Cathepsin K Is Involved in Development of Psoriasis-like Skin Lesions through TLR-Dependent Th17 Activation

Toshide Hirai, Takashi Kanda, Kenji Sato, Mikiro Takaishi, Kimiko Nakajima, Mayuko Yamamoto, Reiko Toshitake Hirai, Takashi Kanda, Kenji Sato, Mikiro Takaishi, Kimiko Nakajima, Mayuko Yamamoto, Reiko Kamijima, John DiGiovanni and Shigetoshi Sano

J Immunol 2013; 190:4805-4811; Prepublished online 29 March 2013;
doi: 10.4049/jimmunol.1200901
http://www.jimmunol.org/content/190/9/4805

References
This article cites 37 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/190/9/4805.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Errata
An erratum has been published regarding this article. Please see next page or:
/content/192/10/4933.full.pdf
Cathepsin K Is Involved in Development of Psoriasis-like Skin Lesions through TLR-Dependent Th17 Activation

Toshitake Hirai,* Takashi Kanda,* Kenji Sato,* Mikiko Takaishi,* Kimiko Nakajima,* Mayuko Yamamoto,* Reiko Kamijima,* John DiGiovanni,†‡ and Shigetoshi Sano*†

Cathepsins (CTSs) are lysosomal cysteine proteases that play an important role in the turnover of intracellular proteins and extracellular proteins, such as the degradation of extracellular matrices and the processing of antigenic proteins. A CTS inhibitor, NC-2300, not only suppresses bone erosion by inhibition of cathepsin K (CTSK), but also ameliorates paw swelling at inflamed joints in adjuvant-induced arthritis in rats. It has been demonstrated that the amelioration of joint inflammation by NC-2300 is mediated by the downregulation of cytokine expression in dendritic cells, which are essential for Th17 activation. In this work, we studied the role for CTSs in the pathogenesis of psoriasis-like lesion in K5.Stat3C mice, a mouse model of psoriasis, in which Th17 contributes to lesion development similar to psoriasis. Psoriatic lesions expressed increased levels of Ctsk and Cts mRNA compared with uninvolved skin and normal control skin. Similarly, the epidermis and dermis in K5.Stat3C mice demonstrated increased CTSK activities, which were sensitive to NC-2300. Topical treatment with NC-2300 significantly ameliorated 12-O-tetradecanoylphorbol-13-acetate–induced psoriasis-like lesions in K5.Stat3C mice, and downregulated the expression of IL-23, Th17 cytokines. In vitro experiments revealed that TLR7 activation of bone marrow–derived myeloid dendritic cells led to increased IL-23 at mRNA and protein levels, which were downregulated by NC-2300. These results suggest that CTSK plays a role in development of psoriatic lesions through TLR7-dependent Th17 polarization. The Journal of Immunology, 2013, 190: 4805–4811.

Psoriasis is a common chronic inflammatory skin disease characterized by increased proliferation, altered differentiation of the epidermis, and inflammatory cell infiltrates (1). Those cell infiltrates are composed of T cells, neutrophils, and dendritic cells (DCs). A number of recent studies have demonstrated that IL-23, which is essential for the development of Th17 cells, is functionally involved in the pathogenesis of psoriasis (2). A human mAb against the p40 subunit shared by IL-12 and IL-23, termed ustekinumab, shows a therapeutic efficacy in psoriasis (3).

Strikingly, IL-23, which is essential for the development of Th17 cells, is functionally involved in the pathogenesis of psoriasis. Psoriatic lesions expressed increased levels of Ctsk and Cts mRNA compared with uninvolved skin and normal control skin. Similarly, the epidermis and dermis in K5.Stat3C mice demonstrated increased CTSK activities, which were sensitive to NC-2300. Topical treatment with NC-2300 significantly ameliorated 12-O-tetradecanoylphorbol-13-acetate–induced psoriasis-like lesions in K5.Stat3C mice, and downregulated the expression of IL-23, IL-23, and Th17 cytokines. In vitro experiments revealed that TLR7 activation of bone marrow–derived myeloid dendritic cells led to increased IL-23 at mRNA and protein levels, which were downregulated by NC-2300. These results suggest that CTSK plays a role in development of psoriatic lesions through TLR7-dependent Th17 polarization. The Journal of Immunology, 2013, 190: 4805–4811.

Received for publication March 22, 2012. Accepted for publication February 27, 2013.

This work was supported in part by a grant from the Ministry of Education, Science, Sports, and Culture in Japan.

Address correspondence and reprint requests to Prof. Shigetoshi Sano, Department of Dermatology, Kochi Medical School, Kochi University, Kochi 783-8505, Japan; †Division of Pharmacology and Toxicology, University of Texas, Austin, TX; and ‡Department of Nutritional Sciences, University of Texas, Austin, TX.

Abbreviations used in this article: AEP, asparagine endopeptidase; CTS, cathepsin; CTSK, cathepsin K; DC, dendritic cell; mDC, myeloid DC; pDC, plasmacytoid DC; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1200901
In this study, we investigated whether CTSK is involved in the development of psoriasis-like lesions in TPA-treated K5.Stat3C mice. The results show that CTSK activity is higher in the epidermis and the dermis of K5.Stat3C mice than in wild-type mice. Furthermore, TPA-induced psoriasis-like lesions are ameliorated by topical treatment with a CTSK inhibitor. Our data suggest that CTSK plays a role in development of psoriatic lesions through the TLR7-mediated IL-23/Th17 pathway.

Materials and Methods

Patients and skin samples

The study protocol was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki and was approved by the Institute Ethical Review Board of the Kochi Medical School. Skin biopsy specimens were taken from lesions and from contiguous uninvolved skins from 10 patients with plaque psoriasis (9 males, 1 female). Before taking skin biopsy, the patients had not been treated with topical steroids, vitamin D3, UV irradiation, or systemic therapy for at least 1 mo. Normal control skins were obtained from four donors who had plastic surgery.

Animals used, topical application of NC-2300, and determination of CTSK activity

All experimental procedures performed on mice were approved by the Institutional Animal Care and Use Committee of Kochi Medical School. K5.Stat3C mice were generated as previously reported (17). Briefly, Stat3C cDNA (a gift from Dr. J. Bromberg, Memorial Sloan Kettering Cancer Center, New York, NY) was ligated into the pBK5 construct, followed by digestion with EcoRI. The construct was then used to generate transgenic founder mice on an FVB background. Transgenic mice were identified by PCR of their genomic DNAs with primers specific for the gene encoding rabbit β-globin, as follows: 5′-TTACAGGTGTGTGTAGAACTGTG-3′ and 5′-CAATAAGAATATCCAGCCA-3′. In some experiments, the transgenic mice were backcrossed at least 10 generations with C57BL/6 mice to obtain spontaneous psoriasis-like lesions. The CTSK inhibitor, NC-2300, was provided by Nippon Chemiphar (Saitama, Japan) and has been previously described (13, 18). To examine the in vivo effect of NC-2300, mice were treated subcutaneously with 50 mg ml−1 acetone on the right ear of each mouse, and with 20 μl NC-2300 in acetone at the indicated concentrations at every 12 h or acetone alone. Skin samples were taken at 2 h after the last treatment with NC-2300. The ears were placed dermal side down on PBS containing 20 mM EDTA for 80 min at 37°C. The epidermis was separated from the underlying dermis using forceps and washed several times in PBS. The CTSK activity was determined using a Cathepsin K Activity Assay Kit (BioVision), according to the manufacturer’s instruction.

TPA-induced psoriasis-like lesions in the ear skin of K5.Stat3C mice

The generation of psoriasis-like lesions in the ears of K5.Stat3C mice was as described previously (15, 16). In brief, the early TPA response was conducted by topical treatment with 0.68 nmol TPA (Sigma-Aldrich) in 20 μl acetone on the right ear of each mouse, and with 20 μl acetone on the left ear as a control once per day at days 1 and 3. NC-2300 (1, 5, 10, and 50 mg ml−1, twice per day) or vehicle alone (acetone) were applied on the ears for consecutive 3 d, and ear skin was biopsied at day 4. All mouse experiments were performed with strict adherence to institutional guidelines for minimizing distress.

Histology and immunostaining

Ear skin tissues were fixed in 20% buffered formalin and embedded in paraffin. Sections were obtained from the paraffin blocks and stained by H&E. For staining of CTSK in the skin, sections were deparaffinized in xylene and rehydrated through a 100% ethanol, immersed in pH 9.0 Target Retrieval Solution (Dako) in a microwave for 10 min, and then placed in 0.3% H2O2 in PBS for 30 min at room temperature to block endogenous peroxidase activity. After the blocking of nonspecific protein binding by incubation for 30 min with PBS containing 1% BSA (Sigma-Aldrich) and 5% normal goat serum (ImmunoBioScience), sections were incubated overnight at 4˚C with rabbit anti-CTSK polyclonal Ab (BioVision). Subsequently, sections were stained using the EnVision+ System (Dako). For detection of IL-23-producing CD11c+ cells and CTSK-expressing CD11c+ cells in the ear skin that had been topically treated with TPA, snap-frozen sections were treated with 5% BSA for blocking at room temperature for 1 h. They were then treated overnight at 4°C with armenian hamster anti-mouse CD11d mAb (clone N418; Bio-Legend), rabbit anti-CTSK polyclonal Ab, or rabbit anti-mouse IL-23p19 polyclonal Ab (Abcam). This was followed by treatment with goat anti-armenian hamster IgG-FITC (Santa Cruz Biotechnology) and goat anti-rabbit IgG-Alexa 594 (Invitrogen), respectively, and counterstaining with DAPI.

Generation of mDCs from bone marrow cells and in vitro culture

Immature mDCs were generated from mouse bone marrow cells, as previously described (13). In brief, bone marrow cells were cultured with 10 ng ml−1 murine GM-CSF (PeproTech) and 0.3 ng ml−1 murine IL-4 (PeproTech) in RPMI 1640 medium supplemented with 10% FBS for 6 d. Loosely adherent cells were harvested by gentle pipetting and were used as mDCs. To stimulate mDCs, cells were cultured in the presence of either 10 μM imiquimod (Enzo Life Sciences) or 10 nM R848 (resiquimod; Enzo Life Sciences) for 24 h. NC-2300 was added to cells 3 h before stimulation with the TLR ligands. Concentration of IL-12/23p40 and IL-23p19 in the culture supernatant was determined by ELISA kits (eBioscience), according to the manufacturer’s instructions.

RNA isolation and quantitative real-time PCR

Skin tissues were minced with scissors into small pieces on ice, and were disrupted by sonication. Total RNAs were extracted using a RNA isolation kit (Promega), according to the manufacturer’s protocol, and were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) with random oligonucleotide hexamers (Invitrogen). PCs

FIGURE 1. CTS expression in psoriatic skin and CTSK activity in K5.Stat3C mouse. (A) Quantitative mRNA expression of CtsK, CtsB, CtsL, and CtsS from control skin from normal donors, lesion, and uninvolved skin from patients (pso). The data are shown as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, n = 5–10 per group. Tukey-Kramer test. (B) Enzymatic activity of CTSK in the epidermis and dermis from the ear skin of K5.Stat3C mice (black bars) and wild-type mice (white bars) before and after treatment with NC-2300. The ears of the mice were treated with NC-2300 at concentrations of 1, 5, 10, and 50 mg ml−1 (stepwise increments indicated by triangles). The data represent mean ± SD, ***p < 0.001 versus vehicle-treated FVB/N mice; *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treated K5.Stat3C mice, n = 5–8 per group. Tukey-Kramer test.
were performed using Power SYBER Green PCR Master Mix (Applied Biosystems), and amplification conditions were as follows: 50°C for 2 min, 90°C for 10 min for 1 cycle, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers for mouse genes are as previously listed (16), except for human Ctsk, sense, 5′-GCTGGAGGACCTTTCTCTG-3′ and antisense, 5′-TGGGGGCAAAGCTCCAGAC-3′; human Ctsb, sense, 5′-TGAGGAGGCTTCTCGG-3′ and antisense, 5′-TGACGTGGTG-GTACACTCTCTG-3′; human Ctsl, sense, 5′-GGAGACCTCAGGGAATCA-3′, and antisense, 5′-GGAAGGCAATGAGG-3′. The quantity of each transcript was analyzed using the 7300 Fast System Software (Applied Biosystems), and amplification conditions were as follows: 50°C for 2 min, 90°C for 10 min for 1 cycle, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers for mouse genes are as previously listed (16), except for human Ctsk, sense, 5′-GCCAGACAAACGATTTGCA-3′ and antisense, 5′-CGAGGAAAGCTCACCAACAG-3′; human Ctsb, sense, 5′-TGAGGAGGCTTCTCTTG-3′ and antisense, 5′-TGACGTGGTG-GTACACTCTCTG-3′; human Ctsl, sense, 5′-GGAGGCAATGAGGAC-3′, and antisense, 5′-GGAAGGCAATGAGG-3′. The quantity of each transcript was analyzed using the 7300 Fast System Software (Applied Biosystems) and was normalized to Hprt according to the ΔΔCt method.

Flow cytometry and Abs
Cervical lymph nodes were collected as the skin-draining lymph nodes from K5.Stat3C mice following TPA treatment. The single-cell suspensions were stimulated for 4 h with 0.2 mg ml⁻¹ PMA (WAKO), 2.7 μM ionomycin (Sigma-Aldrich), and 40 mg ml⁻¹ brefeldin A (Sigma-Aldrich). Cells were then stained for 20 min with FITC-conjugated anti-CD3 and Cy5-conjugated anti-CD4 mouse Abs (BD Pharmingen), followed by permeabilization with Cytofix/Cytoperm buffer (BD Pharmingen) and intracellular staining with PE-conjugated anti–IL-17A mAb (BD Pharmingen). Samples were separated using a FACSCalibur flow cytometer, and data analysis was conducted using Cell-Quest Pro software (BD Biosciences).

Statistical analysis
Statistical analysis was conducted using GraphPad Prism (version 4.03; GraphPad Software). Statistical differences were evaluated by either unpaired t test or ANOVA, wherever applicable, followed by the Tukey or Tukey-Kramer.

Results
Increased cathepsin K expression in psoriatic lesion and the skin of K5.Stat3C mice
Previous studies demonstrated increased levels of CTSs B, D, L, and S in psoriasis (19, 20). We analyzed the expressions of Ctsk, Ctsb, Ctsl, and Ctss in human psoriasis (Fig. 1A). These four genes were all upregulated significantly in psoriatic lesions compared with normal skin. In particular, transcript levels of Ctsk and Ctss were significantly increased in the psoriatic lesions compared with uninvolved skin. We next examined the enzymatic activity of CTSK in the skin from K5.Stat3C, in which the psoriasis-like lesion develops spontaneously or is induced by wounding stimuli or topical treatment with TPA (14). Interestingly, the CTSK activity from K5.Stat3C mice was higher than that from wild-type mice both in the epidermis and dermis (Fig. 1B). Because the epidermis

**FIGURE 2.** Effect of topical treatment with NC-2300 on the development of the psoriasis-like lesions in K5.Stat3C mice. (A) Immunostaining of K5.Stat3C mouse skin treated for 3 d with acetone (top) and TPA (middle and bottom). Note that TPA-treated epidermis (ep) is positive for CTSK compared with vehicle treatment. Arrows indicate inflammatory cells. Asterisks, unspecific staining in the cornified layer. Immunofluorescence double staining of psoriasis-like lesion with anti-CD11c and anti-CTSK Abs (bottom panel). Arrows indicate CTSK-expressing CD11c⁺ cells, which were both positive for CTSK (red) and CD11c (green). Scale bars, 50 μm. (B) The dose-dependent effect of NC-2300 on the psoriasis-like lesion. The ear skin of K5.Stat3C mice was treated with acetone (open circles, n = 4) or TPA (filled circles, n = 4) and then acetone instead of NC-2300, respectively. NC-2300 was topically applied at 1 mg ml⁻¹ (open squares, n = 5), 5 mg ml⁻¹ (filled squares, n = 5), 10 mg ml⁻¹ (open triangles, n = 6), and 50 mg ml⁻¹ filled triangles, n = 4) on TPA-treated K5.Stat3C mice. Results are shown as mean increased ear thickness (μm) ± SD. **,** **,** p < 0.001 versus vehicle-treated mice; **,** **,** p < 0.001 versus TPA-treated mice. Tukey-Kramer test. (C) Representative histological features of TPA-treated ear skin following treatment with 10 mg ml⁻¹ NC-2300. H&E staining. Scale bar, 100 μm. (D) Assessment of the epidermal thickness. White bar, vehicle control (n = 6); black bar, TPA control (n = 6); gray bar, TPA plus 10 mg ml⁻¹ NC-2300 treatment (n = 6). The data are reported as mean thickness (μm) ± SD, **,** p < 0.001. Tukey test.
and arrowheads in Fig. 2A bottom cells, were strongly positive for CTSK (arrows in Fig. 2A). In particular, the dermal-infiltrating cells, including CD11c+ that the expression of CTSK was increased in the epidermis and of anti-IL17A, anti-IL12/23p40, or anti-IL23p19 Abs. We found lesion demonstrated cytokine and antimicrobial profiles that closely We previously established the psoriasis-like skin lesion in the ear swelling over time (Fig. 2B) and attenuated the epidermal the psoriasis-like lesions in K5.Stat3C mice (15, 16). Notably, topical treatment with NC-2300 dose dependently reduced the TPA-induced Th17 cell activation. Upon TPA stimulation, Th17 cells were increased skin-draining lymph nodes, but this increase in Th17 cells was reversed by NC-2300 treatment (Fig. 3B, 3C). These results imply that CTSK might facilitate the Th17 polarization/activation induced by topical TPA treatment.

**NC-2300 downregulates IL-12/IL-23 transcripts and decreases IL-23-producing DCs in K5.Stat3C mice**

Because IL-23 stimulation is essential for Th17 development, we next studied the effect of NC-2300 on IL-23 expression in TPA-induced psoriasis-like lesions. We have previously demonstrated that TPA-induced skin lesions in K5.Stat3C mice have increased mRNA transcript levels of both subunits of IL-23, namely IL-12/23p40 and IL-23p19 (Il12b and Il23a, respectively) (Fig. 3A) (16). The expression of Il12a and Il23a mRNAs was significantly inhibited by topical treatment with NC-2300. This result suggests a role for CTSK in facilitating IL-12/IL-23 production in the skin lesions. Because the psoriasis-like lesions did not show increased IFN-γ gene expression, Th1 skewing did not occur in K5.Stat3C mice (16). In psoriasis, inflammatory mDCs have been identified as Tip-DCs (TNF-α and inducible NO synthase–producing DCs) that produce IL-23, contributing to Th17 cell differentiation and proliferation (5, 21). Similar to human psoriasis, there were increased numbers of CD11c+ cells that produce IL-23 in the dermis of TPA-induced lesions in K5.Stat3C mice (Fig. 4B, 4C). Notably, IL-23–producing CD11c+ cells were significantly decreased in the dermis by topical treatment with NC-2300 (Fig. 4B, 4D). This result suggests that the impairment in Th17 induction by CTSK inactivation might be, at least in part, attributed to a decrease of IL-23–producing mDCs that accumulate in the lesions.

**TLR7/8 ligand induces IL-12 and IL-23 expression in bone marrow–derived DCs, and this effect is inhibited by NC-2300**

It has been shown that the cathelicidin antimicrobial peptide LL37, which is produced by keratinocytes, is a key factor that mediates pDC activation in psoriasis (6). LL37 binds to self-DNA fragments that are released by stressed or dying cells in the skin, which then trigger pDC activation through TLR9, resulting in production of IFN-α and activation of the adaptive immune response toward psoriasis. Self-RNA-LL37 complexes not only induce IFN-α production by pDCs through TLR7, but also trigger mDC maturation and activation through TLR8, leading to cytokine production and upregulation of CD80 and CD86 expression (7). Furthermore, self-RNA-LL37 complexes are associated with maturation of mDCs through activation of TLR5 in psoriatic skin (7). We next examined whether NC-2300 affected TLR activation within mDCs. TLR7/8 ligands, imiquimod, and R848 induced transcriptional upregulation of all subunits of IL-12 and IL-23 in

---

**FIGURE 3.** Effect of NC-2300 (10ng mL−1) on Th17 cytokines in the psoriasis-like lesions. (A) The mRNA expression levels of Th17 cytokines in the lesions. The values are shown by mean mRNA levels relative to Hprt transcripts ± SD. *p < 0.05, **p < 0.01, n = 4–10 per group. Tukey-Kramer test. (B) Intracellular expression of Th17 cells in skin-draining cervical lymph nodes. Representative data are shown from three independent experiments. Number represents percentages of Th17 cells. (C) Bar graphs represent the frequency of Th17 cells in the cervical lymph nodes. The data are shown as mean ± SD. *p < 0.05, n = 3. Tukey test.

**Topical treatment with NC-2300 inhibits the development of the psoriasis-like lesions in K5.Stat3C mice**

We previously established the psoriasis-like skin lesion in the ear of K5.Stat3C mouse with short-term TPA treatment (16). This lesion demonstrated cytokine and antimicrobial profiles that closely resembled human psoriatic lesions, and sensitive to administration of anti-IL17A, anti-IL12/23p40, or anti-IL23p19 Abs. We found that the expression of CTSK was increased in the epidermis and dermis of K5.Stat3C mice in a dose-dependent manner. The same trend of inhibitory effect of NC-2300 on the enzyme activity was obtained in those from wild-type mice (Fig. 1B).

**Effect of NC-2300 on Th17 cell activation in TPA-treated skin of K5.Stat3C mice**

Th17 cells have been shown to play a crucial role in psoriasis (5). Similarly, the skin lesions of K5.Stat3C mice depend on activation of the IL-23/Th17 axis, and the inflamed skin has Th17 cell infiltrates (16). We examined whether NC-2300 affects Th17 cytokine mRNA levels in the psoriasis-like lesions in TPA-treated K5.Stat3C mice. TPA treatment induced the transcript levels of Il17a, Il17f, and Il22 in the skin of K5.Stat3C mice, indicating the activation of Th17 cells (Fig. 3A). Topical application of NC-2300 tended to reduce expression levels of those Th17 cytokine genes, suggesting that CTSK activity might be required for TPA-induced Th17 activation. Upon TPA stimulation, Th17 cells were increased in skin-draining lymph nodes, but this increase in Th17 cells was reversed by NC-2300 treatment (Fig. 3B, 3C). These results imply that CTSK might facilitate the Th17 polarization/activation induced by topical TPA treatment.

---

**Downloaded from [http://www.jimmunol.org/](http://www.jimmunol.org/) on April 16, 2017**
mDCs most likely through activation of TLR7, because TLR8 is nonfunctional in mice (Fig. 5A, 5B). However, pretreatment with NC-2300 inhibited the transcriptional activation of these genes induced by imiquimod or R848. In addition, ELISA revealed that imiquimod-induced production of IL-12/23p40 and IL-23p19 by mDCs was significantly inhibited by NC-2300 (Fig. 5C). Taken together, the impaired induction of Th17 cells and the resulting attenuation of TPA-induced psoriasis-like lesions by CTSK inactivation are caused, at least in part, by downregulation of TLR7-dependent production of IL-23 from mDCs.

Discussion

CTSs are members of the lysosomal cysteine protease family and show strong collagenolytic and elastolytic activities. CTSK is expressed by osteoclasts, where it mediates bone resorption (22). In this regard, CTSK has been considered to be a pharmacological target for the treatment of osteoporosis (23). CTSK is also expressed by fibroblasts in keloids and in dermatofibromas (24). Recent studies demonstrated that stromal CTSK is involved in the invasive nature of cancers, such as squamous cell carcinoma, Paget disease, and melanoma, presumably through its ability to promote matrix degradation. Furthermore, it has been demonstrated that CTSK also participated in adjuvant-induced arthritis in rat (13).

In the current study, we found that Ctsk was upregulated in the psoriatic lesions compared with uninvolved skin and normal control skin. This is the case with the skin of K5.Stat3C mice, a mouse model of psoriasis. Similar to psoriatic epidermis, in which Stat3 is activated, K5.Stat3C mice express constitutively activated Stat3 in the epidermis (14). Previous studies demonstrated that Stat3 directly suppresses cystatin C, which is an endogenous inhibitor of cysteine proteases (25), and that expression of cystatin C is detected in keratinocytes (26). The absence of cystatin C in K14-HPV16 transgenic mice enhances epidermal dysplasia and increases the expression and activities of CTSs in the skin (27). It might be speculated that cystatin C levels would be reduced in keratinocytes in psoriatic lesions as well as those in K5.Stat3C mice, and thereby the CTSK activity might be enhanced. Notably, CTSK activities were higher not only in the epidermis, but in the dermis of K5.Stat3C mice than those in wild-type mice. This might be attributed to an indirect influence from Stat3C-expressing keratinocytes. Topical application of NC-2300 indeed reduces CTSK activity in the epidermis and the dermis. Immunohistochemical study revealed CTSK expression was upregulated in the infiltrating cells, including the CD11c+ cells, in the dermis as well as the epidermal keratinocytes when topically treated with TPA, suggesting that an increased CTSK expression in the skin is associated with psoriasis-like change.

NC-2300 ameliorates adjuvant-induced arthritis in rats, which represents an animal model for rheumatoid arthritis (13). Furthermore, CTSK-deficient mice are resistant to experimental autoimmune encephalomyelitis, in which Th17 cells play an important role (13, 28). Th17 cytokines also play a critical role in the immunopathogenesis of psoriasis and in TPA-induced psoriasis-like lesions in K5.Stat3C mice (16). Treatment with NC-2300 attenuated psoriasis-like lesions, and simultaneous downregulation of Il17a, Il17f, Il12a, and Il23a occurred. The decrease in IL-23 transcripts in the skin is in part due to the reduced recruitment of IL-23–producing DCs. It should be noted that IFN-γ is not upregulated in psoriasis-like lesions, whereas Th17 cytokines are highly upregulated (16). Therefore, Th17 cells have predominant roles over Th1 cells in developing skin lesions, suggesting that the IL-23/Th17 axis is one of the targets of NC-2300 in our experimental setting. We consider that NC-2300 inhibited the early phase of lesion development, whereas the chronic, persistent lesions were resistant to topical NC-2300 (data not shown). This result may suggest the presence of an opportunistic window for the treatment with NC-2300, because the dermal inflammation is less prominent in the chronic lesions of this mouse model. In this regard, the therapeutic effect of NC-2300 is required to validate using more relevant models, such as the SCID-hu xenogeneic transplantation model (1).

---

**FIGURE 4.** Topical treatment of NC-2300 (10mg ml−1) suppressed IL-23 production in the psoriasis-like lesions. (A) Expression of Il12a, Il12b, and Il23a mRNAs in the lesions. The data are shown as mean ± SD, *p < 0.05, n = 4–10 per group. Tukey-Kramer test. (B and C) Immunostaining of the ear skin of K5.Stat3C mice treated with TPA demonstrated IL-23–producing CD11c+ cells, which were stained with anti–IL-23p19 (red), anti-CD11c (green), and DAPI. Rectangular area in (B) is shown at a higher magnification in (C). Arrowheads indicate IL-23p19+CD11c+ cells. Scale bars, 100 μm (B), 20 μm (C). (D) The number of IL-23p19+CD11c+ was counted in the ears of K5.Stat3 mice treated with vehicle alone (white bar, n = 6), TPA (black bar, n = 6), and TPA plus NC-2300 (gray bar, n = 6). NC-2300 treatment significantly reduced the number of IL-23p19+CD11c+ cells. The data represent mean ± SD, **p < 0.001. Tukey test.**
lesions compared with uninvolved skin or normal healthy skin (6). Moreover, LL37 forms complexes with self-DNA and mediates pDC activation through the TLR9 pathway that might drive the autoimmunity in psoriasis. LL37 also forms complexes with self-RNA, by which TLR8-dependent mDC activation occurs in psoriatic skin lesions (7). We in this study demonstrate that triggering with TLR7/8 ligands upregulates IL-12 and IL-23 in mDCs. Because TLR8 is nonfunctional in mice, this result suggests that TLR7 activation in mDCs contributes to upregulation of IL-23. Recombinant CTSs and asparagine endopeptidase (AEP) could cleave TLR7 or TLR9, leading to TLR signaling (33, 34, 35). The proteolysis of TLR7 and TLR9 occurs in a multistep fashion. The first step includes a processing of the TLR ectodomain by AEP and CTSs. However, NC-2300 did not affect the AEP activity (data not shown), suggesting that NC-2300 prevents CTSK from TLR cleavage. We do not exclude the possibility that species of CTSs other than CTSK could also be pharmacological targets of NC-2300, in particular, at higher concentrations (13).

Psoriasis is one of the most common skin diseases and is considered to develop through the crosstalk between the epidermal keratinocytes and immunocytes. Recently developed biologics are the most promising therapeutics for psoriasis. They include TNF inhibitors and anti–IL-12/23p40 Abs (ustekinumab), both of which target the IL-23/Th17 axis (36). The trend for anticytokine therapies depends on the development of Abs to more specific components of this axis, including IL-23p19, IL-17, IL-17R, and IL-22 (37). However, there is a risk for systemic adverse events with anticytokine therapies, including sepsis, opportunistic infections, and reactivation of latent tuberculosis. Whereas inhibitors of CTSK have been used in studies of patients with osteopenia/osteoporosis, they might also be applicable for the treatment of rheumatoid arthritis because of its inhibitory effect on Th17 activation (13). The current study using a psoriasis mouse model revealed that NC-2300 efficiently penetrated the skin and exerted an antipsoriatic effect through downregulation of TLR7/TLR23/Th17 cascade. Thus, topical targeting CTSK by specific inhibitors might be an alternative therapy, with less risk of systemic adverse reactions, for psoriasis.

Acknowledgments
We thank Drs. M. Tarutani and T. Yamakawa for discussion and technical assistance. We are grateful to Nippon Chemiphar for generously providing the NC-2300.

Disclosures
The authors have no financial conflicts of interest.

References

**FIGURE 5.** Effect of NC-2300 on IL-12 and IL-23 in bone marrow–derived mDCs after treatment of TLR7/8 ligands. (A) The mDCs were pretreated with vehicle alone or 10 μM NC-2300 for 3 h prior to stimulation with 10 nM R848 for 24 h at 37°C. Treatment of R848-induced transcripts of Il12a, Il12b, and Il23a, all of which were inhibited by pretreatment with NC-2300. The data are shown as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, n = 6. Tukey test. (B) R848 was replaced with imiquimod (IMQ, 10 μM) under the same experimental protocol. The results were mostly reproduced when IMQ was used. The data are shown as mean ± SD, *p < 0.01, ***p < 0.001, n = 6. Tukey test. (C) The mDCs were pretreated with vehicle alone or 1, 10, and 30 μM NC-2300 for 3 h prior to stimulation with 10 μM IMQ for 24 h at 37°C. The data represent mean ± SD, **p < 0.01 versus vehicle-treated mDCs; *p < 0.05, **p < 0.01 versus IMQ-treated mDCs, n = 5. Tukey test.

The ability of cathelicidin to function as an antimicrobial peptide is required for optimal host defense against infection (29, 30). A lack of cathelicidin antimicrobial peptides has been associated with increased microbial growth both in mouse models and in vitro cell cultures (29, 30). Furthermore, recent in vitro studies indicate that cathelicidin also modulates innate immunity. Deficiencies in cathelicidin and/or other antimicrobial peptides correlate with increased opportunities of infection in patients with atopic dermatitis or Kostman syndrome (31, 32). LL37, an active peptide of cathelicidin, is highly expressed in psoriatic-involved...
Corrections


There was an error in the data of Fig. 5 as published. Data shown in Fig. 5A as *Il12a* gene expression was duplicated from the data for *Il23a*. The correct data for *Il12a* gene expression is now shown in the revised Fig. 5 below. The figure legend was correct as published and is shown below for reference. This error does not change the conclusion of the paper.

**FIGURE 5.** Effect of NC-2300 on IL-12 and IL-23 in bone marrow–derived mDCs after treatment of TLR7/8 ligands. (A) The mDCs were pretreated with vehicle alone or 10 μM NC-2300 for 3 h prior to stimulation with 10 nM R848 for 24 h at 37˚C. Treatment of R848-induced transcripts of *Il12a, Il12b,* and *Il23a,* all of which were inhibited by pretreatment with NC-2300. The data are shown as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, n = 6. Tukey test. (B) R848 was replaced with imiquimod (IMQ, 10 μM) under the same experimental protocol. The results were mostly reproduced when IMQ was used. The data are shown as mean ± SD, **p < 0.01, ***p < 0.001, n = 6. Tukey test. (C) The mDCs were pretreated with vehicle alone or 1, 10, and 30 μM NC-2300 for 3 h prior to stimulation with 10 μM IMQ for 24 h at 37˚C. The data represent mean ± SD, ###p < 0.001 versus vehicle-treated mDCs; *p < 0.05, **p < 0.01 versus IMQ-treated mDCs, n = 5. Tukey test.