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

Toshitake Hirai,* Takashi Kanda,* Kenji Sato,* Mikiro 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 Ctss 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-12, IL-23, and Th17 cytokines. In vitro experiments revealed that TLR7 activation of bone marrow–derived myeloid dendritic cells led to increase in 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).

In the majority of human psoriatic lesions (14), K5.Stat3C transgenic mice, in which Stat3 is constitutively active in keratinocytes, develop psoriasis-like lesions following wounding stimuli or topical treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), which strongly suggests that Stat3 activation is required for the development of psoriasis. Furthermore, topical treatment with a Stat3 inhibitor attenuates human psoriatic plaques as well as the development of psoriasis-like lesions in TPA-treated K5.Stat3C mice (15). Human psoriatic lesions and TPA-induced psoriasis-like lesions in K5.Stat3C mice both show increased transcript levels for IL-23, IL-17A, IL-17F, and IL-22, which all belong to the IL-23/Th17 pathway. Furthermore, psoriatic lesions in human and TPA-induced psoriasis-like lesions in K5.Stat3C mice are sensitive to administration of anti–IL-12/23p40 or anti–IL-23p19 Abs (16), suggesting an essential role for the IL-23/Th17 axis.
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’s Declaration of Helsinki and was approved by the Institute Ethical Review Board of the Kochi Medical School, Kochi University. Skin biopsy specimens were taken from lesions and from contiguous uninvolved skins of 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'-TTCAAGGATGGTTTGAAGTG-3' and 5'-CAATAAGAGATATTCCACGCA-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 on the CTSK activity in skin, we topical treated K5.Stat3C mouse onto the ear skin with either 20 μl acetone on the right ear of each mouse, and with 20 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). PCRs

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. Immunohistochemical staining was performed with serial sections. Paraffin 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 with rabbit anti-CTSK polyclonal Ab (BioVision). Sequentially, 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 CD11 (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.

FIGURE 1. CTS expression in psoriatic skin and CTSK activity in K5.Stat3C mouse. (A) Quantitative mRNA expression of CtsK, CtsB, CtsL, and CtsS of 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 increases indicated by triangles). The data represent mean ± SD, #p < 0.01 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′-GCCAGACAAAGATTCCATC-3′ and antisense, 5′-CACAGGAAAGCTACCAGAG-3′; human Ctb, sense, 5′-TGAGGGAGCTTTTCTTCTGTG-3′ and antisense, 5′-TGACGTGTGTGTTACACTCTTGTG-3′; human Cts, sense, 5′-TCAGAGCTTGCAGCAGAGCAGA-CC-3′ and antisense, 5′-GGGAGGCGGTGGGAACCTCA-3′; and human Ctsl, sense, 5′-GGGAGGCGGTGGGAAGGAC-3′ and antisense, 5′-GCCAGGACATTCCAGAG-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 (14). The ear skin of K5.Stat3C mice was treated with acetone (open circles, which were both positive for CTSK (red) and CD11c (green). Scale bars, 50 μm.

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).

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 Cts 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 Cts 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

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.

Fig. 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 control epidermis. Arrows indicate inflammatory cells. Asterisks, unspecific staining in the cornified layer. Immunofluorescence double staining of psoriasis-like lesion with anti-CD11c and anti-CTS K 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 topicaly 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+ of anti-IL17A, anti-IL12/23p40, or anti-IL23p19 Abs. We found resembled human psoriatic lesions, and sensitive to administration lesion demonstrated cytokine and antimicrobial profiles that closely of K5.Stat3C mouse with short-term TPA treatment (16). This psoriasis-like lesions in 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.

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 (Fig. 4A) (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 TLR8 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
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

CTSSs 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 mDCs model of psoriasis. Similar to psoriatic epidermis, CTSK is 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).

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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 CTSSs. 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 CTSSs 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/IL-23/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 experimental compounds. We thank Drs. M. Tarutani and T. Yamakawa for discussion and technical assistance. We are grateful to Nippon Chemiphar for generously providing experimental compounds.

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. The data are shown for 3 h prior to stimulation with 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 CTSSs. 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 CTSSs other than CTSK could also be pharmacological targets of NC-2300, in particular, at higher concentrations (13).

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Disclosures
The authors have no financial conflicts of interest.

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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.