

Cathepsin G cleaves and activates IL-36 γ and promotes the inflammation of psoriasis

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Background: IL-36 γ is considered to be a valuable biomarker in psoriatic patients, which is expressed as an inactive precursor that needs to be proteolytically processed and activated, and neutrophil-derived proteases seemed to be potent activating enzymes of IL-36 γ .

Objectives: This study aims to investigate the activation of IL-36 γ by cathepsin G (CG) and neutrophil elastase (NE).

Materials and methods: We used inactive recombinant full-length (FL)-IL-36 γ with different doses of NE or CG to stimulate HaCaT cells; neutrophil extracellular traps (NETs) were prepared to act on FL-IL-36 γ and then stimulate HaCaT cells. Real-time quantitative PCR and ELISA were performed to detect CXCL-1 and CXCL-8 expression. We developed imiquimod-induced psoriasis-like mouse model to evaluate the effect of hypodermic injection of neutrophil-derived protease or its inhibitor. Histopathology and Western blotting were conducted for effect assessment.

Results: Purified CG cleaved and activated recombinant human FL-IL-36 γ to promote CXCL-1 and CXCL-8 expression by human keratinocytes, and NETs activated FL-IL-36 γ and the activation was inhibited by serpin A3. CG induced expression of a more truncated IL-36 γ in psoriasisiform lesion of mice and aggravated the psoriasis-like lesion induced by imiquimod, whereas recombinant serpin A3 alleviated the severity of the psoriasis-like mouse mode.

Conclusion: CG has the ability to cleave and activate IL-36 γ and aggravate imiquimod-induced mouse psoriasisiform lesion. Thus, CG-specific inhibitors might be promising therapeutic drugs for psoriasis.

Keywords: psoriasis, IL-36, neutrophil, cathepsin, elastase

Introduction

The vital role of IL-36 in the pathogenesis of psoriasis including psoriasis vulgaris (PV) and pustular psoriasis, especially generalized pustular psoriasis (GPP), has been extensively elucidated in recent years.^{1,2} IL-36 cytokines include three receptor agonists (IL-36 α , IL-36 β , IL-36 γ) and a receptor antagonist (IL-36Ra), which use the same receptor complex IL-36R (IL-1Rrp2)/IL-1RAcP. In skin, IL-36 is predominantly expressed in epidermal keratinocytes,³ and IL-36R is widely expressed by keratinocytes and antigen presenting cells (APCs).^{4,5} IL-36 induces the expression of proinflammatory cytokines and chemokines by keratinocytes and drives APC activation to promote psoriatic inflammation.⁵

Both mRNA and protein expressions of all IL-36 cytokines were significantly higher in human PV lesions compared with healthy skin, where the expression of IL-36 β was far weaker than IL-36 α and IL-36 γ in PV.^{6,7} Low-level IL-36 β and increased expression of IL-36 α , IL-36 γ , and IL-36Ra were also seen in GPP lesions, and significantly greater expression of IL-36 α and IL-36 γ were found in GPP compared with PV lesions.²

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As an IL-36R agonist, IL-36 γ is now considered to be a valuable biomarker in psoriatic patients, which correlates closely with disease activity.⁸ Similar to IL-36 α and IL-36 β , IL-36 γ is expressed as an inactive precursor, which needs to be proteolytically processed and activated, and neutrophil-derived proteases seemed to be potent activating enzymes of IL-36 γ .⁹ Henry et al reported neutrophil elastase (NE) could cleave recombinant full-length (FL)-IL-36 γ and activate the protein;¹⁰ however, Johnston et al found cathepsin G (CG) but not NE had the ability to activate IL-36 γ in vitro, and NE could cleave FL-IL-36 α .²

This study aims to further investigate the potential of CG and NE to activate IL-36 γ in vitro and vivo.

Materials and methods

Study design

Human keratinocyte line HaCaT cells were purchased from KeyGEN BioTECH (Nanjing, China) and cultured as previously described¹¹ and seeded in 6-well culture plates and divided into 15 groups as follows: control, 100 ng/mL recombinant human FL-IL-36 γ (2320-IL-025, R&D Systems, Inc., Minneapolis, MN, USA), 100 ng/mL recombinant human truncated (T)-IL-36 γ (6835-IL-010, R&D Systems, Inc.), 10 ng/mL recombinant human NE (R&D Systems, Inc.), 10 ng/mL NE + FL-IL-36 γ , 100 ng/mL NE, 100 ng/mL NE + FL-IL-36 γ , 1 μ g/mL NE, 1 μ g/mL NE + FL-IL-36 γ , 10 ng/mL purified human CG (Enzo Life Sciences, Inc., Ann Arbor, MI, USA), 10 ng/mL CG + FL-IL-36 γ , 100 ng/mL CG, 100 ng/mL CG + FL-IL-36 γ , 1 μ g/mL CG, 1 μ g/mL CG + FL-IL-36 γ . The cells were incubated for 24 hours, and then prepared for real-time quantitative PCR and ELISA. Experiments were repeated independently at least three times. Recombinant FL-IL-36 γ was incubated with a neutrophil-derived protease in PBS for 1 hour at 37°C and then prepared for Western blotting.

Neutrophils were isolated from peripheral blood from healthy volunteers using ficoll and 3% dextran as described.¹² Neutrophil extracellular traps (NETs) were generated by stimulating one million neutrophils in 0.5 mL RPMI without FBS with 20 nmol/L phorbol 12-myristate 13-acetate for 3 hours at 37°C.² After washing with PBS for three times, 1 mL of 100 ng/mL FL-IL-36 γ and/or 1 μ g/mL recombinant serpin A3 (Enzo Life Sciences, Inc.) in PBS were incubated with the NETs for 1 hour at 37°C. The processed solutions were added to cultured HaCaT cells in 12-well culture plates for 24 hours. T-IL-36 γ at 100 ng/mL was used as positive control. Real-time quantitative PCR was subsequently performed.

Thirty female BALB/c mice (6–8 weeks old) were purchased from animal core facility of Nanjing Medical University and kept under specific pathogen-free conditions. The mice were fed with standard laboratory water and food ad libitum and kept on a 12-hour light–dark cycle. All mice were randomly divided into six groups of five mice each as follow: control, imiquimod (Aldara, 3M Health Care Limited, Loughborough, UK), imiquimod + CG, imiquimod + serpin A3, CG, and serpin A3. Topical imiquimod 5% (31.25 mg) was applied on the right dorsal skin, and/or multipoint hypodermic injection with 0.1 mL PBS containing CG (200 ng) or serpin A3 (500 ng) was administered on the right dorsal skin daily. After being treated for 6 days, the mice were killed by cervical dislocation at Day 7, and right dorsal skin from each mouse was collected for Western blotting, and formalin fixed and paraffin embedded for H&E staining. The thickness of mouse epidermis was measured using Olympus cellSens, version 1.16.

Real-time quantitative PCR

After 24 hours of incubation, the supernatant was collected and prepared for ELISA, and the HaCaT cells attached to the culture plates were washed three times using PBS and then lysed in TRIzol (Zoonbio Biotechnology, Nanjing, Jiangsu, China), and total RNA was isolated. First-strand cDNA was synthesized from 2 μ g of total RNA using Prime Script II First Strand cDNA Synthesis Kit (Takara Biotechnology (Dalian) Co. Ltd., Dalian, Liaoning, China) using Oligo dT Primer. PCR amplification was performed in a total volume of 20 μ L containing 1 μ L template cDNA and 10 μ L SYBR Premix Ex Taq (SYBR Green) (Takara Biotechnology (Dalian) Co. Ltd.), and transcripts were quantified using StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA).¹³ All values were normalized to the expression of β -actin. Primer sequences are shown in Table 1.

ELISA

Measurement of secretory protein in supernatant was performed using CXCL-1 ELISA Kit (CUSABIO, Wuhan, Hubei, China) and CXCL-8 ELISA Kit (Proteintech, Wuhan, Hubei, China). This assay employs the quantitative sandwich enzyme immunoassay technique.

Western blotting

We incubated 20 μ g recombinant human FL-IL-36 γ with 20 μ g CG in 5 mL PBS for 1 hour at 37°C, and then protein concentration in PBS was measured using the Bradford method, and SDS-PAGE performed. The primary antibody was anti-IL-36 γ antibody (R&D Systems, Inc.).

Table 1 Primers for the target genes in real-time quantitative PCR

Target genes	Primers
β -actin (103 bp)	Sense: 5'-CATTCCAAATATGAGATGCGTTGT-3' Antisense: 5'-TGTGGACTTGGGAGAGGACT-3'
CXCL-1 (173 bp)	Sense: 5'-TGCTGCTCCTGCTCCTGGTA-3' Antisense: 5'-TGTGGCTATGACTTCGGTTTGG-3'
CXCL-8 (236 bp)	Sense: 5'-TGGCAGCCTTCCTGATTT-3' Antisense: 5'-AACCTCTGCACCCAGTT-3'

The collected mouse dorsal skin was homogenized in cold lysis buffer containing protease inhibitor. Centrifugal separation was conducted at 4°C, at 14,000 rpm for 15 minutes. The upper layer of the solution was tested for protein as above mentioned. The primary antibody was added as below: anti-IL-36 γ antibody (Cloud-Clone Corp., Wuhan, Hubei, China) and anti- β -actin antibody (Boster Biological Technology Co., Ltd, Wuhan, Hubei, China), following the manufacturer's instructions. Gel-Pro 32 (Media Cybernetics, Rockville, MD, USA) was used to detect protein expression.

Statistical analyses

All data were analyzed using GraphPad Prism for Windows (GraphPad Software, San Diego, CA, USA) and presented as mean \pm SD. Statistical significance was calculated using a Student's *t*-test, Mann-Whitney *U*-test, or Friedman's test, as appropriate.¹³ $P < 0.05$ was defined as statistical significance.

Ethics statement

This study was carried out in accordance with the recommendations of institutional guidelines and Local Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Healthy volunteers were recruited for blood draws for neutrophil isolation and all subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocols including animal experiment were approved by the Local Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. The institutional guidelines of the Animal Care and Use of Nanjing Medical University were followed for the welfare of the animals.

Results

Purified CG cleaves and activates recombinant human full-length-IL-36 γ

We incubated recombinant FL-IL-36 γ with different doses of purified CG or recombinant NE to stimulate HaCaT cells, and we found 100 ng/mL FL-IL-36 γ alone had low activity to stimulate

HaCaT cells, whereas 100 ng/mL CG used with FL-IL-36 γ had significant synergistic effect on CXCL-1 and CXCL-8 mRNA expression in HaCaT cells ($P < 0.05$; Figure 1A), which was confirmed at the protein level by ELISA analysis of supernatant ($P < 0.001$; Figure 1B). T-IL-36 γ had significantly higher activity compared with FL-IL-36 γ ($P < 0.05$). Either CG or NE alone activated HaCaT cells to varying degrees (Figure 1A). Western blot showed purified CG could cleave FL-IL-36 γ from 18.7 to 17 KDa (Figure 1C).

NETs activate full-length-IL-36 γ and the activation is inhibited by serpin A3

The DAPI staining of DNA confirmed the formation of NETs under fluorescent microscopy (Figure 2A and B). Exposure to NETs for 1 hour significantly increased the activity of FL-IL-36 γ to induce CXCL-1 and CXCL-8 mRNA expression by HaCaT cells in comparison with untreated FL-IL-36 γ ($P < 0.01$). Serpin A3, a specific inhibitor of CG, inhibited significantly the action of NETs on FL-IL-36 γ ($P < 0.01$; Figure 2C).

Purified CG aggravates psoriasis-like lesion induced by imiquimod whereas recombinant serpin A3 alleviates the severity of psoriasis-like mouse model

Topical imiquimod successfully induced psoriasis-like mouse model, and hypodermic injection of purified CG or recombinant serpin A3 alone had no effect on skin appearance of mice (Figure 3A). Imiquimod used with CG induced severer psoriasis-like lesion compared with imiquimod alone, whereas serpin A3 relieved the psoriasiform lesion induced by imiquimod (Figure 3A).

Imiquimod-induced psoriasis-like lesion had thicker epidermis and more dermal inflammatory cells infiltration than control group, whereas imiquimod used with CG induced more obvious change in both epidermis thickness and dermal cell infiltration compared to the imiquimod group; by contrast, serpin A3 alleviated the imiquimod-induced skin pathological changes in mice (Figure 3B and C).

Western blotting showed that CG promoted but serpin A3 inhibited the expression of truncated IL-36 γ (16 KDa) in imiquimod-induced psoriasis-like lesion in mice ($P < 0.05$; Figure 4A and B).

Discussion

The IL-36 cytokines are involved in the pathogenesis of psoriasis where IL-36 can act on keratinocytes and immune cells to induce a powerful inflammatory response.¹⁴ IL-36

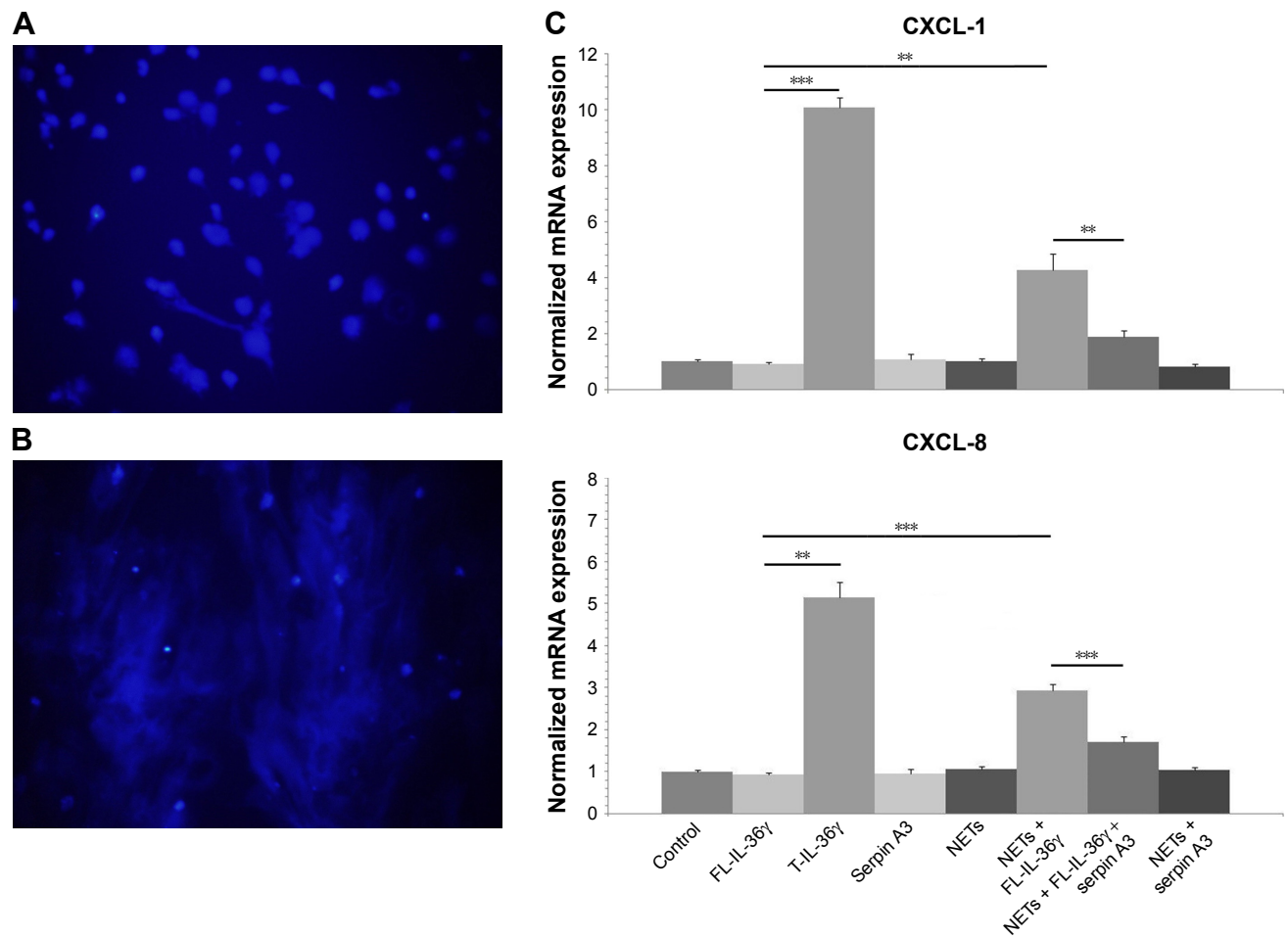


Figure 2 DAPI staining of DNA, and real-time quantitative PCR detecting mRNA expression of CXCL-1 and CXCL-8.
Notes: (A) DAPI staining shows neutrophils without PMA stimulation, original magnification $\times 200$. (B) DAPI staining confirms the formation of NETs by stimulating neutrophils with 20 nmol/L PMA, original magnification $\times 200$. (C) NETs increase the activity of FL-IL-36 γ (100 ng/mL) to induce significant CXCL-1 and CXCL-8 mRNA expression by HaCaT cells, whereas serpin A3 (1 μ g/mL) can inhibit significantly the action of NETs on FL-IL-36 γ . T-IL-36 at 100 ng/mL as positive control for IL-36 activity. The normalized data are from representative experiment conducted in triplicate. Statistical significance indicated: ** $P < 0.01$, *** $P < 0.001$.
Abbreviations: FL-IL-36 γ , full-length-IL-36 γ ; NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate 13-acetate; T-IL-36, truncated IL-36 γ .

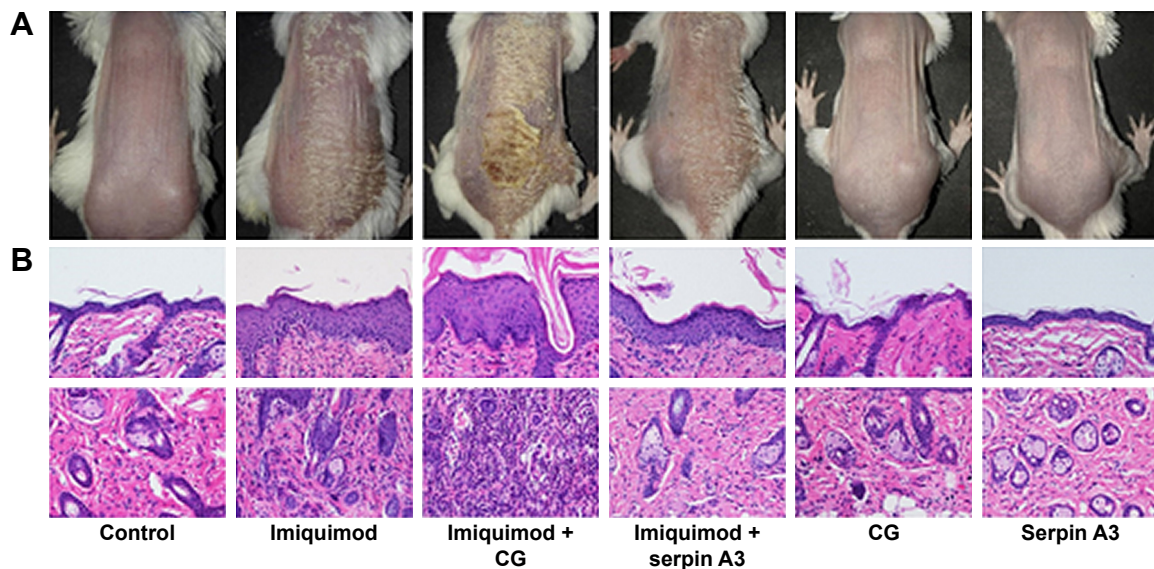


Figure 3 (Continued)

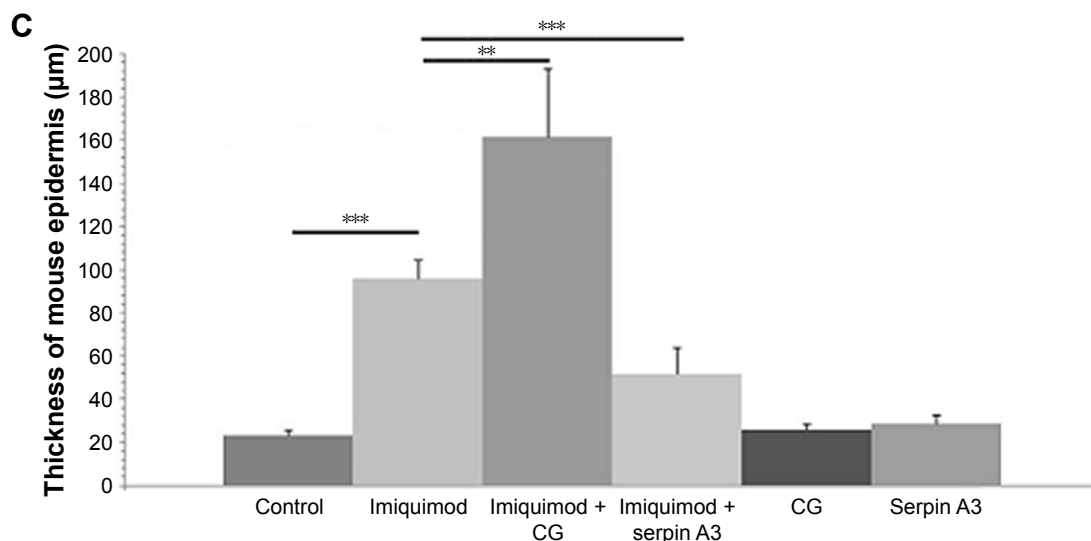


Figure 3 Imiquimod-induced psoriasis-like mouse model, evaluating the effect of hypodermic injection of CG or serpin A3 using histopathology analysis. **Notes:** (A) Topical imiquimod successfully induces psoriasis-like mouse model, and hypodermic injection of CG used with topical imiquimod displays significantly thicker lesion and more scales compared with imiquimod group, whereas serpin A3 relieves the psoriasiform lesion induced by imiquimod. (B) Histopathology shows that imiquimod + CG group has thickest epidermis and maximum inflammatory cells infiltration, whereas imiquimod + serpin A3 group has thinner epidermis and less cells infiltration than imiquimod group, original magnification $\times 200$. (C) The thickness of mouse epidermis was measured, and the results confirm the epidermal histopathological change. Statistical significance indicated: $**P < 0.01$, $***P < 0.001$. **Abbreviation:** CG, cathepsin G.

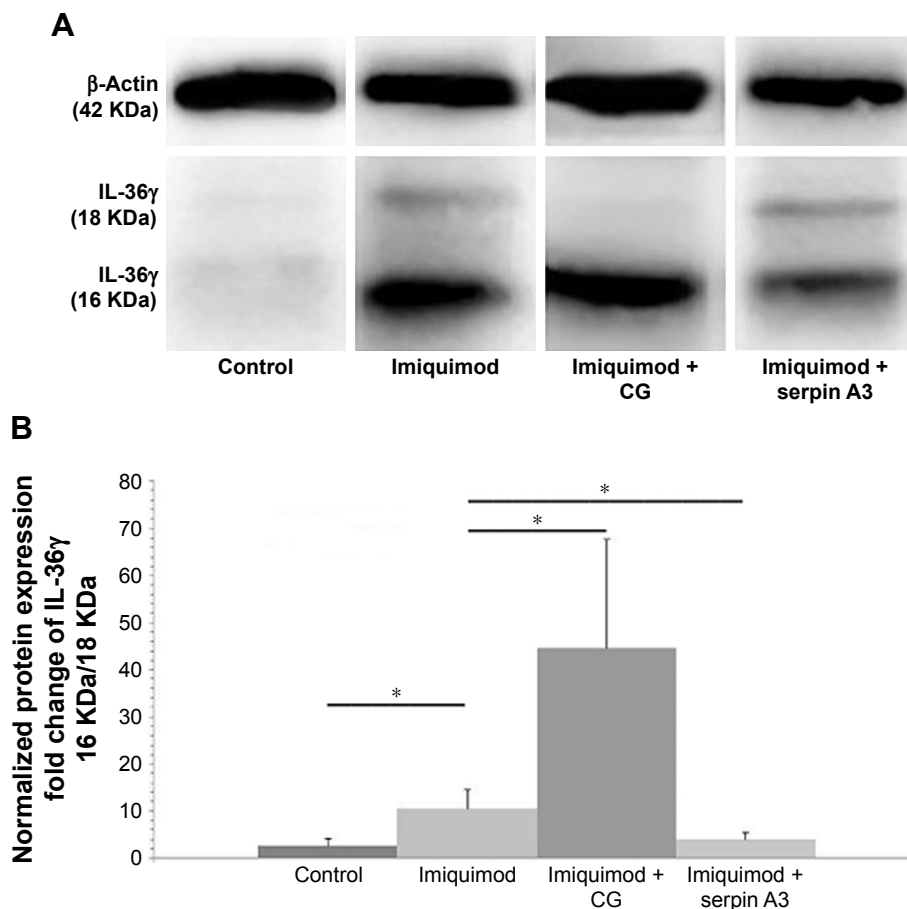


Figure 4 IL-36 γ protein detection by using Western blotting. **Notes:** (A) Western blotting shows that imiquimod + CG group has more significant expression of truncated IL-36 γ (16 KDa) in comparison with imiquimod group, whereas imiquimod + serpin A3 group has lower truncated IL-36 γ expression. (B) Fold change of IL-36 γ protein (16 KDa) to IL-36 γ protein (18 KDa) indicates that imiquimod used with CG has most notable cleaving effect on IL-36 γ . Statistical significance indicated: $*P < 0.05$. **Abbreviation:** CG, cathepsin G.

Most studies of imiquimod-induced lesions are performed using female BALB/c or C57BL/6 (B6) mice, and expression of IL-36 α , IL-36 β , and IL-36 γ was elevated in imiquimod-treated B6 mice and female BALB/c mice,^{23,24} which are both suitable for our study on protease processing of IL-36. Swindell et al reported that imiquimod increased IL-17 cytokine expression in B6 mice but not BALB/c mice;²³ however, other studies showed that IL-17A expression was induced by imiquimod with a faster response in BALB/c mice than B6 mice, and BALB/c lesions develop more rapidly than B6 lesions.^{25,26} Milora et al reported that unprocessed IL-36 α could cooperate with IL-1 α to promote psoriasis-like disease, using an imiquimod-induced mouse model on B6 background.²⁷ Our study showed hypodermic injection of purified CG aggravated imiquimod-induced psoriasis-like lesion in female BALB/c mice, which displayed thicker lesion and more scales in appearance and presented thicker epidermis and more dermal inflammatory cells infiltration in histopathology. Western blotting confirmed that CG could induce more truncated IL-36 γ expression in psoriasiform lesion. Conversely, hypodermic injection of serpin A3 significantly relieved the severity of imiquimod-induced mice lesion.

Conclusion

Taken together, our study has confirmed that CG has the ability to cleave and activate IL-36 γ to promote CXCL-1 and CXCL-8 expression by human keratinocytes, and CG can aggravate imiquimod-induced mouse psoriasiform lesion, and contrarily serpin A3 alleviates the severity of the mouse model. Neutrophil-derived CG proteolytically processes inactive IL-36 γ and then induces neutrophil chemokines secretion from keratinocytes that further recruits surrounding neutrophils, which is a typical amplification cycle very likely involved in the pathogenesis of psoriasis. The specific inhibitors of CG might be promising therapeutic drugs for psoriasis.

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Disclosure

The authors report no conflicts of interest in this work.

References

- Baliwag J, Barnes DH, Johnston A. Cytokines in psoriasis. *Cytokine*. 2015; 73(2):342–350.
- Johnston A, Xing X, Wolterink L, et al. IL-1 and IL-36 are dominant cytokines in generalized pustular psoriasis. *J Allergy Clin Immunol*. 2017;140(1):109–120.
- Gresnigt MS, van de Veerdonk FL. Biology of IL-36 cytokines and their role in disease. *Semin Immunol*. 2013;25(6):458–465.
- Kumar S, McDonnell PC, Lehr R, et al. Identification and initial characterization of four novel members of the interleukin-1 family. *J Biol Chem*. 2000;275(14):10308–10314.
- Foster AM, Baliwag J, Chen CS, et al. IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin. *J Immunol*. 2014;192(12):6053–6061.
- Johnston A, Xing X, Guzman AM, et al. IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. *J Immunol*. 2011;186(4): 2613–2622.
- Li B, Tsoi LC, Swindell WR, et al. Transcriptome analysis of psoriasis in a large case-control sample: RNA-seq provides insights into disease mechanisms. *J Invest Dermatol*. 2014;134(7):1828–1838.
- D'Erme AM, Wilschmann-Theis D, Wagenpfeil J, et al. IL-36 γ (IL-1F9) is a biomarker for psoriasis skin lesions. *J Invest Dermatol*. 2015; 135(4):1025–1032.
- Sullivan GP, Henry CM, Clancy DM, et al. Suppressing IL-36-driven inflammation using peptide pseudosubstrates for neutrophil proteases. *Cell Death Dis*. 2018;9(3):378.
- Henry CM, Sullivan GP, Clancy DM, Afonina IS, Kulms D, Martin SJ. Neutrophil-derived proteases escalate inflammation through activation of IL-36 family cytokines. *Cell Rep*. 2016;14(4):708–722.
- Zhou BR, Zhang JA, Zhang Q, et al. Palmitic acid induces production of proinflammatory cytokines interleukin-6, interleukin-1 β , and tumor necrosis factor- α via a NF- κ B-dependent mechanism in HaCaT keratinocytes. *Mediators Inflamm*. 2013;2013:530429.
- Clark RA, Nauseef WM. Isolation and functional analysis of neutrophils. *Curr Protoc Immunol*. 2001.
- Yin L, Hu Y, Xu J, Guo J, Tu J, Yin Z. Ultraviolet B inhibits IL-17A/TNF- α -stimulated activation of human dermal fibroblasts by decreasing the expression of IL-17RA and IL-17RC on fibroblasts. *Front Immunol*. 2017;8:91.
- Bassoy EY, Towne JE, Gabay C. Regulation and function of interleukin-36 cytokines. *Immunol Rev*. 2018;281(1):169–178.
- Capon F. The genetic basis of psoriasis. *Int J Mol Sci*. 2017;18(12):2526.
- Pfaff CM, Marquardt Y, Fietkau K, Baron JM, Lüscher B. The psoriasis-associated IL-17A induces and cooperates with IL-36 cytokines to control keratinocyte differentiation and function. *Sci Rep*. 2017; 7(1):15631.
- Wang W, Yu X, Wu C, Jin H. IL-36 γ inhibits differentiation and induces inflammation of keratinocyte via Wnt signaling pathway in psoriasis. *Int J Med Sci*. 2017;14(10):1002–1007.
- Towne JE, Garika KE, Renshaw BR, Virca GD, Sims JE. Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF- κ B and MAPKs. *J Biol Chem*. 2004;279(14):13677–13688.
- Berekéri A, Latzko A, Alase A, Macleod T, Ainscough JS, Laws P. Detection of IL-36 γ via non-invasive tape stripping reliably discriminates psoriasis from atopic eczema. *J Allergy Clin Immunol*. 2018;142(3): 988–991.
- Towne JE, Renshaw BR, Douangpanya J, et al. Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36 α , IL-36 β , and IL-36 γ) or antagonist (IL-36Ra) activity. *J Biol Chem*. 2011;286(49): 42594–42602.
- Ainscough JS, Macleod T, McGonagle D, et al. Cathepsin S is the major activator of the psoriasis-associated proinflammatory cytokine IL-36 γ . *Proc Natl Acad Sci U S A*. 2017;114(13):E2748–E2757.
- Sullivan GP, Davidovich PB, Sura-Trueba S, et al. Identification of small-molecule elastase inhibitors as antagonists of IL-36 cytokine activation. *FEBS Open Bio*. 2018;8(5):751–763.
- Swindell WR, Michaels KA, Sutter AJ, et al. Imiquimod has strain-dependent effects in mice and does not uniquely model human psoriasis. *Genome Med*. 2017;9(1):24.

24. Tortola L, Rosenwald E, Abel B, et al. Psoriasiform dermatitis is driven by IL-36-mediated DC-keratinocyte crosstalk. *J Clin Invest*. 2012; 122(11):3965–3976.
25. van der Fits L, Mourits S, Voerman JS, et al. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol*. 2009;182(9):5836–5845.
26. Flutter B, Nestle FO. TLRs to cytokines: mechanistic insights from the imiquimod mouse model of psoriasis. *Eur J Immunol*. 2013;43(12): 3138–3146.
27. Milora KA, Fu H, Dubaz O, Jensen LE. Unprocessed Interleukin-36 α regulates psoriasis-like skin inflammation in cooperation with interleukin-1. *J Invest Dermatol*. 2015;135(12):2992–3000.

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