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IFN-γ or IFN-α Ameliorates Chronic Proliferative Dermatitis by Inducing Expression of Linear Ubiquitin Chain Assembly Complex

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The linear ubiquitin chain assembly complex (LUBAC) ubiquitin ligase complex, composed of HOIL-1L–interacting protein (HOIP), heme-oxidized IRP2 ubiquitin ligase-1L (HOIL-1L), and SHANK-associated RH domain protein, specifically generates linear polyubiquitin chains and is involved in NF-κB activation. Lack of SHANK-associated RH domain protein, which drastically reduces the amount of HOIP and HOIL-1L, causes chronic proliferative dermatitis (cpdm) in mice. Impaired NF-κB activation and augmented apoptosis have been implicated in the pathogenesis of cpdm in mice. In this study, we found that IFN-γ increased the amount of LUBAC by inducing HOIP and HOIL-1L mRNA transcription and enhanced the signal-induced NF-κB activation in embryonic fibroblasts, keratinocytes, and bone marrow–derived macrophages from wild-type and/or cpdm mice; however, IFN-γ failed to augment NF-κB activation in mouse embryonic fibroblasts lacking linear polyubiquitination activity of LUBAC. Moreover, s.c. injection of IFN-γ for 3 wk into the skin of cpdm mice increased the amount of HOIP, suppressed apoptosis, and ameliorated the dermatitis. Inhibition of keratinocyte apoptosis by IFN-γ injection suppressed neutrophil, macrophage, and mast cell infiltration and the amount of TNF-α in the skin of cpdm mice. Similarly, IFN-α also enhanced the amount of HOIP as well as NF-κB activation, inhibited apoptosis, and ameliorated cpdm dermatitis. These results indicate that the IFNs enhance NF-κB activation and ameliorate cpdm dermatitis by augmenting expression of HOIP and HOIL-1L and linear polyubiquitination activity of LUBAC. The Journal of Immunology, 2014, 192: 3793–3804.

The linear ubiquitin chain assembly complex (LUBAC) ubiquitin ligase complex specifically generates linear polyubiquitin chains and plays a key role in the activation of NF-κB (1, 2). NF-κB is a family of transcription factors involved in a wide variety of cellular processes including cell survival, immune responses, and inflammation induced by various stimuli such as infectious agents or inflammatory cytokines (3–5). Abnormal activation of NF-κB is involved in several diseases including cancer and rheumatoid arthritis (3, 6–8). Thus, signal-induced NF-κB activation pathway has been extensively studied.

NF-κB resides in the cytoplasm of resting cells, where it binds to its inhibitor proteins called IκBs (4, 9, 10). When cells are stimulated by ligands such as TNF-α and IL-1β, the IκB kinase (IKK) complex composed of IKK1 (IKKa), IKK2 (IKKβ), and NF-κB essential modulator (NEMO, also called IKKγ) is activated, which leads to the phosphorylation and degradation of IκBs, followed by the release of NF-κB from IκBs and its translocation to the nucleus (11–13). Linear polyubiquitination of NEMO by LUBAC has been suggested to be involved in the activation of IKK complex (1). LUBAC is composed of HOIL-1L–interacting protein (HOIP), heme-oxidized IRP2 ubiquitin ligase-1L (HOIL-1L), and SHANK-associated RH domain protein (SHARPIN). Lack of SHARPIN causes chronic proliferative dermatitis (cpdm) in cpdm mice, which exhibit systemic chronic inflammation including dermatitis, arthritis, and esophagitis (14, 15).

Although the pathogenesis of chronic dermatitis in cpdm mice has not been precisely elucidated yet, suppressed NF-κB activation and increased apoptosis, which are provoked by the drastic decrease in the amount of the other two components of LUBAC, HOIP and HOIL-1L, are suggested to be involved in the development of dermatitis (16, 17). On the basis of our previous findings, showing that an increase in the amount of LUBAC augments signal-induced NF-κB activation (1), we hypothesized that the induction of LUBAC composed of HOIP and HOIL-1L enhances NF-κB activation, inhibits apoptosis, and ameliorates cpdm dermatitis.

In this paper, we searched for an inducer of LUBAC and identified both IFN-γ and IFN-α as inducers of the expression of HOIP and HOIL-1L mRNA. IFN-γ and less effectively, IFN-α, increased the amount of LUBAC in mouse embryonic fibroblasts (MEFs) and keratinocytes from both wild-type (WT) and cpdm mice. Moreover, s.c. injection of IFN-γ or IFN-α was sufficient to provoke the increase in HOIP levels and mediate the suppression
of TNF-α–induced apoptosis in the skin of cdpm mice. IFN-γ failed to induce signal-mediated NF-κB activation in cells lacking linear polyubiquitination activity of LUBAC, suggesting that IFN-γ enhances NF-κB activation through its augmenting effect on LUBAC. Moreover, s.c. injection of IFN-γ or IFN-α for 3 wk into the skin of cdpm mice led to a partial amelioration of chronic dermatitis. These results imply that LUBAC–activity–inducing agents could be effective therapeutic drugs for diseases in which suppressed NF-κB activation and increased apoptosis are involved.

Materials and Methods

**Mice, preparation of cells, and extracts**

cdpm mice were maintained under specific pathogen-free conditions in the animal facilities of Osaka and Kyoto Universities. Primary keratinocytes were prepared from WT and cdpm mice at the age of 8–10 wk. Tails were excised and tail bones were removed. Tail skins were harvested by incubation with 4 mg/ml dispase in PBS for 1 h at 37˚C. The epidermis was collected, and keratinocytes were then obtained by incubation with 0.5% trypsin for 5 min at 37˚C. Bone marrow (BM) cells were isolated from WT and cdpm mice at the age of 8–10 wk. The femurs and tibias were cut and BM cells were flushed with PBS. The preparation of BM-derived macrophages (BMDMs) was performed as previously reported (18). Skin sections were prepared by homogenization of skin tissues with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 2 mM PMSF containing complete protease inhibitor mixture EDTA-free (Roche) using Multi Beads Shocker (Yasui Kikai). All mouse protocols were approved by Kyoto and Osaka Universities.

**Cells and cell cultures**

Immortalized MEFs were derived from WT and cdpm mice as described previously (19). MEFs were cultured in DMEM (Sigma-Aldrich) containing 10% FBS, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. Keratinocytes were cultured in human keratinocyte serum-free medium (DS Pharma Biomedical) supplemented with bovine pituitary extract. BM cells were cultured in RPMI 1640 medium (Sigma-Aldrich) containing 10% FBS, 100 IU/ml penicillin G, 100 μg/ml streptomycin, and M-CSF derived from CMG14-12 cells.

**Abs and reagents**

Anti-IκBα, anti–p-IκBα, anti–p-IκB and anti-cleaved caspase-3 Abs were purchased from Cell Signaling Technology. Anti–Ki-67 Ab was purchased from Cell Signaling Technology. Anti–HOIP, anti–HOIL-1L, anti–SHARPIN, anti–tubulin, and CD3, anti–CD4, and anti–CD8 Abs were from BioLegend. Anti-p65, anti–Gr-1 and anti–RIP1 Abs were from BD Pharmingen. Anti–Keratin 6, anti–HMGB1, anti–c-kit, anti–IL-10, and anti–TNF-α Abs were purchased from R&D Systems. LPS from *Escherichia coli* O111:B4 was obtained from Sigma-Aldrich.

**Immunoblot analysis**

Cells were lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 2 mM PMSF and complete protease inhibitor mixture EDTA free. For immunoprecipitation, lysates were incubated with the appropriate Abs overnight at 4˚C, followed by incubation with rmp protein A–Sepharose (GE Healthcare). Samples were separated in 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After the membrane was blocked in TBS containing 0.1% Tween 20 and 5% skimmed milk, it was incubated with the appropriate primary Ab, followed by incubation with HRP-conjugated secondary Ab (GE Healthcare). Immunoreactive proteins were visualized using enhanced chemiluminescence. Immunoblots were quantified using an LAS3000 or LAS4000 Mini-Imaging Analyzer (Fujiﬁlm).

**DNA extraction and real-time quantitative PCR**

Total RNA was isolated from cells using RNeasy mini kit (Qiagen) with RNase-free DNase (Qiagen). The product was reverse transcribed into cDNA using high capacity RNA-to-cDNA kit (Applied Biosystems). Total RNA from skin tissues and spleens was extracted by sequazol (Nacalai Tesque) using Polytron (Kinematics) to homogenize the tissues and treated using the RNeasy mini kit (Qiagen) followed by DNase treatment. cDNA was generated with the Power SYBR Green PCR Master Mix (Applied Biosystems) or TaqMan MasterMix (Applied Biosystems) in Applied Biosystems ViiA 7 (Applied Biosystems). GAPDH was used as a control. Sequence-specific primers were designed as follows: mouse myc, 5′-GGGAACCCCC-GGCAAGACGGCAGAGTGC-3′ (sense) and 5′-AACAtGATGTT-AGGAGCAAGGCGCCAGGC-3′ (antisense); mouse IL-5, 5′-GATGAAG-TTTCCTGGTCTCTCT-3′ (sense) and 5′-TGCAAGGTTTGGAAATACAC-TTTCC-3′ (antisense); mouse IL-13, 5′-AACCGGAGCAGTGTGATTTG-3′ (sense) and 5′-TGTCGCTTGATAGATGTGATTGC-3′ (antisense); mouse IL-20, 5′-AGCAATGCGAGAAGACGTC-3′ (sense) and 5′-GC-TTTCCAGAGCACGAACTACG-3′ (antisense); mouse IFN-γ, 5′-ATCCA-GAAGCGCTCAAGCAGTC-3′ (sense) and 5′-GAACAGGCTTATCACA-CGAC-3′ (antisense); mouse IL-1β, 5′-GCCCTTGCCATCCAAGGAT-GC-3′ (sense) and 5′-ACACTGTCTGCTGGTGGAGTTC-3′ (antisense). TaqMan probes for mouse GAPDH, mouse HOIP, mouse HOIL-1L, mouse SHARPIN, mouse TNF-α, mouse IL-6, mouse IFN-γ, mouse IL-2p40, mouse IL-4, and mouse VACM-1 were purchased from Applied Biosystems.

**Immunocytochemical staining**

Skin tissue samples were fixed in 10% formaldehyde for 24 h, followed by paraffin embedding and microtome sectioning. Slides were stained with H&E or toluidine blue. For immunohistochemical analysis, sections were hydrated by passage through xylene and graded ethanol. After Ag retrieval for 10 min at 99˚C in citric buffer (pH 6), the slides were blocked with 1% BSA and 10% PBS/PBS at room temperature for 1 h. Cells were incubated with the appropriate primary Ab for 16 h at 4˚C followed by incubation with the appropriate secondary Ab labeled with Alexa Fluor 488 (Invitrogen) and DAPI (Wako Pure Chemical Industries) for nuclear counterstaining. Images were obtained using a confocal microscope (FV1000; Olympus).

**Immunohistochemical analysis**

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**TUNEL staining**

Staining of paraffin-embedded sections of mouse back skin was performed with In Situ Cell Death Detection Kit, Fluorescin (Roche). Sections were incubated in proteinase K solution (20 μg/ml) for 30 min at 37˚C. Frozen sections of ear skins were permeabilized by incubation in 0.3% Triton X-100/PBS for 15 min at room temperature. The sections were then incubated in TUNEL reaction mixture, according to the manufacturer’s protocol.

**Cytokine ELISA**

The concentration of TNF-α was measured using Mouse TNF-α ELISA MAX purchased from BioLegend. Nunc MaxiSorp flat-bottom 96-well plates were used.
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Therapeutic intervention

Back skins of 6-wk-old cpdm mice were injected s.c. with 2 ml IFN-γ (300 U/ml) or PBS. This treatment was performed six times per week for 3 wk. For etanercept treatment, back skins of 6-wk-old cpdm mice were s.c. injected with 0.5 μg/g body weight etanercept three times per week for 3 wk.

Evaluation of dermatitis severity

The severity of dermatitis was evaluated before and after the treatment as described previously (20). The development of 1) erythema/hemorrhage, 2) dryness/scaling, 3) edema, and 4) erosion/excoriation was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The evaluated areas were rostral back and caudal back. The dermatitis score was the sum of each score obtained for rostral back and caudal back. Each dermatitis score was assessed by three observers in a blinded fashion.

Measurement of epidermal thickness

Intrafollicular epidermal thickness was calculated by averaging five locations in each section, which included at least one full-length hair follicle. Three sections from each mouse were evaluated.

Statistical analysis

The data are expressed as mean values ± SD. Statistical analysis was performed by Student t test. A p value < 0.05 was considered statistically significant.

Results

IFN-γ augments NF-κB activation by increasing the amount of the LUBAC

The Rnf31 gene, which encodes HOIP, is located between the Psme2 gene and If9 gene on chromosome 14 in mice. Because the expression of both Psme2 and If9 genes is activated by IFN-γ (21–24), we examined whether IFN-γ regulates the expression of HOIP mRNA by real-time PCR. IFN-γ promoted the expression of HOIP mRNA in MEFs established from WT mice. In addition, IFN-γ induced the expression of HOIL-1L (Rbck1) transcripts to a small degree but did not induce the expression of SHARPIN (Sharpin) mRNA (Fig. 1A). We next investigated the cis-acting element for IFN-γ-mediated HOIP mRNA induction, using a luciferase assay in NIH3T3 fibroblastic cells. We found that the IFN regulatory factor 1 (IRF-1) (−773 to −762) binding sequence in the upstream region and the IFN-γ-activated site (+12416 to +12424) and γ-IFN-activated transcriptional element (+12434 to +12458) sequences, both of which are IFN-γ-responsive regulatory elements (23–26), in the downstream region of the Rnf31 gene, were involved in IFN-γ-mediated HOIP mRNA induction (Supplemental Fig. 1A–C). We also found that the IFN-stimulated response element (−37 to −23) sequence in the upstream genomic fragment of Rbck1 gene, which is involved in IFN-γ-mediated transcription (25, 27), was responsible for IFN-γ-induced induction of HOIL-1L mRNA (Supplemental Fig. 1D).

Immunoblotting analyses revealed that IFN-γ increased the amounts of not only HOIP and HOIL-1L but also SHARPIN proteins in WT MEFs, BMDMs, and keratinocytes (Fig. 1B). To determine the extent of not only HOIP and HOIL-1L but also SHARPIN (23–26), in the downstream region of the sequences, both of which are IFN-γ-responsive regulatory elements (23–26), we examined whether IFN-γ augmented NF-κB-mediated HOIP mRNA induction, using a luciferase assay in NIH3T3 fibroblastic cells. We found that the IRF-1 (−773 to −762) binding sequence in the upstream region and the IFN-γ-activated site (+12416 to +12424) and γ-IFN-activated transcriptional element (+12434 to +12458) sequences, both of which are IFN-γ-responsive regulatory elements (23–26), in the downstream region of the Rnf31 gene, were involved in IFN-γ-mediated HOIP mRNA induction in primary keratinocytes (Supplemental Fig. 1A–C). We also found that the IFN-stimulated response element (−37 to −23) sequence in the upstream genomic fragment of Rbck1 gene, which is involved in IFN-γ-mediated transcription (25, 27), was responsible for IFN-γ-induced induction of HOIL-1L mRNA (Supplemental Fig. 1D).

Immunoblotting analyses revealed that IFN-γ increased the amounts of not only HOIP and HOIL-1L but also SHARPIN proteins in WT MEFs, BMDMs, and keratinocytes (Fig. 1B). To investigate the mechanism underlying the increase of SHARPIN mediated by IFN-γ in the absence of SHARPIN mRNA expression induction (Fig. 1A), gel filtration analyses were performed. The amount of HOIP, HOIL-1L, and SHARPIN proteins with high m.w. (fractions 9–11) was higher in IFN-γ-treated cells than in nontreated cells. Moreover, the amount of SHARPIN with low m.w. (fractions 13 and 14), which is present as a free form, was decreased in IFN-γ-treated cells (Fig. 1C). In addition, the amount of HOIL-1L and SHARPIN communoprecipitated with anti-HOIP Ab was increased in IFN-γ-treated cells, whereas the amount of SHARPIN and HOIL-1L was decreased in the unbound fraction (Fig. 1D). These results implied that SHARPIN becomes more stable in the LUBAC and the increase in the amount of SHARPIN in LUBAC contributes to the increase in the total amount of SHARPIN by IFN-γ.

LUBAC plays a key role in signal-induced NF-κB activation (1). Indeed, IFN-γ augmented TNF-α–induced phosphorylation and degradation of IκBα, which are the hallmarks of NF-κB activation, in both WT keratinocytes and MEFs (Fig. 1E, Supplemental Fig. 2A), and TNF-α–induced expression of NF-kB target genes in MEFs (Fig. 1F). Moreover, IFN-γ enhanced the IL-1β–mediated phosphorylation and degradation of IκBα in MEFs (Supplemental Fig. 2B) and LPS-mediated NF-κB activation in BMDMs (Fig. 1G). LPS-mediated expression of TNF-α mRNA, an NF-kB target gene, also was promoted by pretreatment with IFN-γ (Fig. 1H). To confirm whether IFN-γ–mediated enhancement of NF-kB activation is due to the increase in the amount of LUBAC by IFN-γ, HOIP Δlinear MEFs, expressing a HOIP mutant (HOIP Δlinear) lacking the catalytic domains for linear polyubiquitination, were used (28). TNF-α–mediated phosphorylation and degradation of IκBα was profusely suppressed in HOIP Δlinear MEFs. Importantly, IFN-γ pretreatment did not reverse the suppressed TNF-α–mediated phosphorylation and degradation of IκBα or induce the expression of NF-kB target mRNAs, although IFN-γ did increase the expression of the HOIP Δlinear protein (truncated HOIP) (Fig. 1I, 1J). Considering that the HOIP Δlinear protein does not exhibit linear polyubiquitination activity, these results strongly indicated that IFN-γ–mediated NF-kB activation is mediated by the increase in the linear polyubiquitination activity of the LUBAC ligase complex.

IFN-γ–mediated increase of HOIP and HOIL-1L–protected cpdm cells from TNF-α–mediated apoptosis both in vitro and in vivo

cpydm mice are spontaneous mutant mice lacking SHARPIN (15, 29). As observed in the WT cells, IFN-γ increased the expression of HOIP and HOIL-1L mRNA in MEFs from cpdm mice (Fig. 2A). The amount of HOIP and HOIL-1L proteins also was increased by IFN-γ in MEFs and primary keratinocytes prepared from cpdm mice (Fig. 2B). Signal-induced NF-kB activation is suppressed in cpdm cells because of the drastic decrease of the LUBAC caused by the lack of SHARPIN (16, 17, 19). IFN-γ pretreatment accelerated TNF-α– and IL-1β–mediated phosphorylation and degradation of IκBα in cpdm MEFs (Fig. 2C, Supplemental Fig. 2C). TNF-α–induced NF-kB activation also was enhanced by IFN-γ in primary keratinocytes prepared from cpdm mice (Fig. 2D). Moreover, TNF-α–induced expression of NF-kB target genes and the nuclear translocation of p65 were augmented in cpdm MEFs pretreated with IFN-γ (Fig. 2E, 2F). LUBAC-mediated linear polyubiquitination of NEMO may play a crucial role in NF-κB activation (1). We therefore examined the effect of IFN-γ on TNF-α–induced linear polyubiquitination of NEMO. Immunoblotting analysis showed that TNF-α–induced linear polyubiquitination of NEMO was accelerated by pretreatment with IFN-γ (Fig. 2G).

The cells derived from cpdm mice are more susceptible to TNF-α–induced apoptosis than WT cells (19). Our immunostaining analyses revealed that IFN-γ suppressed TNF-α–induced apoptosis in cpdm MEFs (Fig. 2H). Ubiquitination of RIP1 in the TNFR1 signaling complex (TNF-RSC) protects the cells from TNF-α–mediated apoptosis (30–32). The amount of RIP1 coprecipitated with TRADD, which is recruited to TNF-RSC upon TNF-α stimulation, was increased in IFN-γ–pretreated cpdm MEFs, suggesting that IFN-γ treatment increased the amount of RIP1 recruited to TNF-RSC by TNF-α. Moreover, TNF-α–induced RIP1 polyubiquitination was also promoted by pretreatment with IFN-γ (Fig. 2I). Taken together, our findings suggest that IFN-γ enhances NF-kB activation and suppresses apoptosis induced by TNF-α by augmenting NEMO and RIP1 ubiquitination, respectively.
Next, we examined whether IFN-γ enhances NF-κB activation and suppresses apoptosis mediated by TNF-α in vivo. Subcutaneous injection of IFN-γ for 2 consecutive days increased the amount of HOIP and HOIL-1L in the skin of cpdm mice (Fig. 3A). We next injected TNF-α into the ear of cpdm mice after pretreatment with PBS or IFN-γ. Immunostaining analysis showed that IFN-γ enhanced TNF-α–induced phosphorylation of IKK1/2, which is one of the hallmarks of NF-κB activation (Fig. 3B). Moreover, HOIP expression was induced and cleaved caspase-3 and TUNEL–positive cells were reduced in the ear treated with IFN-γ (Fig. 3C, 3D). Collectively, these findings suggest that IFN-γ enhances NF-κB activation and protects cpdm cells from TNF-α–induced apoptosis by increasing the amount of HOIP both in vitro and in vivo.

Amelioration of dermatitis by s.c. injection of IFN-γ in cpdm mice

Although the precise mechanism underscoring chronic dermatitis in cpdm mice has not been clarified yet, both TNF-α and increased keratinocyte apoptosis have been suggested to be involved in the pathogenesis of dermatitis (16, 29). Because we observed that IFN-γ protects cpdm keratinocytes from TNF-α–induced apoptosis and activates NF-κB both in vitro and in vivo, we injected IFN-γ once a day into the back of cpdm mice for 3 wk to evaluate
the effect of IFN-γ on the severity of cpdm dermatitis. The amount of HOIP was larger in mice treated with IFN-γ than mice treated with PBS, confirming that IFN-γ increased the amount of HOIP even with longer treatment (Fig. 4A, 4B). Before treatment, the average dermatitis scores of PBS and IFN-γ–treated mice were 0.7 and 0.7, respectively (data not shown). The average severity score in mice treated with IFN-γ for 3 wk was much lower than that of mice treated with PBS (Fig. 4C, 4D). The average severity scores in mice treated with PBS and IFN-γ were 6.0 and 2.8, respectively. Epidermal thickening, which is one of the major characteristics of cpdm dermatitis, was significantly lower in IFN-γ–treated mice than in PBS-treated mice (Fig. 4E). In addition, the number of cleaved caspase-3 and TUNEL-positive cells was much higher in the epidermis of PBS-treated mice than that of IFN-γ–treated mice (Fig. 4F, 4G). HMGB1, one of the damage-associated molecular patterns, is normally located in the nucleus and translocates into the cytoplasm prior to its release from keratinocytes at

the later stage of apoptosis (33–35). Immunohistochemical staining showed that the amount of HMGB1 located outside the nucleus of keratinocytes was increased in the skin of cpdm mice, whereas in keratinocytes of WT mice, HMGB1 was detected in the nucleus (Supplemental Fig. 3A). In IFN-γ–treated cpdm mice, the amount of cytoplasmic HMGB1 was decreased (Fig. 4H). An increase in the number of proliferative keratinocytes is another characteristic of cpdm dermatitis (14, 15). Keratinocytes expressing the proliferation markers Ki67 and Keratin 6 were decreased in IFN-γ–treated mice, as compared with PBS-treated mice (Fig. 4I, 4J). These results suggested that IFN-γ treatment ameliorates cpdm dermatitis.

Reduction of macrophage, neutrophil, and mast cell infiltration and the amount of TNF-α in the skin of cpdm mice by IFN-γ

Infiltration of inflammatory cells into the affected skin has been reported in cpdm mice (14). We confirmed that neutrophils (Gr-1+), macrophages (F4/80+), and T cells (CD3+ and CD4+) infiltrated

FIGURE 2. IFN-γ augments NF-κB activation and suppresses TNF-α–mediated apoptosis in cpdm cells. (A) Real-time PCR analyses of HOIP and HOIL-1L mRNA induction by IFN-γ in cpdm MEFs (n = 3). (B) Immunobots showing IFN-γ–mediated increase in HOIP and HOIL-1L in MEFs and primary keratinocytes from cpdm mice. (C) Cpdm MEFs pretreated with or without IFN-γ were stimulated with TNF-α as indicated, and lysates were probed as depicted. (D) Enhanced TNF-α–induced NF-κB activation by IFN-γ pretreatment in cpdm keratinocytes. Primary keratinocytes from cpdm mice were treated and probed as in (C). (E) Real-time PCR analyses of TNF-α (10 ng/ml)–induced NF-κB target gene expression in cpdm MEFs preincubated with or without IFN-γ (n = 3). (F) Immunocytochemical analyses of p65 nuclear translocation by TNF-α (10 ng/ml) stimulation in cpdm MEFs pretreated with or without IFN-γ. Nuclei were stained with DAPI (original magnification ×400). (G) Anti-NEMO immunoprecipitates and lysates of cpdm MEFs preincubated with or without IFN-γ and stimulated with TNF-α as indicated were probed with the depicted Abs. (H) Immunocytochemical analyses of cleaved caspase 3 in cpdm MEFs pretreated with or without IFN-γ and incubated with TNF-α (10 ng/ml) for 4 h. Nuclei were stained with DAPI. The number of cleaved caspase–3-positive cells is also shown (original magnification ×200). (I) Enhanced TNF-α–mediated RIP1 ubiquitination by IFN-γ. Anti-TRADD immunoprecipitates and lysates of cpdm MEFs preincubated with or without IFN-γ and stimulated with TNF-α as indicated were probed with the depicted Abs. IFN-γ was used at 300 U/ml for 24 h unless indicated otherwise. Error bars indicate mean ± SD. *p < 0.05, **p < 0.01.
the skin of cpdm mice, but we did not detect CD8+ cells. The number of mast cells (toluidine blue positive) in the skin was higher in cpdm (202.1 ± 4.7/mm²) than in WT mice (109.7 ± 13.5/mm²) (Supplemental Fig. 3B). The amount of TNF-α, which is suggested to be involved in cpdm dermatitis (16, 17, 19), was greater in the skin of cpdm mice than that of WT mice (Supplemental Fig. 3C, 3D), and macrophages, neutrophils, and mast cells were the major producers of TNF-α in the skin of cpdm mice (Supplemental Fig. 3E). Because IFN-γ treatment reduced the number of infiltrating macrophages, neutrophils, and mast cells (Fig. 5A, 5B), the amount of TNF-α protein and mRNA in the skin of IFN-γ and PBS-treated mice was examined. Both TNF-α protein and mRNA were lower in IFN-γ-treated mice than PBS-treated mice (Fig. 3C, 3D). Immunohistological analysis confirmed that IFN-γ reduced the amount of TNF-α in cpdm skin (Fig. 5E). The increase of cytokines produced by Th2 cells including IL-5 and IL-13 in the skin and spleen of cpdm mice plays a role in the pathogenesis of dermatitis (36). We confirmed that the amount of IL-5 and IL-13 mRNA was higher and that of Th1 cytokines IL-12 and IFN-γ was lower in the spleen of cpdm mice than in WT mice. The amount of IFN-α and IFN-β in the spleen did not differ between WT and cpdm mice. (Supplemental Fig. 3F). There was no significant difference in the amount of IL-12, IFN-γ, IL-6, and IFN-β mRNA, whereas the amount of IL-13 was higher in the skin of cpdm mice than that of WT mice (Supplemental Fig. 3G). We then assessed the effect of IFN-γ injection on the amount of Th1, Th2 cytokines, and type I IFNs mRNA in the spleen and skin of cpdm mice. Subcutaneous IFN-γ injection did not decrease Th2 nor increase Th1 cytokines significantly in either spleen or skin of cpdm mice. Similarly, there was no significant change in type I IFNs mRNA expression in the spleen and skin (Fig. 5F, 5G). These results suggested that s.c. IFN-γ ameliorates cpdm dermatitis by increasing the amount of HOIP but not by suppressing the production of Th2 cytokines.

TNF-α is suggested to be involved in the pathogenesis of cpdm (16). We have observed that the amount of TNF-α is reduced by IFN-γ treatment in the skin of cpdm mice. To confirm the involvement of TNF-α in cpdm, we treated cpdm mice with etanercept, which blocks the effect of TNF-α (37, 38). Etanercept treatment ameliorated cpdm and inhibited keratinocyte apoptosis and infiltration of inflammatory cells such as granulocytes, macrophages, and mast cells (Supplemental Fig. 4). These findings indicated that TNF-α is involved in the pathogenesis of cpdm and that IFN-γ-mediated amelioration of cpdm is partly due to the reduction of TNF-α in the skin.

*IFN-γ suppresses apoptosis but does not effectively augment NF-κB target gene induction mediated by a low dose of TNF-α*

We next dissected the mechanism underscoring the IFN-γ-mediated amelioration of cpdm. As shown in Figs. 1 and 2, IFN-γ increased the amount of LUBAC and TNF-α–mediated expression of NF-κB target genes in keratinocytes, fibroblasts, and macrophages in vitro. Subcutaneous injection of IFN-γ also augmented LUBAC in not only keratinocytes but also in cells in the dermis such as fibroblasts and macrophages (Fig. 6A). However, s.c. injection of IFN-γ for 2 d failed to enhance the production of transcripts of NF-κB target genes including TNF-α, IL-6, and VCAM-1 in the skin of cpdm mice (Fig. 6B). The amount of IL-6 mRNA was increased by IFN-γ slightly, although the increase was not statistically significant. Previous studies suggested that the physiological levels of TNF-α are much lower than those that we use in in vitro experiments (10 ng/ml) (39, 40). Thus, we stimulated cpdm MEFs with 0.1 ng/ml TNF-α and found that IFN-γ pretreatment did not increase the amount of TNF-α and VCAM-1 mRNA, although it significantly induced IL-6 mRNA (Fig. 6C). IL-6 mRNA expression in vivo was not induced by IFN-γ treatment, and there are no previous studies suggesting that IL-6 is important in the pathogenesis of cpdm in mice. However, IFN-γ pretreatment inhibited the activation of caspase-3 in cpdm MEFs.

**FIGURE 3.** IFN-γ augmented the amount of LUBAC and TNF-α–mediated NF-κB activation and suppressed TNF-α–mediated apoptosis in the skin of cpdm mice. (A) Skin extracts from two cpdm mice injected with PBS (left back) or IFN-γ (right back) were probed as indicated. (B) Immunohistochemical analyses of p-IKK1/2 in the ear of cpdm mice treated with a single s.c. injection of PBS or IFN-γ, followed by TNF-α (5 μg/ml for 45 min) stimulation 24 h later. Nuclei were stained with DAPI. (C) Immunohistochemical staining with anti-cleaved caspase-3, anti-HOIP Abs, and TUNEL staining of the ear of cpdm mice treated with a single s.c. injection of PBS or IFN-γ, followed by TNF-α (5 μg/ml for 6 h) stimulation 24 h later. (D) The number of cleaved caspase-3 and TUNEL-positive cells shown in (C). Scale bars, 100 μm. Error bars indicate mean ± SD. *p < 0.05.
stimulated with 0.1 ng/ml TNF-α (Fig. 6D). Thus, these results suggest that IFN-γ-mediated increase of LUBAC exhibits a profound effect on the suppression of TNF-α-induced apoptosis compared with the effect on NF-κB activation in cpdm cells.

Apoptosis of keratinocytes and TNF-α are known to be involved in the pathogenesis of cpdm (16, 29), and we found that s.c. injection of IFN-γ for 2 d suppressed TNF-α-mediated apoptosis of keratinocytes (Figs. 3C, 3D). However, the 2-d IFN-γ treatment...
failed to reduce the number of infiltrated inflammatory cells (Fig. 6E). TNF-α, IL-6, and VCAM-1 mRNA expression also was not changed by IFN-γ treatment (Fig. 6B). We thus suggest that the effects of IFN-γ on keratinocytes may be the initial event leading to amelioration of cpdm dermatitis, although both keratinocytes and leukocytes are the targets of IFN-γ.

**IFN-α increased the amount of LUBAC and ameliorated dermatitis in cpdm mice**

We observed that in addition to IFN-γ, a type I IFN, IFN-α also increased the amount of LUBAC in keratinocytes and BMDMs from WT mice (Fig. 7A). IFN-α increased the amount of HOIP and HOIL-1L in cpdm MEFs, although IFN-α was a weaker inducer of LUBAC than IFN-γ (Fig. 7B). In addition, IFN-α augmented TNF-α–mediated IkBa phosphorylation and degradation in cpdm keratinocytes (Fig. 7C). These results indicated that IFN-α also enhances NF-κB activation via an increase of LUBAC composed of HOIP and HOIL-1L in cpdm cells. We also evaluated the in vivo effect of IFN-α. Subcutaneous injection of IFN-α into the ear, as performed with IFN-γ in Fig. 4, increased the amount of HOIP and inhibited TNF-α–mediated apoptosis of keratinocytes (Fig. 7D). Next, IFN-α was s.c. injected into the back of cpdm mice using an identical protocol to IFN-γ injection. Immunoblotting analysis and immunofluorescent staining confirmed that the amount of HOIP was increased by the IFN-α injection (Fig. 7E, 7F). The average dermatitis scores of PBS and IFN-α–treated mice before treatment were 1.1 and 0.9, respectively (data not shown). They were not significantly different. As shown in Fig. 7G, the severity score of cpdm dermatitis was significantly decreased by IFN-α injection. The average scores of PBS and IFN-α–treated mice after treatment were 6.9 and 4.2, respectively. Histological analyses showed a reduction of epidermal thickness and the number of cleaved caspase-3–positive cells in IFN-α–treated cpdm mice (Fig. 7H, 7I). It is of note that s.c.

**FIGURE 5.** IFN-γ decreased macrophage, neutrophil, and mast cell infiltration and the amount of TNF-α in the skin of cpdm mice. (A) Immunofluorescent staining of F4/80, Gr-1, CD3, and CD4-positive cells in the skin of cpdm mice treated with PBS or IFN-γ for 3 wk. Nuclei were stained with DAPI. (B) Toluidine blue staining of skin treated with PBS or IFN-γ for 3 wk. The number of positive cells per square millimeter is also shown. (C and D) The amount of TNF-α protein (C) or mRNA (D) in the skin extract treated with PBS or IFN-γ for 3 wk. (E) Immunofluorescent staining of TNF-α in the skin of cpdm mice treated with PBS or IFN-γ for 3 wk. Nuclei were stained with DAPI. (F and G) Real-time PCR analyses of mRNA expression of Th1 (IFN-γ and IL-12), Th2 (IL-4, IL-5, and IL-13) cytokines, and type I IFNs (IFN-α and IFN-β) in the spleen (F) or skin (G) of cpdm mice treated with PBS or IFN-γ for 3 wk (n = 10). Scale bars, 100 μm. *p < 0.05.
injection of IFN-α did not overtly affect the amount of IL-4, IL-5, IL-12, IL-13, and IFN-γ mRNA (Fig. 7J). These results indicated that enhanced NF-κB activation by IFN-α as well as IFN-γ through the increase of LUBAC composed of HOIP and HOIL-1L ameliorated cpdm dermatitis.

Discussion

Cpdm mice are characterized by various symptoms including chronic proliferative dermatitis (15, 29). Because of the lack of SHARPIN, the amount of the other two subunits of LUBAC is drastically reduced in cpdm mice. Suppressed NF-κB activation and increased apoptosis, which appears to be mediated by the reduction of LUBAC, are thought to provoke chronic proliferative dermatitis (16, 17). In this study, we could ameliorate cpdm dermatitis by augmenting NF-κB activation and suppressing TNF-α-mediated apoptosis via IFN-γ or IFN-α-mediated increase of LUBAC.

In cpdm mice, augmented production of cytokines generated by Th2 cells such as IL-5 and IL-13, and an impaired ability of dendritic cells from cpdm mice to induce Th1 cells, have been reported previously (41). Moreover, suppressed IFN-γ secretion, which has been reported in cpdm mice (36), might be involved in the pathogenesis of dermatitis. Because IFN-γ is produced by Th1 cells and is able to induce Th1 cells, it is possible that in our study, IFN-γ ameliorated the dermatitis by inducing Th1 cells in cpdm mice. Moreover, systemic administration of IL-12, which is known to induce Th1 cells, resolves chronic dermatitis in cpdm mice (36). However, we observed that the amount of type 1 and type 2 cytokines was not significantly affected by s.c. injection of IFN-γ, which has been reported in cpdm mice (36), might be involved in the pathogenesis of dermatitis. Because IFN-γ is produced by Th1 cells and is able to induce Th1 cells, it is possible that in our study, IFN-γ ameliorated the dermatitis by inducing Th1 cells in cpdm mice. Moreover, systemic administration of IL-12, which is known to induce Th1 cells, resolves chronic dermatitis in cpdm mice (36). However, we could not rule out the possibility that an induction of Th1 cells might be involved in the resolution of cpdm dermatitis by IFN-γ, because IFN-γ improved the dermatitis more prominently than IFN-α. A recent study reported that an increased expression of β1-integrin might be involved in the pathogenesis of
cpdm dermatitis (42). However, it may not play a major role in cpdm dermatitis because intercrossing of cpdm mice with TNFR1 knockout (KO) mice drastically ameliorates cpdm dermatitis (16). Therefore, it seems plausible that impaired NF-κB activation and enhanced TNF-α–induced apoptosis are involved in the pathogenesis of cpdm dermatitis.

Keratinocyte-specific ablation of IKK2, the catalytic component of the canonical IKK complex, results in the development of dermatitis, characterized by increased proliferation of keratinocytes. It has been shown that impaired signal-induced NF-κB activation and TNF-α signaling play an essential role in the pathogenesis of dermatitis in the keratinocyte-specific IKK2 KO mice (43). Keratinocyte-specific expression of mutant IkBα, which is resistant to degradation triggered by the IKK-mediated phosphorylation of the protein, also resulted in the development of severe proliferative dermatitis in mice (44). Therefore, in dermatitis characterized by increased proliferation of keratinocytes, suppressed NF-κB activation might augment the inflammation process, even though NF-κB is known to be a proinflammatory transcription factor (45). Enhanced apoptosis of keratinocytes is observed in cpdm skin and keratinocyte apoptosis can be suppressed by intercrossing with TNFR1 KO mice (16). We also observed that the protection of keratinocytes from apoptosis appears to be the initial event leading to the amelioration of skin inflammation in cpdm mice following s.c. injection of IFN-γ. Thus, increased TNF-α–mediated apoptosis of keratinocytes might be involved in the pathogenesis of cpdm dermatitis. Indeed, induced apoptosis of keratinocytes has been observed in pathological skin conditions such as sunburn, toxic epidermal necrolysis, pemphigus, eczematous dermatitis, and graft-versus-host disease (46). However, some unknown mechanism might also be involved in cpdm dermatitis because no overt keratinocyte death could be observed in keratinocyte-specific IKK2 KO mice.

**FIGURE 7.** IFN-α also increases the amount of LUBAC and ameliorates cpdm dermatitis. (A) Immunoblots showing the increase in the amount of HOIP, HOIL-1L, and SHARPIN by IFN-α in primary keratinocytes and BMDMs from WT mice. (B) Immunoblots showing weaker induction of the amount of LUBAC subunits by IFN-α compared with IFN-γ in cpdm MEFs. (C) cpdm keratinocytes pretreated with or without IFN-α (1000 U/ml) for 24 h were stimulated with TNF-α as indicated, and lysates were probed as depicted. (D) Immunofluorescent staining with anti-HOIP and anti-cleaved caspase-3 Abs in the ear of cpdm mice injected with IFN-α or PBS, followed by TNF-α administration. (E and F) Immunoblotting analysis (E) and immunofluorescent staining (F) with anti-HOIP Ab in the skin extract of cpdm mice treated with IFN-α or PBS for 3 wk. (G) Severity scores of dermatitis in cpdm mice treated with PBS or IFN-α (n = 12). (H) Reduced epidermal thickness by IFN-α treatment (n = 12). H&E staining of the skin treated with PBS or IFN-α for 3 wk. (I) Immunohistochemical staining with anti-cleaved caspase-3 Ab in IFN-α–treated or untreated skin of cpdm mice. The number of cleaved caspase-3–positive cells per square millimeter is also shown. (J) Real-time PCR analyses of Th1 (IFN-γ and IL-12) and Th2 (IL-4, IL-5, and IL-13) cytokines mRNA expression in the spleen of IFN-α–treated or untreated cpdm mice (n = 12). IFN-α and IFN-γ were used at 300 U/ml. Scale bars, 100 μm. Error bars indicate mean ± SD. *p < 0.05.
mice, whose dermatitis resembles that of cpdm mice (43). Nevertheless, in HOIL-IL KO mice, dermatitis was not observed, although TNF-α–induced NF-κB activation was heavily suppressed by the reduction of LUBAC, as observed in cpdm mice (1). This finding might be attributed to the differential sensitivity to TNF-α–mediated apoptosis, because HOIL-IL KO MEFs are more resistant to TNF-α–mediated apoptosis than cpdm MEFs (19). Further work will be needed to elucidate the precise mechanism underlying the amelioration of cpdm dermatitis by the increase of LUBAC.

In this study, we demonstrated that IFN-γ induces LUBAC not only in keratinocytes but also in BMDMs. It has been shown that priming with IFN-γ augments NF-κB expression and the activation of NF-κB target genes induced by ligands of TLRs, including LPS in macrophages (47). The molecular mechanism underlying the augmentation of NF-κB activation by IFN-γ pretreatment is not well understood, although the combined presence of the binding sites of STAT1, which is an IFN-γ–inducible transcriptional activator, and the NF-κB binding sites in the promoter region of many NF-κB target genes has been demonstrated (47–49). We also showed that IFN-γ increases the amount of LUBAC and LPS-mediated NF-κB activation in BMDMs. In addition, TNF-α– and IL-1β–mediated NF-κB activation was augmented by IFN-γ in MEFs and keratinocytes. Because IFN-γ failed to augment NF-κB activation in MEFs lacking the catalytic activity of LUBAC, the present results strongly indicate that LUBAC is the main target for IFN-γ–mediated increase of NF-κB activation, involved in LUBAC-mediated linear polyubiquitination by various stimuli such as LPS, TNF-α, and IL-1β.

LUBAC has recently been reported to play a key role in human disease (50). In addition, a strong genetic link with A20 and A20 binding and inhibitor of NF-κB in psoriasis has been reported (51, 52). Both A20 and A20 binding and inhibitor of NF-κB can bind to linear ubiquitin chains that are specifically generated by LUBAC (53–55). Psoriasis is characterized by increased proliferation of keratinocytes as observed in cpdm dermatitis. Thus, it will be of interest to examine the roles of LUBAC-mediated linear ubiquitination in the pathogenesis of psoriasis or other dermatitis conditions with similar characteristics.

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Disclosures
The authors have no financial conflicts of interest.

References

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Figure S1. IFN-γ responsive cis-element of Rnf31 and Rbck1. (A) Promoter analyses of the upstream of Rnf31 with luciferase reporter. 1, -817 to -1; 2, -598 to -1; 3, -161 to -1; 4, -138 to -1 (B) Promoter analyses of the downstream of Rnf31 with luciferase reporter including core promoter (-88 to -1). 1, no fragment of the downstream; 2, +11,927 to +12,496; 3, +11,927 to +12,432; 4, +11,927 to +12,410. (C) Promoter analyses of both the upstream and the downstream of Rnf31. 1, -817 to -1 and +11,927 to +12,496; 2, -817 to -1 and +11,927 to +12,432; 3, -817 to -1 and +11,927 to +12,410; 4, -817 to -1 with mutated IRF1 sequence and +11,927 to +12,410. (D) Promoter analyses of the upstream of Rbck1. 1, -1,201 to -1; 2, -329 to -1; 3, -104 to -1; 4, -1,201 to -1 with mutated ISRE sequence. Nucleotide sequences are as follows: IRF-1 (GCTTTCGCTTTC), mutant IRF-1 (GCCTGCGCTTTC); ISRE (CACTTTCACTTTCTC), mutant ISRE (CACGATCACTTTCTC). IFN-γ was used at 300 U/ml for 6 hours. Error bars indicate mean ± SD (n=3). *p < 0.05; **p < 0.01; ***p < 0.005.
Figure S2. IFN-γ enhanced TNF-α or IL-1β-mediated phosphorylation and degradation of IκBα in WT and cpdm MEFs. (A, B) WT MEFs pretreated with or without 300 U/ml of IFN-γ for 24 hours were stimulated with TNF-α (A) or IL-1β (B) as indicated and lysates were probed with the depicted antibodies. (C) Cpdm MEFs pretreated with or without 300 U/ml of IFN-γ for 24 hours were stimulated with IL-1β as indicated and lysates were probed with the depicted antibodies.
Figure S3. Infiltration of inflammatory cells, elevated levels of cytokines, and cell death of keratinocytes in cpdm and WT mice. (A) Immunohistochemical staining of the skin of WT and cpdm mice with anti-HMGB1 antibody. Nuclei were stained with DAPI. (B) Immunofluorescence staining of the skin of WT and cpdm mice with anti-F4/80 (macrophages), anti-Gr-1 (neutrophils), anti-CD3, anti-CD4, and anti-CD8 (T-cells) antibodies and toluidine blue staining (mast cells). (C) Real-time PCR analyses of TNF-α mRNA in the skin of WT and cpdm mice (n=3). (D) Immunofluorescent staining with anti-TNF-α antibody of the skin of WT and cpdm mice. Nuclei were stained with DAPI. (E) Skins of cpdm mice were multistained with anti-TNF-α and anti-F4/80, anti-Gr-1, anti-CD3, or anti-c-kit (mast cells) antibodies. Nuclei were stained with DAPI. (F) Real-time PCR analyses of Th1 (IFN-γ and IL-12), Th2 (IL-4, IL-5 and IL-13) cytokines, and type I interferons (IFN-α and IFN-β) mRNA expression in the spleen of WT and cpdm mice (n=3). (G) Real-time PCR analyses of Th1 (IFN-γ and IL-12), Th2 (IL-13) cytokines, and type I interferons (IFN-α and IFN-β) mRNA expression in the skin of WT and cpdm mice (n=3). Scale bars: 100 µm. *p < 0.05; **p < 0.005.
Figure S4. Subcutaneous injection of etanercept (EC) ameliorated cpdm dermatitis. (A) Severity scores of dermatitis in cpdm mice treated with PBS or EC for 3 weeks (n=9). Bar indicates the average of the severity scores. (B) Representative mice after treatment with PBS or EC are shown. (C) H-E staining of the skin treated with PBS or EC is shown. Epidermal thickness of treated mice (n=9) was measured. (D, E) Immunohistochemical analyses of cleaved caspase 3 (D) or TUNEL staining (E) in the skin of cpdm mice treated with PBS or EC. The numbers of cleaved caspase 3-positive (D) or TUNEL-positive (E) cells/mm² are also shown. Dotted lines indicate the border between the epidermis and dermis. (F) Immunohistochemical staining with anti-HMGB1 antibody of the skin treated with PBS or EC. (G) Immunofluorescence staining of F4/80, Gr-1, CD3, and CD4 positive cells in the skin of cpdm mice treated with PBS or EC. Nuclei were stained with DAPI. (H) Toluidine blue staining of the skin treated with PBS or EC. The number of positive cells is also shown. Scale bars: 100 µm. Error bars indicate mean ± SD. *p < 0.05.