h). Thus, the data indicate that T cell-induced autoinflammation is a main pathogenesis underlying T cell-elicted immunological changes in Sharpin<sup>fl/fl</sup>Foxp<sup>3cre</sup> mice. However, the comparable skin pathologies suggest that pathological changes in Sharpin<sup>fl/fl</sup>Foxp<sup>3cre</sup> mice are provoked in a manner similar to those in autoinflammatory Sharpin<sup>fl/fl</sup>K5cre mice.

**Skin-oriented T cells induce programmed keratinocyte death**

Next, we examined autoinflammatory manifestations in Sharpin<sup>fl/fl</sup>Foxp<sup>3cre</sup> mice in more detail. Keratinocyte death is a critical event that triggers autoinflammation in cpdm mice since LUBAC protects against TNFα-induced caspase-dependent apoptosis and necroptosis; the latter is a type of programmed necrotic cell death mediated by RIPK1, RIPK3, and MLKL. Immunohistochemical staining of cleaved caspase-3 (apoptosis) or phosphorylated MLKL (necroptosis) confirmed that both types of cell death occur in keratinocytes in the inflamed skin of Sharpin<sup>fl/fl</sup>K5cre mice (Fig. 5a, b). To our great interest, both types of cell death were also observed in the skin
**Fig. 4** Distinct etiologies, autoimmunity in *Sharpin*/*Foxp3Cre* or autoinflammation in *Sharpin*/*K5Cre*, result in similar myeloid cell-dominant inflammatory outcomes. a Pathology underlying skin inflammation. Skin sections were subjected to Masson’s trichrome and immunofluorescence staining. The types of infiltrating immune cell were identified by staining with antibodies specific for CD3e (T cells), F4/80 (macrophages), and MPO (neutrophils). Keratin14 (K14) was used as a structural marker of the epidermis. The outlined areas are magnified in Fig. 7f. Scale bars: 100 μm. b Percentage of skin-infiltrating CD11b+ cells. c Representative plots, percentages, and absolute numbers of eosinophils (CD11b+SiglecF+) and neutrophils (CD11b+GrP+) in the spleen in 6-week-old *Sharpin*/*K5Cre* and 10-week-old *Sharpin*/*Foxp3Cre* and respective control mice. n = 5 biologically independent animals. d Quantitative analysis of skin mRNA encoding inflammation-related cytokines. n = 4 biologically independent animals. e Histogram showing the presence of phosphorylated STAT3 in Gr1+ granulocytes isolated from secondary lymph nodes. As a positive control, cells were incubated for 15 min at 37 °C with IL-6 (30 ng/ml) prior to intracellular staining. f Percentage of activated CD69+ cells in CD45neg keratinocytes, which led to earlier onset of disease among the indicated strains (numbers of mice: control, 8; *Sharpin*/*K5Cre*, n = 15; *Sharpin*/*K5CreFoxp3Cre*, n = 8). Disease scores were recorded up to 60 days after birth. No gender-based differences in the incidence of skin disease were observed. Each score represents an average value from the indicated number of mice. Small horizontal lines indicate the mean (±s.e.m.). ns, p > 0.05; *p < 0.05; **p < 0.01. Two-tailed Mann–Whitney U-test was used for c–f. Data are representative of at least three independent experiments (a, b, e, g), or pooled from two independent experiments (c, d, f). Also, see Supplementary Fig. 4. Source data are provided in a Source Data file.

**Fig. 5** Skin-oriented T cells induce apoptotic and necroptotic keratinocyte death in an inflammatory setting. a TUNEL staining of skin sections from 5-week-old *Sharpin*/*K5Cre*, 10-week-old *Sharpin*/*Foxp3Cre*, and control mice. Scale bars: 50 μm. b Immunofluorescence staining of skin sections. Cleaved caspase-3 and phosphorylated MLKL were used as markers of apoptosis and necroptosis, respectively. Scale bars: 20 μm. Cell death in the epidermis was quantified by counting the total number of bright cells per low-power field (LPF). c Appearance of skin dermatitis. d Progression (upper) and incidence (lower) of skin disease among the indicated strains (numbers of mice: control, n = 12; *Sharpin*/*K5Cre*, n = 15; *Sharpin*/*K5CreFoxp3Cre*, n = 8). Disease scores were recorded up to 60 days after birth. No gender-based differences in the incidence of skin disease were observed. e Representative histogram showing death-prone epidermal cells from the indicated strains. Isolated epidermal cells were incubated with PhiPhiLux-G1D2, a fluorescent substrate of active caspase-3. f Immunofluorescence staining of skin sections to detect skin-infiltrating T cells. Scale bars: 100 μm. The small horizontal lines in b, d indicate the mean (±s.e.m.). Data are representative of at least three independent experiments. Source data are provided in a Source Data file.

of *Sharpin*/*Foxp3Cre* mice; indeed, the phenomena were almost as common in these mice as in *Sharpin*/*K5Cre* mice (Fig. 5a, b). Additional elimination of Sharpin from Treg in *Sharpin*/*K5Cre* mice (*Sharpin*/*K5CreFoxp3Cre*) exacerbated skin inflammation, with a marked increase in caspase-3 activity in CD45neg keratinocytes, which led to earlier onset of disease (Fig. 5c–e). Moreover, a massive T cell infiltrate was observed in the skin of *Sharpin*/*K5CreFoxp3Cre* mice (Fig. 5f). Collectively, these results strongly suggest that skin-oriented activated T cells promote keratinocyte death and induce skin autoinflammation, and that induction of cell death underlies T cell-mediated inflammatory processes.
Fig. 6 A pathogenic role of the TNFα-TNFRI axis during T cell-mediated autoinflammation. a Staining of MEFs co-cultured with T cells. Scale bars: 100 μm. Magnified images are shown on the right. b-d Percentage of surviving cells in a (b), Sharpin−/− MEFs co-cultured with the indicated T cells (c), and the indicated CRISPR/Cas9-targeted MEFs (d) n = 4 biologically independent experiments. e Treatment with a TNFα-neutralizing antibody or solubilized TNFRII. f Treatment with z-VAD-fmk and/or Nec1. The iCelligence system was used to measure cell viability. g-h Before stimulation, 104 CD69+Thy1.2+ cells were co-cultured with the indicated T cells (h). n = 4 independent experiments. i Expression of the indicated receptors on the T cell surface after TCR stimulation. j Disease progression among the indicated strains. M, Male; F, Female. k Immunofluorescence staining of skin sections. Scale bars: 20 μm. l Ratio of CD3ε+CD3γCDγγTCRγδ+CDδδTCRδδ+ cells. n = 6 biologically independent animals. m Number of TCRγδ+ T cells in the skin. n = 4 biologically independent animals. o Percentage of TNFα- and/or IFNγ-expressing TCRγδ+ T cells in the skin. n = 4 biologically independent animals. p Dot plots representing Treg cells. q Data are representative of at least two independent experiments (a, f, i, k, o), or pooled from three to six (b-e, g, h, l-n, p, q) independent experiments. Source data are provided in a Source Data file.
TNFα-TNFRI axis implicates T cell-triggered autoinflammation. To examine the molecular mechanisms underscoring T cell-mediated autoinflammation, we set up co-culture experiments with primary T cells as effectors and fibroblasts as targets. In these experiments, 40–50% of LUBAC-compromised MEFs died within 10 h (Fig. 6a, b). Cell death was not restricted by MHC because both isolated CD4+ and CD8+ T cells induced death at comparable levels. Instead, deletion of Tnfrsf1a genes encoding TNFRI, but not β2m or Tip1, protected target cells from death (Fig. 6c, d). Furthermore, a TNFα-neutralizing antibody (XT3.11) and a soluble Fc-fused TNFRII protein both fold higher than that in control mice, and skin TCR T cell-triggered cell death modulates autoimmune outcomes in vitro experiments confirmed that target cells treated with siRNA targeting the necroptosis regulator RIPK3, but not apoptosis regulators such as caspase-8 or FADD, showed improved survival (Fig. 6h). Moreover, considering that the unstimulated T cells used herein showed only weak expression of TNFα, and that expression increased preferentially in most T cells after TCR stimulation (Fig. 6i), we anticipated that TNFα would be an exacerbating factor for T cell-related inflammatory diseases. Therefore, to determine whether TNFα-induced programmed cell death plays a role in the autoimmune-like pathogenesis in Sharpinβ/−Foxp3Cre mice, we generated Sharpinβ/−Foxp3Cre mice lacking TNFRI or RIPK3. We found that both mouse strains showed clear amelioration of skin disease progression with restricted occurrence of programmed cell death (Fig. 6j, k). Indeed, the number of TCRβ+, but not TCRγδ+, T cells in the skin of Sharpinβ/−Foxp3Cre mice was more than 10-fold higher than that in control mice, and skin TCRβ+ cells expressed much more TNFα and IFNγ, although they were a minor component of the skin infiltrate (Fig. 6l–n). It is interesting that most of the skin-infiltrating T cells appeared to have a CD103+CD69+CD26Lb resident memory T (T RM) phenotype because T RM cells reside long-term and function protectively in the skin (Fig. 6o)26,27. Consistent with the results of in vitro T cell cytotoxicity studies, the TNFα-TNFRII axis, rather than other necroptosis-inducible death signaling pathways via DR5 or Fas receptor, appeared primarily to drive keratinocyte cell death in Sharpinβ/−Foxp3Cre mice (Fig. 6p). Furthermore, we observed upregulated expression of TNFRII and CD40, a receptor for T cell activation co-stimulator CD40L, on keratinocytes (Fig. 6p, q)28–31. Thus, these in vivo and in vitro experiments confirm the pathogenicity of multiple types of T cell-mediated programmed cell death and reveal the underlying role of the TNFα-TNFRII axis during skin autoinflammation.

Discussion

Elimination or suppression of Treg-mediated peripheral tolerance is sufficient to induce T cell-triggered immunological disorders, which are categorized as autoimmune diseases33. Here, we performed comprehensive analysis of mice bearing genetic mutations of subunits of the trimetric LUBAC ubiquitin ligase in Treg and demonstrated that impairment of Treg triggers diverse pathological manifestations. Mice lacking HOIP, a catalytic subunit of LUBAC, in Treg exhibited classical autoimmune disease accompanied by massive lymphocyte infiltration. However, mice lacking an accessory Sharpin subunit specifically in Treg developed a quite distinct form of inflammation, which is an apparently T cell-poor but innate immune cell-dependent inflammatory response, although they still exhibited some autoimmune traits such as activated peripherally circulating T cells and autoantibody production. These results demonstrating the pathological diversity of T cell-mediated sterile inflammatory diseases appear to contradict the current immunopathological definition, i.e., that self-reactive T cells can be the main driver of autoimmunity but not autoinflammation. Our further analyses revealed that loss of an additional HOip locus in mice harboring Treg lacking Sharpin (Sharpinβ/−HOipβ/−Foxp3Cre) resulted in more severe skin inflammation in which both massive T cell infiltration and severe inflammatory response was observed.
Fig. 7 T cell-dependent autoinflammation is a pathogenesis common to several autoimmune diseases. **a** Schematic diagram showing genetic targeting of LUBAC, leading to impairment of both TCR-mediated NF-κB signaling in Treg and Tnfα Stability. **b** Tissues affected during chronic inflammation. Sk, Skin; Lu, Lung; Li, Liver; St, Stomach; Ki, Kidney; Pa, Pancreas. **c** Percentage of CD44hiCD62Llo effector subsets or CD69+ recently activated subsets within the CD4+ T cells. CD4+ T cells isolated from secondary lymphoid tissues were stimulated with PMA and ionomycin for 4 h before intracellular staining for each cytokine. Representative appearance of skin in mice. Skin sections were subjected to Masson’s trichrome and immunofluorescence staining. Keratin14 (K14) was used as a structural marker of the epidermis. Scale bars: 100 μm. The outlined areas are magnified in **f** and Fig. 4e. **g** Stereotypical appearance of skin in 5-week-old Sharpinfl/foxp3Cre mice was more severe than that in Sharpinfl/foxp3Cre mice. **h** Immunofluorescence staining of programmed cell death in skin from the indicated mice. Scale bars: 20 μm. Small circles indicate individual mice. Small horizontal lines indicate the mean (t.s.m.). *p < 0.05, **p < 0.01, ***p < 0.001. Kruskal-Wallis test with Bonferroni correction (α value = 0.05) was used for c and d. Data are representative of at least three independent experiments (b, e, f, h) or pooled from three independent experiments (c, d). Also, see Supplementary Fig. 5. Source data are provided in a Source Data file.
surface in response to TCR-mediated stimulation, along with its direct association with death of epidermal cells in mice. Our co-culture system revealed TNFa-induced T cell-mediated programmed cell death (Fig. 6d, e); therefore, upregulated TNFa should be a primary inducer of cell death mediated by self-reactive T cells. However, in our assays, MEFs and primary keratinocytes from wild-type mice were not killed effectively in a TNFa-dependent manner, even though they were co-cultured with activated T cells. This implies that secondary signaling mediated by inflammatory cytokines may be essential for induction of TNFa-TNFRI-mediated cell death in a physiological setting. For example, simultaneous activation of TNFRII expressed by target cells might play a role since we detected upregulated TNFRII expression on keratinocytes from Sharpinβ/βFoxp3Cre mice. Secreted TNFα activates TNFRII preferentially, whereas membrane-bound TNFα, expression of which is enforced in activated T cells, strongly interacts with both TNFRII and TNFRI to induce distinct signals. Unlike TNFRII, TNFRI cannot induce cell death as it has no cytoplasmic death domain34. However, upon binding to TNFα, TNFRRI recruits TRAF2 and TRAF2-associated protective factors, such as cIAP1 and cIAP2, which may result in reduced recruitment of these signaling molecules to TNFRII, leading to enhanced induction of TNFRII-mediated cell death29,35,36. Also, other surface molecules, such as CD40L, expressed on activated effector T cells via an as-yet-unknown mechanism may cooperate to facilitate TNFRII-mediated necrotic cell death38,39.

The TNFα-TNFRI axis induces two types of programmed cell death under certain conditions: caspase-dependent apoptosis and RIPK1–RIPK3–MLKL-dependent necroptosis. In this context, we detected both types in the inflamed epidermis of Sharpinβ/βFoxp3Cre mice. Necroptosis induces necrosis-like morphological disruption and subsequently releases intracellular factors, such as DAMPs, which can exacerbate inflammation. Also, recent studies implicate necroptosis in the pathogenesis of inflammatory bowel disease, systemic lupus erythematosus, and multiple sclerosis37,38. TNFα-induced cell death in an in vitro co-culture system was suppressed effectively by inhibiting necroptosis (Fig. 6h), which led us to attempt deletion of RIPK3 from Sharpinβ/βFoxp3Cre mice (Sharpinβ/βFoxp3CreRIPK3−/−) to assess the contribution of necroptosis to the pathogenesis of innate inflammation. Amelioration of dermatitis via loss of RIPK3 indicated a significant role for RIPK3-mediated necroptosis in driving T cell-induced innate inflammation. However, apoptosis may also contribute to some extent because loss of RIPK3 failed to fully cure dermatitis in Sharpinβ/βFoxp3Cre mice (Fig. 6j, k). These data may support previous reports showing that deleting either RIPK3 or MLKL does not rescue cdpm mice from autoinflammatory skin disease completely, whereas RIPK3−/−Caspase-8−/−/cdpm mice do not show overt symptoms of dermatitis13,14. As shown in Sharpinβ/βFoxp3CreFoxp3Cre mice, overactivation of self-reactive T cells in autoinflammatory settings induces apoptosis in most keratinocytes (Fig. 5f). Thus, apoptosis induced by T cell-expressed TNFα in the skin might exhibit inflammatory potential, although apoptosis is generally regarded as a non-immunogenic form of cell death. It is unclear how T cells would induce different types of cell death in vivo, or which forms of cell death would contribute to different inflammatory conditions. Therefore, further detailed studies are needed if we are to gain better understanding of the mechanisms underlying T cell-induced inflammation; such studies may lead to effective therapeutic strategies for T cell-mediated (but apparently T cell-poor) systemic inflammatory diseases.

In summary, we provide strong evidence that autoimmune-prone T cells can initiate innate inflammation via TNFα-induced cell death; as far as we are aware, this is a new concept with respect to immune-mediated inflammatory mechanisms. Consistent with our conclusions, TNFα plays crucial roles not only in human autoinflammatory diseases but also in autoimmune diseases such as rheumatoid arthritis, psoriasis, and Crohn’s disease. In addition, based on our observation of apparent pathological conversion of innate inflammatory skin in Sharpinβ/βK5Cre mice into autoimmune skin in Sharpinβ/βK5CreFoxp3Cre mice, we suggest that TNFα-mediated cytotoxicity via slightly activated T cells may trigger innate-type inflammation, which is involved in surprising diverse pathological outcomes depending on the environment within the affected tissue. Thus, aberrant T cell immune tolerance could induce autoimmunity and/or innate inflammatory mechanisms that lead to complex T cell-mediated immunopathophysiology. Our findings may explain the paradoxical etiological outcomes and the propounded continuum model for development of autoimmune and autoinflammatory disorders19-41.

Methods

Mice. All animal use and care were performed according to protocols approved by the Institutional Research Committee, Graduate School of Medicine, Kyoto University, and we complied with all the ethical regulations. All mice were housed at the Institute of Laboratory Animals, Kyoto University, under specific pathogen-free conditions, and all animal experiments were conducted in accordance with the guidelines for animal experiments at Kyoto University and RIKEN Kobe Branch. C57BL/6 (B6) mice were obtained from SLAC, Japan. Cpdata mice were described previously42. Rag2−/− mice were obtained from the Central Institute of Experimental Animals, Japan. TNFα−/− (Stock number: 005308) and B6 (CD45.1) (Stock number: 002014) mice were obtained from the Jackson Laboratory. Lek-Cre (Model number: 4197) transgenic mice were obtained from Taconic. SJL-Cre knock-in mice and Foxp3-YFPCre knock-in mice were kindly provided by You-suke Takahama (University of Tokushima, Japan) and Alexander Y. Rudensky (Memorial Sloan Kettering Cancer Center, USA), respectively43,44. Ripk3−/− mice were kindly provided by Vishva Dixit (Genentech, USA)45. K5-Cre mice were obtained from CARD-Kumamoto University, Japan46. Sharpin conditional KO, HOIP conditional KO, and Sharpin conditional transgenic mice were generated in house, and are available from RIKEN BRC, Japan.

Generation of conditional Sharpin-KO mice. A targeting vector for floxed Sharpin mutant mice was constructed by inserting loxP sequences bracketing exons 3–9 of the Sharpin gene, which encodes the ubiquitin-like (UBL) domain and a NZF domain of the Sharpin protein and is deleted after expression of Cre recombinase. A neomycin resistance gene flanked by FRT sites was inserted into intron 2. Bruce 6 C57BL/6 embryonic stem (ES) cells were transfected with the targeting vector and screened as neomycin-resistant colonies. Appropriate homologous recombination was confirmed by Southern blot analysis. The targeted ES cells were injected into recipient blastocysts to generate germline-transmitting Sharpin-KO mice. Mouse carrying the locus were crossed with Fip Deleter mice to delete the neomycin cassette and then backcrossed onto the C57BL/6N strain for at least five generations before analysis. The following PCR primers were used to genotype the conditional Sharpin-KO mice: Forward (Fw-Intron2), GTGACAAGTGCTCATAATGGAAT; Reverse (Rv-Intron2), CTTGATACCCGAGTGTCAAT; and Reverse (Rv-5), GCTGCTGGCTTCTCAGGCG. Size of floxed allele: 500 bp; size of wild-type allele: 370 bp.

Generation of conditional HOIP-KO mice. A targeting vector for floxed Hoip mutant mice was constructed by inserting loxP sequences bracketing exons 7–11 of the Hoip gene, which encodes the zinc finger domains and ubiquitin-associated (UBA) domain of the HOIP protein and is deleted after expression of Cre recombinase. A neomycin resistance gene flanked by FRT sites was then inserted into intron 6. IMR C57BL/6 N ES cells were then transfected with the targeting vector. The following steps were the same as those used to generate conditional Sharpin-KO mice. The PCR primers used to genotype the conditional HOIP-KO mice were as follows: Forward (8–f4), ACATAACAGAAGTACGCAGC; and Reverse (8–r5), GTCTGGCCCTTCCGATGCG. Size of floxed allele: 370 bp; size of wild-type allele: 220 bp.

Generation of conditional Sharpin transgenic mice. A targeting vector for conditional Sharpin transgenic mice was constructed by insertion of the Sharpin sequence fused to an N-terminal FLAG-His tag, followed by the FRT-banking IRES-EGFP cassette plus the polyA sequence, into the Rosa26 locus. The neomycin resistance gene and Stop sequences were flanked by loxP sequences and placed upstream of the sequence encoding Sharpin such that Sharpin was expressed under control of the Rosa promoter after Cre recombinase-mediated excision. TT2 (C57BL/6N × CBA) ES cells were transfected with the targeting vector. This strain

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Cells and generation of CRISPR/Cas9-mediated KO cell lines. HOIP-KO Jurkat cells and HOIP−/− MEFs were generated as described previously46.47. Gene-targeting KO B3Z, kindly provided from Takayuki Kanaseki (Sapporo Medical University, Japan), Jurkat, and Sharpin−/− MEFS were generated using the CRISPR/Cas9 system. The CRISPR design tool (crispr.mit.edu) was used to generate a list of unique and synthesized sgRNA sequences (see below) were phosphorylated, annealed, and inserted into the pair of BbsI sites in pSpCas9(BB)-2A-GFP (PX458, Addgene). The plasmids were transfected into cells by electroporation. After 24 h, GFP-expressing cells were purified using a FAC-Saria III cell sorter (BD Biosciences), according to the manufacturer’s protocol, and single cell clones were obtained by limiting dilution. Disappearance of targeted proteins was determined by western blot analysis or flow cytometry analysis. The CRISPR sgRNA sequences (gene name, sgRNA sequence, and number) as follows: human Sharpn sgRNA1, CAGGTCGAGGACGCTTAGCG; mouse Sharpn sgRNA1, GTGGAGATACGCGACCTTTCCTC; Reverse (mutant allele), GCAATATGGTGGAAAATAAC. Size of wild-type allele: 350 bp; size of floxed mutant allele: 300 bp.

In vitro T cell-inducible cell death assay. Primary T cells were isolated from secondary lymphoid tissues using anti-CD4, CD8α, or Thy1.2 MACS microbeads (Miltenyi Biotec). The T cells (1 × 10^5) were co-cultured for 10 h with MEFS (target cells; 5 × 10^4) in a 96-well plate (200 μl/well). Floating T cells were removed by washing three times with medium. Survival of target cells was measured in a MTT assay (Dojindo). After 48 h, the puriﬁed organs were embedded in parafﬁn, sectioned (5 μm), and stained with Mayer’s hematoxylin and eosin (H&E), trichrome stain to visualize collagen deposition (Sigma-Aldrich), or Sirius Red F3B to detect collagen (VWR). The xed organs were embedded in parafﬁn for a further 48 h. The xed organs were sectioned (5 μm) and stained with H&E. Skin sections were stained with Masson’s trichrome stain to visualize ﬁbrosis in inﬂamed areas. Photomicrographs were acquired under an microscope. Image analysis was performed using the ImageJ (NIH) and ImageQuant LAS 4000 Image reader (Fujiﬁlm) software.

Luciferase assay for measurement of NF-κB signaling. The luciferase reporter plasmids pGL-NF-κB-Luc (10 ng) and pGL-Tk-Luc (10 ng) (Promega) were transfected into Jurkat cells (5 × 10^6). After 48 h, cells were lysed in a luciferase assay lysis buffer (Roche) and then clariﬁed by centrifugation at 10,000 ×g for 30 min at 4 °C. The luciferase activity was measured (in terms of relative light units) using a LuKL-CR assay (Promega).
transcription factors, including Foxp3, surface-labeled cells were stained overnight at 4 °C with the following antibodies in blocking buffer: anti-CD3e (IH3E, DIA-303; DiaNovia), anti-keratin14 (BR15-155P, Covance), anti-F4/80 (CL5-A-1, MCA497GA; BIO-RAD), anti-myeloperoxidase (MPO) (RB-373-A1, ThermoFisher Scientific), anti-cleaved caspase-3 (Asp175, 9661; Cell Signaling Technology), and anti-phospho (S345) MLKL (EPR9515(2), ab196436; Abcam). The stained samples were then incubated for 1 h at room temperature with a mixture of the following fluorescent dye-conjugated antibodies in blocking buffer: goat-anti-rat IgG-AlexaFluor 647 (A-21247) and goat-anti-rabbit IgG-AlexaFluor 488 (A-11034) (ThermoFisher Scientific). For preservation, labeled sections were mounted in ProLong Gold Antifade Mountant (ThermoFisher Scientific) containing DAPI. Photomicrographs were acquired under a Keyence Fluorescence Microscope BZ-9000 using CF2 Plan Apo ×40 0.95/0.14 mm objective lenses (Nikon). The observed images of apoptotic cells were acquired under a Keyence Fluorescence Microscope BZ-9000 using the BZ-II image analysis application (Keyence).

**TUNEL staining.** Formalin-fixed, paraffin-embedded skin sections were used for TUNEL staining. After deparaffinization, sections were treated for 1 h at room temperature with Proteinase K (10 μg/ml in 10 mM Tris-HCl, pH 7.4). The TUNEL reaction was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche). For preservation, labeled sections were mounted in ProLong Gold Antifade Mountant (ThermoFisher Scientific) containing DAPI. Fluorescent images of apoptotic cells were acquired under a Keyence Fluorescence Microscope BZ-9000 using CF2 Plan Apo ×40 0.95/0.14 mm objective lenses (Nikon). The observed signals were processed using the BZ-II image analysis application (Keyence).

**Cell isolation from tissues and staining for flow cytometry.** Thymocytes from 4- to 5-week-old mice, and splenocytes and lymph node cells from 4- to 10-week-old mice were stained with a mixture of antibodies (see above). Cells infiltrating the liver of 8- to 10-week-old mice were isolated by mechanical dissociation using a μm cell strainer (Falcon), followed by purification on a 33% Percoll solution (Sigma). Skin-resident cells were isolated separately from the dermis or epidermis. After shaving, the skin was immersed in 0.5 % trypsin Dissape II (Wako) diluted in PBS and left at 4 °C overnight. The epidermal layer was then peeled from the dermis. Epidermal tissues were treated at 37 °C for 10 min with 0.05% DNase I (Roche) containing trypsin solution. Dermal tissues were incubated with 0.13 units/ml Epidermal tissues were treated at 37 °C for 10 min with 0.05% DNase I (Roche) at 96 °C, and then boiled for 20 min. Amino-terminal caspase activity in epidermal cells by fluorometry, isolated cells were incubated at 37 °C for 90 min with Phospho-L-Glu-DG (OncoImmunin, Inc.). All antibodies were used at 1:200, except for the anti-CD3e antibody (used at 1:100). Flow cytometry analysis was performed using a FACSCan II flow cytometer, according to the manufacturer’s protocol. Data were analyzed using FlowJo software (Tomy Digital Biology).

**T cell-induced colitis and in vivo Treg suppression assay.** Splenocytes and LN cells from 4- to 5-week-old B6 (CD45.1+) mice were depleted with anti-CD19 and anti-CD8a MACs microbeads (Miltenyi) to enrich CD4+ T cells. Next, pre-enriched cells were stained with a mixture of antibodies, including anti-CD25, anti-CD4, anti-CD10, and anti-CD45RB, followed by staining of dead cells with LIVE/DEAD Fixable Dead Cell Stains (Molecular Probes). Labelled cells were used for FACS sorting on a FACSAria III cell sorter (BD Biosciences). Sorting purity of CD4+CD45RB+CD25- naive T cells. The sorted population was >98% pure. Foxp3-YFP+CD25+CD4+ Treg from control or Treg cells were stained with a mixture of antibodies andthen fixed, paraf-}

Quantitative PCR analysis of skin. After removing hair with an electric shaver, part of the dorsal skin was dissected. Pre-cleaned RNAs were extracted using Sepasol-RNA I Super G (Nacalai Tesque, Japan), according to the manufacturer’s protocol, and then subjected to column-based purification using a RNeasy Mini Kit (Qiagen). Skin cDNA was obtained by reverse transcription of RNA using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by qPCR analysis using SYBR Green PCR Master Mix (Applied Biosystems) and a Viia7 Real-time PCR system (Applied Biosystems). The qPCR amplification conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression of each gene was normalized against that of hypoxanthine-guanine phosphoribosyl transferase (HPRT). The following primers were used: mouse HPRT1, Forward, GATTTACCTGATGAAACCCAGGT; and Reverse, CTCGCCCATCTTTCAAGCAGA; mouse IL-6, Forward, TACCCTACTCAGAAGAGGAG; and Reverse, CTCGGAATGTACATGTGTGGTTC; mouse TNFa, Forward, GTGTCGCTATGCTCAGGCTTCT; and Reverse, GCCGATAGAACGTGAGAGGAGG; mouse IL-1β, Forward, TGGACCTTCCAGGAAGAGGACAAG; and Reverse, GTTCACCTTACCTTGGAGGCG; and mouse IL-4, Forward, ATCGAGATCGTTTTGAGCCAGGTC; and Reverse, ACCGTGAAAGCCCTA CACAGCGA.

Scoring for dermatitis. The disease scoring system was described in previous studies. Briefly, the skin was divided into four regions (head and/or neck; front limbs and/or ventral area; and hind limbs and/or rump area). Each inflamed region scored 1 point, except for the head, which scored 2 points. Additionally, the severity of each affected area was graded as follows: 0 = no disease; 1 = hair loss and mild scaling; 2 = extensive scaling and erosion; 3 = ulceration. The final score for each individual mouse was the sum of the scores for each affected region plus the severity grade (maximum score, 17 points). All mice were scored two or three times per week during the experiment.

B cell or myeloid cell depletion. To deplete B cells or myeloid cells, 4-week-old mice received an intraperitoneal injection of both anti-CD19 (1D3) and anti-B220 (RA3.31A1/6.1), or anti-Gr-1 (RB6-8C5), or Isotype control (LTF2) antibodies; these injections were given five times per week.

In vivo TNFa neutralization. A total of 200 μg of an anti-TNFα Ab (clone XT3.11) or isotype control IgG (clone HRFN) was intraperitoneally administrated to 3-week-old mice. The mice received one injection every 3 days, and that was repeated eight times.

Microarray analysis. Total RNA was extracted from FACs-sorted Treg using an RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions. The purity of the RNA was checked using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) and a 2100 Bioanalyzer (Agilent Technologies). Biotinylated cRNA was synthesized from 250 ng of total RNA using the GeneChip 3' IVT PLUS Reagent Kit (Affymetrix), according to the manufacturer’s instructions. The yield of biotinylated cRNA was checked using the NanoDrop 2000 spectrophotometer. Following fragmentation, 15 μg of cRNA was hybridized to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix) for 16 h at 45 °C. Arrays were washed and stained in a GeneChip Fluidics Station 450 (Affymetrix). Arrays were scanned using GeneChip Scanner 3000 7G. Single Array Analysis was calculated using Microarray Suite version 5.0 (MAS5.0), with the Affymetrix default setting and global scaling as the normalization method. The trimmed mean target intensity of each array was arbitrarily set to 500.

Statistical analysis. Statistical analysis was performed using Prism 5.0 (GraphPad software). Statistical significance was determined using a two-tailed Mann–Whitney U-test, a paired t-test, or the Kruskal-Wallis test, followed by pairwise comparisons with Bonferroni-corrected Dunn’s test. P-value of 0.05 was deemed significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The Gene Expression Omnibus accession number for the transcriptional data reported in this study is GSE108793. The authors declare that all other data of this study are available within the paper or upon reasonable request. The source data are provided in a Source Data file.
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Additional information

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