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Title: Tyk2 is a therapeutic target for psoriasis-like skin inflammation

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Abstract

Tyrosine kinase 2 (Tyk2), a member of the Jak kinase family, mediates signals triggered by various cytokines, which are related to the pathogenesis of psoriasis. In the present study, we investigated the role of Tyk2 in IL-23-induced psoriasis-like skin inflammation. Tyk2^{-/-} mice when injected with IL-23 showed significantly reduced ear skin swelling with epidermal hyperplasia and inflammatory cell infiltration compared to wild type mice. In addition, Tyk2-deficiency reduced production of proinflammatory cytokines and psoriasis-relevant antimicrobial peptides. More noteworthy is that Tyk2 directly regulated IL-22-dependent inflammation and epidermal hyperplasia. Taken together with the inhibition of IL-23-induced inflammation by treatment with neutralizing antibodies against IL-17 or IL-22, Tyk2 participates in both IL-23- and IL-22-signal transductions to mediate psoriasis-like skin inflammation. Based on these findings, we demonstrated for the first time that a small molecule Tyk2 inhibitor significantly inhibited IL-23-induced inflammation and cytokine production in the skin. These observations demonstrate the important role of Tyk2 in experimental skin inflammation and indicate the therapeutic potential of Tyk2 inhibition in human psoriasis.

Keywords: Psoriasis; Tyk2; IL-17; IL-22; IL-23

Introduction

Tyrosine kinase-2 (Tyk2) is a member of the Jak kinase family and is activated in response to various cytokines (1). Especially in IL-12 and IL-23 which share a common subunit p40, these lead receptors dimerize IL-12R β 1/IL-12R β 2 and IL-12R β 1/IL-23R, respectively. IL-12R β 1 associates with Tyk2 and their heterotypic subunits IL-12R β 2 and IL-23R binds to Jak2 as a counter partner (2). In T-cells, IL-12 induces IFN- γ production, and its signals are highly dependent on Tyk2 (3,4). IL-23 enhances the differentiation of activated T-cells into Th17 cells, and both Tyk2 and Jak2 are required for this activity (5). Importantly, we and others have reported that Tyk2 is required for IFN- α/β -mediated signals to suppress hematopoietic cell growth, although not for signals that induce antiviral activities (6,7). Thus, Tyk2 has different contributions among several cytokines. The physiological role of Tyk2 in humans is evident from the information about two patients lacking Tyk2 (8,9). They experienced high susceptibility to viral and/or mycobacterial infections, thereby indicating that Tyk2 plays an essential role in the regulation of human immune systems.

Psoriasis is the most common immune-related skin disease, characterized by excessive keratinocytes and leukocyte infiltration in thickened epidermal layers (10). Although the pathogenesis of psoriasis is not fully understood, accumulating evidence suggests that the IL-23/Th17 cell axis and Th17 cell produced cytokines (IL-17 and IL-22) play important roles in skin abnormalities in human psoriasis (11-16). There are some animal models which share many features of psoriasis (17-20). The imiquimod (IMQ)-applied dermatitis model, which is a recently established mouse model for psoriasis-like skin inflammation, shows erythema, scaling, and swelling of skin. The

inflammation response to IMQ is largely absent in IL-23p19- and IL-17A-deficient mice (20). Besides this, Tyk2 has been identified in a genome wide association study (GWAS) as a psoriasis susceptibility (21). Tyk2-deficiency abolishes IMQ-induced ear thickening resulting from epidermal hyperplasia with inflammatory cell infiltration, suggesting that signals through Tyk2 are highly involved in IMQ-induced psoriasis-like skin inflammation (22).

In the present study, we investigated the *in vivo* involvement of Tyk2 in IL-23-induced psoriasis-like skin inflammation which has been linked to IL-23/Th17 axis-dependent features of disease pathogenesis. Our results revealed that Tyk2-deficient mice showed diminished skin inflammation and psoriasis-like pathology after IL-23 injection into their ear pinna. Moreover, we have found that Tyk2 plays a pivotal role in the severity of the inflammatory process and keratinocyte pathology induced by IL-22. Therefore, Tyk2 is essential in the models of IL-23-induced and IL-22-mediated dermatitis, which develops in sequential T-cell-independent and T-cell-dependent phases. We also demonstrated that the treatment of mice with tyrphostin A1, a Tyk2 inhibitor, significantly reduced IL-23-induced skin inflammation, indicating that Tyk2 may represent an important candidate for drug development by targeting both the IL-23/IL-22-mediated signaling.

Methods

Mice

Gene-targeted $Tyk2^{-/-}$ mice were backcrossed for at least eight generations onto BALB/c mice (3). Mice were kept under specific pathogen-free conditions and provided with food and water *ad libitum*. All experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Hokkaido University and Daiichi-Sankyo.

Cytokine injection into ear pinna and evaluating severity of skin inflammation

The IL-23-induced psoriasis-like skin inflammation model was established as previously described (19,23). $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice received an intradermal injection of either 1 μ g/ear recombinant murine IL-23 (R&D Systems, Minneapolis, MN) or 1 μ g/ear BSA in the ear pinna on Day 0, 1, 2 and 3. In some studies, recombinant murine IL-22 (R&D Systems) was also used instead of IL-23 (24). The reagents used *in vivo* did not contain endotoxins in the LAL test (<0.06 EU/ml). In the cytokine neutralization experiment, 0.1 mg of neutralizing antibody and negative control antibody were intraperitoneally injected before the first IL-23 injection. The following antibodies were used for neutralization *in vivo*; anti-IL-17 Ab (50104), anti-IL-22 Ab (AF582) (R&D Systems); anti-IFN- γ Ab (XMG1.2), anti-IL-12/IL-23p40 Ab (C17.8) (BD Pharmingen, San Diego, CA) (25). In the $Tyk2$ inhibition experiment, $Tyk2$ inhibitor tyrphostin A1 (Santa Cruz Biotechnology, Santa Cruz, CA) was intraperitoneally injected twice a day (26-28). The skin inflammatory response was measured as an increase in the ear thickness using a thickness gauge (Peacock, Tokyo,

Japan) on the days indicated. The ear swelling was calculated by subtracting Day 0 thickness from the thickness measured each day. For further characterization, ear pinnas and ear draining lymph nodes (LNs) were collected from sacrificed mice.

Extraction of tissue RNA and TaqMan analysis of gene expression

RNA was extracted from the ear pinna and whole ear draining LN samples using ISOGEN (Nippon Gene, Tokyo, Japan). Using 5 µg of a total RNA template, cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR analyses of the respective genes, as well as the control GAPDH mRNA transcripts, were carried out using TaqMan Gene Expression assay probe/primer mixture and TaqMan Gene Expression Master Mix. PCR amplification and evaluation were performed using an Applied Biosystems 7900HT Fast Real-Time PCR System. The reverse transcription and PCR conditions were in accordance with the manufacturer's instructions, and PCR was performed for 40 cycles.

Preparation of cells from tissues and flow cytometric analysis

LN cell suspensions were prepared by passing the ear draining LN cells through a sterile nylon mesh filter. For the isolation of inflammatory cells in the skin, ear pinnas were split using forceps, followed by incubation with 2.4 U/mL dispase II (Roche Applied Science, Indianapolis, IN) at 37°C for 2 h to isolate the dermal sheet, and a single cell suspension was prepared for dissociation with 0.28 U/mL Liberase TM (Roche) for 1 h in serum-free medium.

In flow cytometry analysis, isolated cells were stained with anti-CD3 (145-2C11), anti-CD4 (L3T4), anti-γδTCR (GL3), anti-Ly6G (RB6-8C5) (BD Pharmingen) or

anti-F4/80 (BM8) (BioLegend, San Diego, CA). For intracellular cytokine staining, cells were stimulated by PMA (Sigma-Aldrich, St. Louis, MO) and ionomycin (Sigma-Aldrich) with GolgiPlug for 4 h and stained with cell surface markers, then fixed and permeabilized cells were stained with anti-IL-17 Ab (TC11-18H10) (BD Pharmingen) or anti-IL-22 Ab (Poly5164) (BioLegend) using the Cytotfix/Cytoperm Plus Kit (BD Biosciences, San Jose, CA), as indicated.

Cytokine contents in ear homogenate

Twenty-four hours after 4 consecutive days of IL-23 injection, the ear pinna was collected using a biopsy punch, and immediately homogenized in a polytron homogenizer with PBS containing 0.1% Tween-20 (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride (Nacalai Tesque, Kyoto, Japan). The supernatant was collected after centrifugation for 20 min at $10,000 \times g$. Total protein content was measured using a protein assay kit (Dojindo, Kumamoto, Japan). IL-17 and IL-22 contents in the skin tissue homogenates were measured by ELISA (R&D Systems) according to the manufacturer's instructions.

Cytokine productions from LN cells

Prepared ear draining LN cell suspensions from Tyk2^{+/+} and Tyk2^{-/-} mice, as mentioned above, were stimulated with IL-23 under CD3 activation for 72 h. Cytokine production in culture supernatants was measured by ELISA (R&D Systems).

TYK2 knockdown in human keratinocyte cell

A human keratinocyte cell line, HaCaT, was maintained in DMEM containing 10%

FCS. HaCaT cells were plated on 48-well plates at 1×10^4 cells/well and incubated with a siRNA–Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37°C for 4 h, followed by the addition of fresh medium containing 10% FCS. After 48 h of transfection, the cells were left untreated or treated with IL-22 (10 ng/ml) for different periods. The siRNA used in this study was as follows: TYK2, 5'-GGAGAAACCUCCAAUCUCATT-3'(sense), 5'-UGAGAUUGGAGGUUUCUCCTG-3' (antisense). Negative control siRNA was purchased from Qiagen (Cat. 1022076) (Tokyo, Japan).

Western blotting

Harvested ear pinna and cells were lysed and subjected to a Western blotting assay, as described previously (29). Immunoreactive proteins were visualized using an ECL detection system (Millipore, Billerica, MA), and the images were obtained using an LAS 4010 system (GE Healthcare Life Sciences, Buckinghamshire, UK). Antibodies against STAT3 (sc-7179) (Santa Cruz Biotechnology), pSTAT3(Tyr705) (9131) (Cell Signaling Technologies, Beverly, MA), TYK2 (610174) (BD Biosciences) and actin (A1978) (Sigma-Aldrich) were used.

Histology

Sections from formalin-fixed, paraffin-embedded ears were stained with H&E, and epidermal thickness was measured using a BIOREVO BZ-9000 microscope and measurement module BZ-H1ME (KEYENCE, Tokyo, Japan). Epidermal thickness was measured at six positions per section and averaged (22).

Statistical analyses

Results are presented as the mean \pm SD. All unpaired data were analyzed by the *F*-test of variance, followed by Student's *t*-test or Welch's *t*-test by the variance. For paired comparisons, the Student paired *t*-test was performed. For multiple comparisons, the non-parametric Dunnett's multiple comparison test with a joint ranking method was performed. The value of $p < 0.05$ was chosen as an indication of statistical significance. A statistical comparison was performed using statistical software (SAS System Release 8.2; SAS Institute, Cary, NC).

Results

Tyk2 is involved in IL-23-induced skin inflammation

Tyk2 has a critical role in the pathology of the IMQ-induced psoriasis model as shown in our previous report (22). In the IMQ-induced model, IL-23 and IL-23-mediated cytokines including IL-17 and IL-22 are key players in the pathology (20). To analyze molecular mechanisms underlying the pathogenesis of psoriasis, we employed a more direct psoriasis model in which mice were administrated with IL-23 as an inducer of psoriasis-like skin inflammation (19,23). Tyk2^{+/+} mice showed marked ear swelling, gradually increasing in parallel to the injection (Fig. 1A). Importantly, Tyk2^{-/-} mice did not show severe ear swelling during the observation period. Reduction of IL-23-induced ear swelling of Tyk2^{-/-} mice was also confirmed by the evaluation of ear tissue weight (Fig. 1B). Tyk2^{+/+}, but not Tyk2^{-/-}, mice developed epidermal hyperplasia with massive inflammatory cell infiltration by IL-23-injection (Fig. 1C and 1D). These data indicate that signals through Tyk2 are likely to be involved in IL-23-induced psoriasis-like skin inflammation.

Inflammatory cell infiltration and cytokine production in IL-23-induced skin inflammation

There was no difference in the basal population of CD4⁺IL-17⁺ or CD4⁺IL-22⁺ T cells in LN between Tyk2^{+/+} and Tyk2^{-/-} mice (data not shown). When infiltrating cells were analyzed with flow cytometry on Day 4 of the IL-23-injection, CD4⁺ T cells, $\gamma\delta$ TCR⁺ T cells, Ly6G⁺ neutrophils and F4/80⁺ macrophages had invaded into the ear skin (Fig. 1E). Although the total infiltrated cells were significantly fewer in Tyk2^{-/-}

mice than in $Tyk2^{+/+}$ mice (Supplementary Fig.1A), the $\gamma\delta$ T-cells and neutrophils were the major types of invaded cells both in $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice. We then evaluated the infiltrated IL-17- or IL-22-expressing cells, because both of these play important roles in IL-23-induced psoriasis-like skin inflammation. As shown in Fig. 1F, the proportion of IL-17⁺ or IL-22⁺ cells in $\gamma\delta$ T-cells was found to be much higher than that in CD4⁺ $\alpha\beta$ T-cells in the ear skin. The absolute numbers and percentages of CD4⁺IL-17⁺ and CD4⁺IL-22⁺ T-cells after IL-23-injection in $Tyk2^{-/-}$ mice were significantly reduced as compared to $Tyk2^{+/+}$ mice. However, the $\gamma\delta$ T-cells, which have potential to produce IL-17 and IL-22 under the PMA/ionomycin stimulation, similarly recruited and expanded in both $Tyk2^{-/-}$ and $Tyk2^{+/+}$ mice. Next, we created IL-23-induced skin inflammation on an intermissive schedule to sustain the inflammation because psoriasis is known as a chronic inflammatory disease. In this case, we injected 1 μ g/ear of IL-23 on Day 0, 2, 4, 7, and 9 into the ear pinna. Due to the intermissive IL-23 injection, $Tyk2^{+/+}$ mice showed more obvious inflammatory cell infiltration and epidermal hyperplasia than they did after 4-consecutive-day injections, but $Tyk2^{-/-}$ mice displayed limited symptoms again (Supplementary Fig. 2A and 2B). Although the infiltrated F4/80⁺ macrophage population showed a 3-fold increase and a large amount of cells infiltrated in $Tyk2^{+/+}$ mice on the schedule compared to 4-consecutive-day injections, the reductions were observed in $Tyk2^{-/-}$ mice (Supplementary Fig. 2C and 2D). These data likely indicate that the invasion of $\alpha\beta$ T-cells and $\gamma\delta$ T-cells into the ear skin after IL-23-injection may be differently regulated, and that signals through Tyk2 may play a role in differentiation, recruitment or expansion of IL-17⁺ or IL-22⁺ cells in $\alpha\beta$ T-cells, but not $\gamma\delta$ T-cells.

The impaired inflammatory cell invasion in $Tyk2^{-/-}$ mice led us to investigate the expression levels of chemokines in their ear skin. As shown in Fig. 2A, *Ccl2*, *Ccl20* and *Cxcl1*, macrophage/neutrophil-attracting chemokines, were up-regulated by IL-23-injection in $Tyk2^{+/+}$ mice; however, their induction was impaired in $Tyk2^{-/-}$ mice.

The different degree of inflammation between $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice after IL-23-injection also suggested that we examine expression patterns of proinflammatory cytokines. Among these, the induction of IL-17A, IL-22, IFN- γ , IL-1 β , IL-6, IL-12A(p35) and IL-12B(p40) by IL-23-injection was greatly impaired in $Tyk2^{-/-}$ mice ear skin (Fig. 2B). TNF induction was similar in $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice, indicating a limited influence of *Tyk2*. In ear draining lymph nodes, only IL-22 gene expression showed marked induction in response to IL-23-injection in $Tyk2^{+/+}$ mice while no cytokines were elevated in $Tyk2^{-/-}$ mice (Fig. 2C and Supplementary Fig. 1B). These data indicate that although inflammatory cytokines are induced by IL-23-injection, *Tyk2* dependency was dispensable and that cytokine production was completely different between ear skin and draining lymph nodes.

In addition to gene expression, the ear skin of $Tyk2^{+/+}$ mice contained higher concentrations of IL-17 and IL-22 than those of $Tyk2^{-/-}$ mice (Fig. 2D). To clarify and confirm the roles of *Tyk2* in IL-23-induced IL-17 and/or IL-22 production during skin inflammation, we analyzed the productivity of IL-17 and IL-22 in response to IL-23 using lymphocytes isolated from $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice. Although $Tyk2^{+/+}$ lymphocytes markedly produced both IL-17 and IL-22 in a dose dependent manner, $Tyk2^{-/-}$ lymphocytes did not (Fig. 2E). Therefore, signals through *Tyk2* are involved in IL-23-induced IL-17 and IL-22 production in the skin inflammation model.

Tyk2 is a critical factor for psoriasis-related molecular expression in IL-23-induced skin

Antimicrobial proteins are vital components of the innate immune system and protect against the invasion of pathogenic microorganisms (30,31). Skin is one of the body's first-line defensive tissues and produces antimicrobial proteins, such as defensin and S100A proteins during inflammatory events. In psoriatic regions, massive antimicrobial peptide production is observed, and the levels are correlated with the severity of inflammation (32,33). IL-22 is recognized as a key molecule in antimicrobial peptide production (34). As shown in Fig. 3A, large amounts of gene expression of defensin and S100A8 was detected in ear skin samples of $Tyk2^{+/+}$ mice after IL-23-injection; in addition, production of keratin16 (K16), a keratinocyte proliferation marker in psoriasis, was elevated (Fig. 3B). However, their gene expression was greatly impaired in $Tyk2^{-/-}$ mice. These data indicate that induction of antimicrobial protein in ear skin by IL-23 stimulus greatly requires signals through Tyk2.

IL-22 is a key player in IL-23-induced skin inflammation

There are some reports telling that IL-22 is a critical cytokine rather than IL-17 (19,24). Therefore, we focused on the importance of Tyk2 in IL-22-mediated reactions because no one has revealed the functional contribution of Tyk2 in IL-22 signaling. To clarify how IL-22 is involved in IL-23/Tyk2-induced skin inflammation, we first investigated the ear skin inflammation using the same mice model, injecting IL-22 instead of IL-23. IL-22 receptors are expressed on non-hematopoietic cells, including keratinocyte in the skin, but not on hematopoietic immune cells (35). Therefore, the primary target cells of the injected IL-22 are likely to be keratinocytes. As shown in Fig.

4A, IL-22-injection induced marked ear swelling in Tyk2^{+/+} mice while marginal ear swelling was observed in Tyk2^{-/-} mice. Histological analysis also revealed that only the ear skin of Tyk2^{+/+} mice injected with IL-22 developed epidermal hyperplasia with inflammatory cell infiltration (Fig. 4B). These phenomena were identical, but inflammatory cell infiltration was mild although the epidermal hyperplasia occurred similarly to the case of the IL-23-injection (Fig. 1C and 1D). Just as with the IL-23-injection, the IL-22-injection significantly induced IL-22 and IL-17A expression within 1 hour in the ear skins of Tyk2^{+/+} mice, but not in those of Tyk2^{-/-} mice (Supplementary Fig. 3A). The TNF induction by IL-22-injection was independent on Tyk2, and the IL-22 receptor gene was constitutively expressed and did not change after IL-23-injection (Supplementary Fig. 3A). These data suggest that IL-22 has direct effects on acute skin inflammation and that IL-22 and IL-23 show some overlapping in their molecular events. Furthermore, we clarified that administration of IL-22 to Tyk2^{+/+} mice rapidly induced antimicrobial proteins and K16 expression (Supplementary Fig. 3B). In the case of Tyk2^{-/-} mice, induction of these genes by IL-22 was significantly impaired.

To confirm the involvement of IL-22 in IL-23-induced skin inflammation, we intraperitoneally injected the neutralizing Abs or control Abs into Tyk2^{+/+} mice before IL-23-injections. We checked several doses of antibody injection (0.01-0.3 mg/body) and found that 0.1 mg/body antibody showed maximal efficacy (data not shown); in addition, there was a report in which similar doses of the antibodies were used *in vivo* (25). Pretreatment of mice with anti-IL-22 or anti-IL-17 IgGs significantly inhibited IL-23-induced ear swelling. Also pretreatment with anti-IL-23(p40) IgG (Fig. 4C) and anti-IL-22 IgG inhibited epidermal hyperplasia with inflammatory cell infiltration (Fig.

4D). In the case of an IL-17 neutralizing experiment, anti-IL-17 IgG suppressed the skin inflammation including IL-6 production, but not epidermal hyperplasia in histology and IL-22 production in ear tissue homogenate (Supplementary Fig. 3C, data not shown). Pretreatment with anti-IFN- γ IgG failed to inhibit IL-23-induced ear swelling (Fig. 4C).

Therefore, IL-22 acts as a major downstream mediator of IL-23-induced skin pathology and Tyk2 is directly involved in both IL-23- and IL-22-mediated signals.

Requirement of Tyk2 for IL-22- mediated signaling in keratinocyte

To confirm the involvement of Tyk2 in IL-22-mediated signal transduction, IL-22-induced STAT3 phosphorylation in ear skin was examined. Whole skin lysates were prepared 1 h after the IL-22 injection and analyzed by Western blotting. Tyk2^{+/+} mice skin lysate showed marked STAT3 phosphorylation compared with the Tyk2^{-/-} mice lysate (Fig. 5A). However, a low level of STAT3 phosphorylation was detected in the ears of Tyk2^{-/-} mice after the IL-22-injection. To elucidate the direct effects of IL-22-Tyk2 signaling cascade on keratinocytes, a human keratinocyte cell line, HaCaT was utilized. A specific siRNA for Tyk2 or a non-target control siRNA was transfected into HaCaT cells, and the cells were stimulated with IL-22. The total cell lysates were analyzed by Western blotting. Tyk2 siRNA-transfected HaCaT cells showed a great reduction of IL-22-induced STAT3 phosphorylation as compared to control siRNA transfectants (Fig. 5B). Our densitometry analysis indicated that the reduction was approximately 95% compared with wild-type mice (Fig. 5C), indicating that Tyk2 is directly involved in IL-22-induced signals. Therefore, Tyk2 is required for IL-22 dependent skin inflammation.

Promising treatment with Tyk2 inhibitors for IL-23-induced skin inflammation

As mentioned above, our data suggest that signals through Tyk2 play a central role in IL-23-induced ear swelling, a model of psoriasis-like skin inflammation. To understand the therapeutic potential of Tyk2 kinase inhibition, we investigated the efficacy in IL-23-induced skin inflammation using a traditional Tyk2 inhibitor, tyrphostin A1 (26,27). We checked the inhibition and selectivity of tyrphostin A1 against the Jak family proteins. As shown in Fig. 6A, tyrphostin A1 selectively inhibited Tyk2-mediated, but not Jak1- or Jak2-mediated STAT3-responsible luciferase activity. In addition, tyrphostin A1 suppressed autophosphorylation of Tyk2, but not Jak2 in HeLa cells while autophosphorylation of Jak1 was mildly suppressed at high concentration. Tyrphostin B42, another tyrphostin-related derivative, widely suppressed autophosphorylation of the Jak family proteins (Fig. 6B). In an *in vivo* study, IL-23 was injected into the ear pinna on Day 0 to Day 3 along with tyrphostin A1. The doses of tyrphostin A1 used for injection were similar to those of tyrphostin B42 in a previous report (28). Tyk2^{+/+} mice injected with IL-23 showed significant ear swelling. When 2 or 10 mg/kg tyrphostin A1 was given to mice, there was no decrease in IL-23-induced ear swelling (Fig. 6C). However, the treatment with 50 mg/kg tyrphostin A1 resulted in a significant decrease of ear swelling. The inhibition of IL-23-induced skin inflammation by tyrphostin A1 was also confirmed by the evaluation of ear tissue weight (Fig. 6D). In an ear homogenate sample, treatment with 50 mg/kg tyrphostin A1 showed significant reduction of IL-17 production and a trend to reduce IL-22 production as shown in Tyk2^{-/-} mice (Fig. 6E). In addition, the increase of ear weight was well correlated with the IL-17 or IL-22 cytokine production in the ear, and mice treated with 50 mg/kg tyrphostin A1 exhibited a lower ear tissue weight and IL-17- or

IL-22-production (Fig. 6F). Therefore, Tyk2 seems to be a potential target to treat IL-23-induced skin inflammation.

Discussion

Tyk2 associates with various receptor subunits for IFNs, IL-6, IL-10, IL-12, IL-13, and IL-23, and plays a critical role in immune and/or inflammatory responses. However, a different level of dependence on Tyk2 is evident for certain cytokines; therefore, *in vivo* roles of Tyk2 in immune and/or inflammatory diseases should be clarified. In the present study, we evaluated how Tyk2 participates in IL-23-induced psoriasis-like skin inflammation using Tyk2-deficient mice. We showed that Tyk2^{-/-} mice had an attenuated response to the IL-23-induced ear swelling response. Tyk2 deficiency decreased the ability to recruit Th17 and Th22 cells into inflammatory sites. Also, IL-22 and IL-23 cooperated to induce skin inflammation, and signals through Tyk2 were involved in the skin inflammation. Whereas Tyk2 is associated with the IL-22 receptor as well as Jak1, little is known about its role in IL-22 signaling. Therefore, this is the first report to state that Tyk2 has a functional contribution in IL-22-mediated reactions.

A high gene expression level of IL-23 is reported in human psoriatic skin (13,14), and specific polymorphisms in *IL-23p19* and *IL-23R* genes are associated with an increased risk of developing psoriasis (15,16). The downstream impact of IL-23 on Th17 activation also contributes to psoriasis by secreting inflammatory cytokines, such as IL-17 and IL-22. In IL-23-transgenic mice, inflammation of multiple organs, including the skin, occurs systemically (36). Notably, the intradermal delivery of recombinant IL-23 protein in mice results in psoriasis-like skin inflammation (19,23,25). In addition, IL-22 activates keratinocytes to proliferate and produce antimicrobial peptides, such as defensin and S100A molecules (34,35,37). Thus, IL-23, IL-17 and IL-22 are likely to be the key cytokines to induce psoriatic skin phenotypes, including

erythema, hyperplasia of epidermis, parakeratosis, and leukocyte infiltration. Among these three cytokines, IL-23 is produced by dermal dendritic cells and macrophages in psoriatic skin (13). IL-23 promotes Th17 cell expansion and survival; in addition, IL-23 together with IL-1 β activates $\gamma\delta$ T-cells to produce IL-17 (38). IL-22 is another Th17 cell cytokine to mediate skin inflammation induced by IL-23 (23). IL-22 expression is restricted to the cells of lineages of the innate and adaptive immune systems, whereas the functional IL-22R seems to be restricted to non-hematopoietic cells of skin, pancreas, intestine, liver, lung and kidney (39). IL-22-IL-22R interactions can induce the expression of gene encoding molecules involved in tissue inflammation, immunosurveillance and homeostasis. Consistent with this, dysregulated expression of IL-22 or IL-22R has been reported in certain human diseases, and studies of mouse models have identified crucial roles for IL-22-IL-22R in regulating homeostasis of epithelial cells at barrier surfaces (34,35).

Our data showed that IL-23 and IL-22 cooperated to induce skin inflammation and that signals through Tyk2 were critical in the induction of a massive infiltration of leukocytes as well as inflammatory cytokine production. Excess keratinocyte proliferation and differentiation is a known effect of IL-17 and IL-22 (40). The Tyk2 kinase is involved in the IL-22 signaling cascade (41), but the dependence of Jaks in cytokine signaling is not similar and the Tyk2 role in IL-22 signaling, especially *in vivo*, and is not well understood. In the current study, we revealed that Tyk2 plays an important role in the IL-22 signaling cascade and IL-22-dependent epidermal hyperplasia. In addition, keratinocytes produce some chemokines in response to IL-17, IL-22, and IFN- γ (42), and leukocyte infiltration may be a result of changing chemokine expression, such as Cxcl1, Ccl2, and Ccl20. Mouse epidermis and dermis contain large

numbers of $\gamma\delta$ T-cells, which constitutively express the IL-23 receptor unlike $\alpha\beta$ T-cells. In addition, dermal $\gamma\delta$ T-cells constitutively express a number of chemokine receptors including Ccr1, Ccr2, Ccr4, Ccr5, Cxcr3, and Cxcr4 (43). Thus, the constitutive existence of $\gamma\delta$ T-cells in skin and different sensitivity to chemokines may be the reason for rapid trafficking into inflammatory regions.

Our data indicated that Tyk2 is mainly involved in IL-22 signaling, but also suggested that IL-22 has a Tyk2-independent pathway because the phenotype in skin development is limited in Tyk2^{-/-} compared with IL-22^{-/-} mice. Actually, there is evidence that Jak1 and Jak2 are also associating Jak family proteins with IL-22 receptors, and Tyk2 and Jak1, but not Jak2, mainly contribute to receptor signaling (41). Thus each Jak may have some indispensable functions in receptor signaling. On the other hand, it is certain that we do not know how Jak1 contributes to the function of IL-22 *in vivo* because Jak1^{-/-} mice often die perinatally (44). Therefore, Tyk2 clearly plays a role in the IL-22 pathway despite the existence of Tyk2-independent mechanisms; however, whether different contributions between each Jak in the receptor signaling exist remains an open question.

The proportion of IL-17⁺ cells was much higher in $\gamma\delta$ T-cells than in $\alpha\beta$ T-cells; therefore, a major source of IL-17 in the inflammation is likely to be $\gamma\delta$ T-cells that infiltrate into the ear skin. In our data, $\gamma\delta$ T-cells also increased in the IL-23-treated Tyk2^{-/-} mice as well as WT mice. In Tyk2^{-/-} mice, $\gamma\delta$ T-cells hold basal function because $\gamma\delta$ T-cells produced Th17 cytokines by Tyk2-independent PMA/ionomycin stimulation as shown in Fig. 1F. There is a report telling that Tyk2^{-/-} $\gamma\delta$ T-cells isolated from some tissues reduced IL-23-dependent but not PMA-dependent IL-17 production (45); therefore, infiltrating $\gamma\delta$ T-cells are actually diminished the cytokine production in

the situation where Tyk2 signals are required as shown in Fig. 2E. Moreover, IL-23-treated ear tissue from Tyk2^{-/-} mice strongly diminished IL-17 and IL-22 production as shown in Fig. 2D, suggesting that Tyk2 has an indispensable role in IL-23-dependent cytokine production from invaded $\gamma\delta$ T-cells in ear tissue not only in Tyk2^{+/+} mice but also in Tyk2^{-/-} mice. Importantly, $\alpha\beta$ T-cells, but not $\gamma\delta$ T-cells, require signals through Tyk2 for differentiating into IL-17⁺ cells although infiltration of both types of cells was dependent on Tyk2 (22). Interestingly, the pathological roles of $\gamma\delta$ T-cells are shown to be limited in human psoriasis, which is probably dependent on $\alpha\beta$ T-cells rather than $\gamma\delta$ T-cells (17). Thus, the selective involvement of Tyk2 likely suggests that great advantage to use a Tyk2 inhibitor for human psoriasis. Recent reports have suggested that CD27 is a critical determinant of the balance between IFN- γ ⁺ and IL-17-producing $\gamma\delta$ T-cell subsets (46). Skin dermal and epidermal $\gamma\delta$ T-cells are exclusively CD27-negative and produce large amounts of IL-17, but not IFN- γ . This unique phenotype of skin $\gamma\delta$ T-cells may explain why the influence of Tyk2 deficiency on IL-17-induction was smaller than that on IFN- γ -induction.

There are some therapeutic options to treat patients with severe psoriasis, however, a safe, highly effective, convenient systemic therapy is still required. Recently, a monoclonal antibody, which recognizes p40 and neutralizes both IL-12 and IL-23 biological activity, was generated and improved the psoriasis therapy (47). This antibody therapy is highly effective for ameliorating psoriatic plaques, pruritus, and nail psoriasis. The neutralizing antibody against IL-12 and IL-23 seems to be a promising option for clinical utility, and these facts also suggest that IL-23 plays an important role in the pathogenesis of human psoriasis. Similarly, Jak inhibitors are believed to confer great therapeutic benefits through disease control in patients with other autoimmune

diseases, which presumably result from high levels of circulating cytokines that signal through Jak kinases (48,49). Jak inhibitors should have some characteristic differences by their Jak selectivity: A Jak3 inhibitor inhibits signals mediated by γ_c cytokines (*e.g.* IL-2, -7, and -15) and then shows potent suppressive effect on immune cells, but it also suppresses T_{reg} cells which play an endogenous regulatory role; a Jak2 inhibitor inhibits not only IL-23 signals but also erythropoietin signals critical for hematopoiesis. We can not speculate on the benefit of Jak1 inhibitors because of the severe phenotype observed in $Jak1^{-/-}$ mice (44). We demonstrated here that a Tyk2 inhibitor could inhibit IL-23-induced skin inflammation and Th17 cell cytokine production in a dose-dependent manner. It should be emphasized that Tyk2 inhibitors could directly suppress IL-22 signals. In these observations, $Tyk2^{-/-}$ mice largely decreased IL-23-mediated IL-17 and IL-22 production, indicating that Tyk2 is important in the cytokines production. However, $Tyk2^{-/-}$ mice also reduced IL-22-mediated reactions in psoriasis-like skin, therefore, Tyk2 seems like a rationally efficient target in psoriasis therapy. Furthermore, we have demonstrated that Tyk2 does not affect T_{reg} cell differentiation (22). Our data propose that a selective Tyk2 inhibitor will be a new class of Jak inhibitor which suppresses IL-22 and IL-23 and such an inhibitory profile cannot be achieved by other Jak inhibitors. However, tyrphostin A1 shows insufficient potential as a Tyk2 inhibitor, because tyrphostin A1 is also shown to suppress Jak1 at a high concentration (Fig. 6B). In clinical study, Jak inhibitors show some safety risk dependent upon on- or off-target events such as anemia, neutropenia and tumors (50,51). As $Tyk2$ -deficient mice grow normally and do not exhibit any immunosuppressive phenotypes under steady state conditions, we may be able to use Tyk2 specific inhibitors as a new therapeutic option without severe adverse effects in clinical fields.

Thus, our results strongly suggest that Tyk2 could be a promising drug target for psoriasis treatment.

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Figure legends

Figure 1. Involvement of Tyk2 in IL-23-induced skin inflammation. (A - D) The ear pinna of Tyk2^{+/+} and Tyk2^{-/-} mice was intradermally injected with 1 µg/ear of IL-23 or BSA for 4 consecutive days. Ear swelling was evaluated by a dial thickness gauge (A). Ear tissue weight uniformly collected by a biopsy punch on Day 4 was measured (B). Epidermal hyperplasia was evaluated by measuring the epidermal thickness by imaging analysis (C). Data represents mean ± SD (n=5). **p*<0.05, ***p*<0.01, ****p*<0.001 compared with BSA; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 compared with Tyk2^{-/-}. Representative H&E stained histological features of IL-23-treated ear pinna with inflammatory cell infiltration (D). Scale bar, 100 µm. (E and F) Dermal cells were isolated from pooled Tyk2^{+/+} and Tyk2^{-/-} ear skin on day 4, and subjected to flow cytometry. Surface markers of major inflammatory cells (E) and intracellular IL-17 and IL-22 in CD4⁺ cells and γδTCR⁺ cells stimulated with PMA (30 ng/ml) and ionomycin (1 µg/ml) for 4 h (F) were shown. Similar results were obtained in at least two independent experiments.

Figure 2. Role of Tyk2 in inflammatory cytokine expression during IL-23-injections. (A - C) Tyk2^{+/+} and Tyk2^{-/-} mice were intradermally injected with 1 µg/ear of IL-23 or BSA each day. mRNAs were prepared from the ear pinna (A, B) or the ear draining LN (C) of Tyk2^{+/+} and Tyk2^{-/-} mice on Day 2 (A, B, C), and subjected to quantitative RT-PCR with the indicated primers. Gene expression level was normalized by GAPDH expression. (D) IL-17 and IL-22 contents in the ear pinna. After 4 consecutive days of IL-23 injection, the ear pinna was homogenated and cytokine contents were measured

by ELISA as indicated. Data represents mean \pm SD (n=5-6). (E) Ear draining LN cells isolated from naïve Tyk2^{+/+} and Tyk2^{-/-} mice were stimulated with various concentration of recombinant mouse IL-23 (0 – 10 ng/ml) using an anti-CD3 Abs coated plate for 72 h and measured the secreted cytokines by ELISA. Data represents mean \pm SD (n=4). * p <0.05, ** p <0.01, *** p <0.001 compared with BSA; # p <0.05, ## p <0.01, ### p <0.001 compared with Tyk2^{-/-}. Similar results were obtained in two independent experiments.

Figure 3. Psoriasis-related molecular expression in IL-23-induced skin inflammation model. (A, B) Tyk2^{+/+} and Tyk2^{-/-} mice were intradermally injected with 1 μ g/ear of IL-23 or BSA each day. mRNAs were prepared from the ear pinna of Tyk2^{+/+} and Tyk2^{-/-} mice on Day 4, and subjected to quantitative RT-PCR with the indicated primers. Gene expression levels of β 2-defensin and S100A8 (A) and keratin 16 for keratinocyte proliferation marker (B) were normalized by GAPDH expression. Data represents mean \pm SD (n=5-6). ** p <0.01, *** p <0.001 compared with BSA; ## p <0.01 compared with Tyk2^{-/-}. Similar results were obtained in two independent experiments.

Figure 4. IL-22 is a key player in IL-23-induced skin inflammation. (A, B) The ear pinna of Tyk2^{+/+} and Tyk2^{-/-} mice was intradermally injected with 1 μ g/ear of IL-22 for 4 consecutive days. Ear swelling (Day 1 - 4) (A) and epidermal thickness and H&E staining (Day 4) (B) were evaluated. Data represents mean \pm SD (n=5). ** p <0.01, *** p <0.001 compared with BSA; ## p <0.01, ### p <0.001 compared with Tyk2^{-/-}. Scale bar, 100 μ m. (C) Cytokine neutralization in IL-23-induced skin inflammation. The indicated neutralization antibody or negative control antibody (0.1 mg/mouse) was

intraperitoneally injected on Day 0, then the ear pinna of Tyk2^{+/+} mice was intradermally injected with 1 µg/ear of IL-23 or BSA for 4 consecutive days. Ear swelling (C) was measured on Day 1 - 4, and epidermal thickness and histological features (D) were evaluated on Day 4. Data represents mean ± SD (n=5). **p*<0.05, ***p*<0.01, ****p*<0.001 compared with BSA; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 compared with neutralization Abs. Scale bar, 100 µm. Similar results were obtained in two independent experiments.

Figure 5. STAT3 phosphorylation by IL-22 in keratinocytes. (A) The phosphorylation of STAT3 in the ear pinna by IL-22 stimulation *in vivo*. The ear pinna from Tyk2^{+/+} and Tyk2^{-/-} mice was harvested at 1 h after the single IL-22 injection and STAT3 phosphorylation in Tyk2 cascade was analyzed by Western blotting. Each lane represents data from individual mouse. (B) The phosphorylation of STAT3 in human keratinocyte cell line HaCaT by IL-22 stimulation *in vitro*. Two days before the stimulation, TYK2 knockdown in HaCaT cell was conducted. Cells were harvested 5 min and 30 min after IL-22 stimulation and STAT3 phosphorylation was analyzed by Western blotting. Similar results were obtained in three independent experiments. (C) Densitometric analysis of western blots shown in B. pSTAT3 band intensity was quantified and normalized to the density of the STAT3 band. ***p*<0.01 compared with siRNA control.

Figure 6. Efficacy of tyrphostin A1 in IL-23-induced skin inflammation. (A) HeLa cells were transfected with STAT3-responsive APRE-luciferae and a Renilla luciferase, together with TYK2, JAK1 or JAK2 using Metafectene. At 24 h after transfection, the

cells were treated with vehicle (DMSO) or Tyrphostin A1 (30 μ M) for an additional 7 h and assayed for their luciferase activities. Data represents mean \pm SD (n=3). * p <0.05.

(B) HeLa cells were plated on 24-well plates and transfected with a vector coding for TYK2, JAK1 or JAK2 as indicated using Metafectene. At 36 h after transfection, the cells were treated with vehicle (DMSO), Tyrphostin A1 or Tyrphostin B42 at indicated concentration for an additional 1 h. The cells were lysed, and lysate was immunoblotted with the indicated antibodies and assessed the autophosphorylation levels of JAKs. Similar results were obtained in three independent experiments. (C and D) Tyrphostin A1 was intraperitoneally administrated twice a day into IL-23-treated Tyk2^{+/+} mice. Ear swelling (C) and ear tissue weight on Day 4 (D) was measured. Data represents mean \pm SD (n=5). ** p <0.01, *** p <0.001 compared with BSA; # p <0.05, ## p <0.01 compared with IL-23. (E) IL-17 and IL-22 contents in the ear pinna. After 4 consecutive days of IL-23 injection and tyrphostin A1 administration, cytokine contents in the ear pinna were measured by ELISA as indicated. Data represents mean \pm SD (n=5). ** p <0.01 compared with BSA; # p <0.05 compared with IL-23. (F) IL-17 and IL-22 production and ear tissue weight that were shown in Fig. 5D and 5E in each treated mice were plotted. Similar results were obtained in two independent experiments.

Figure 1

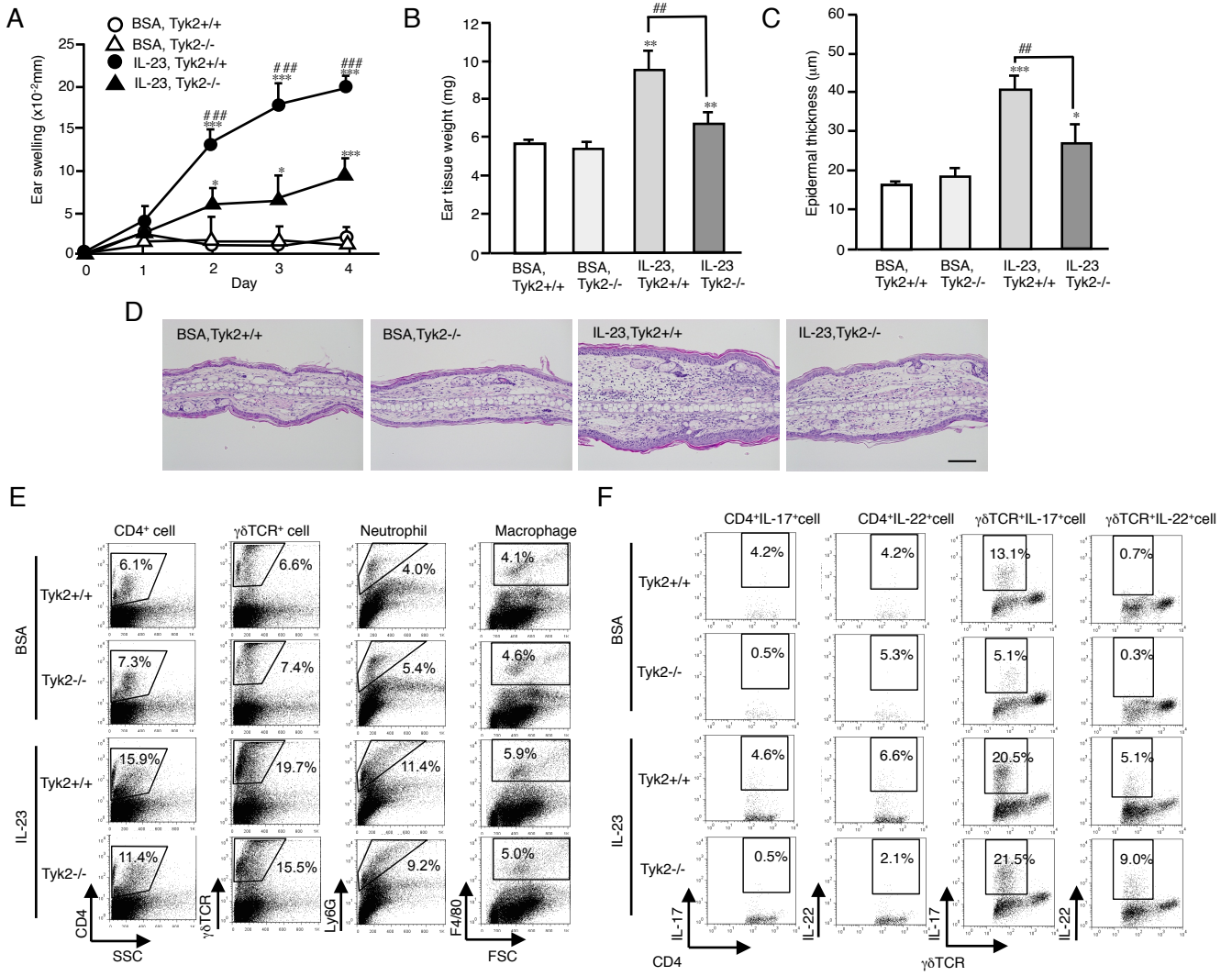


Figure 2

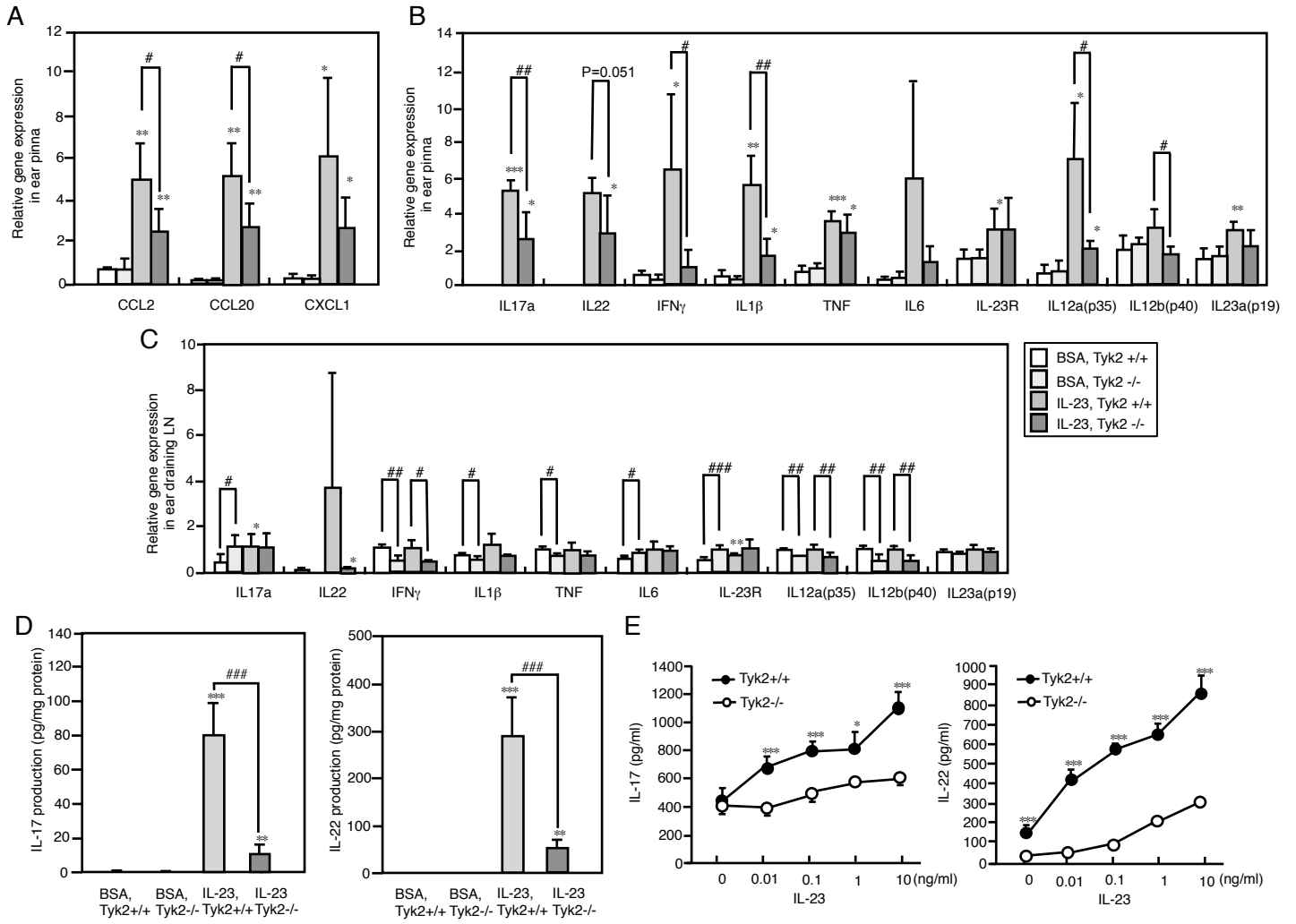


Figure 3

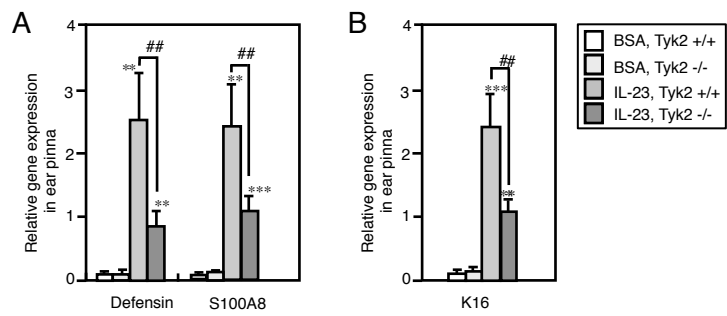


Figure 4

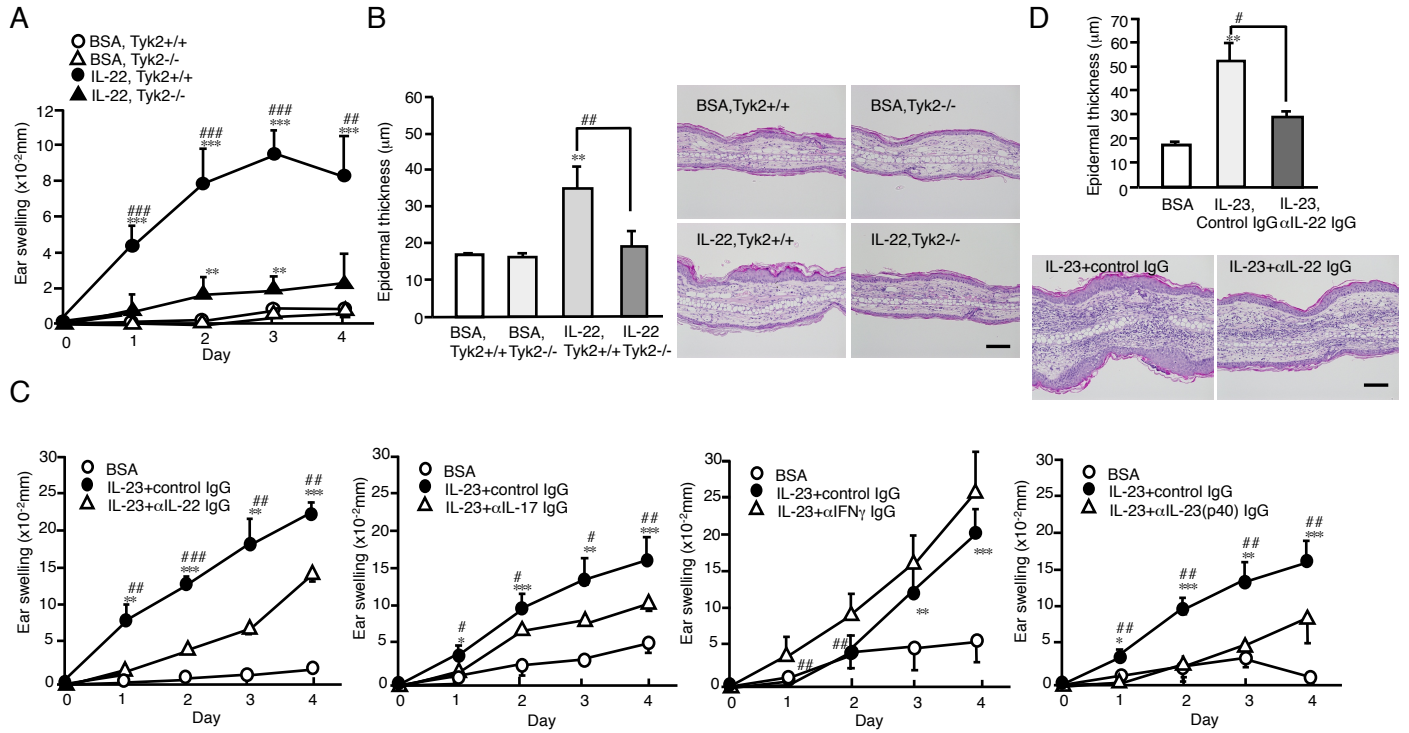


Figure 5

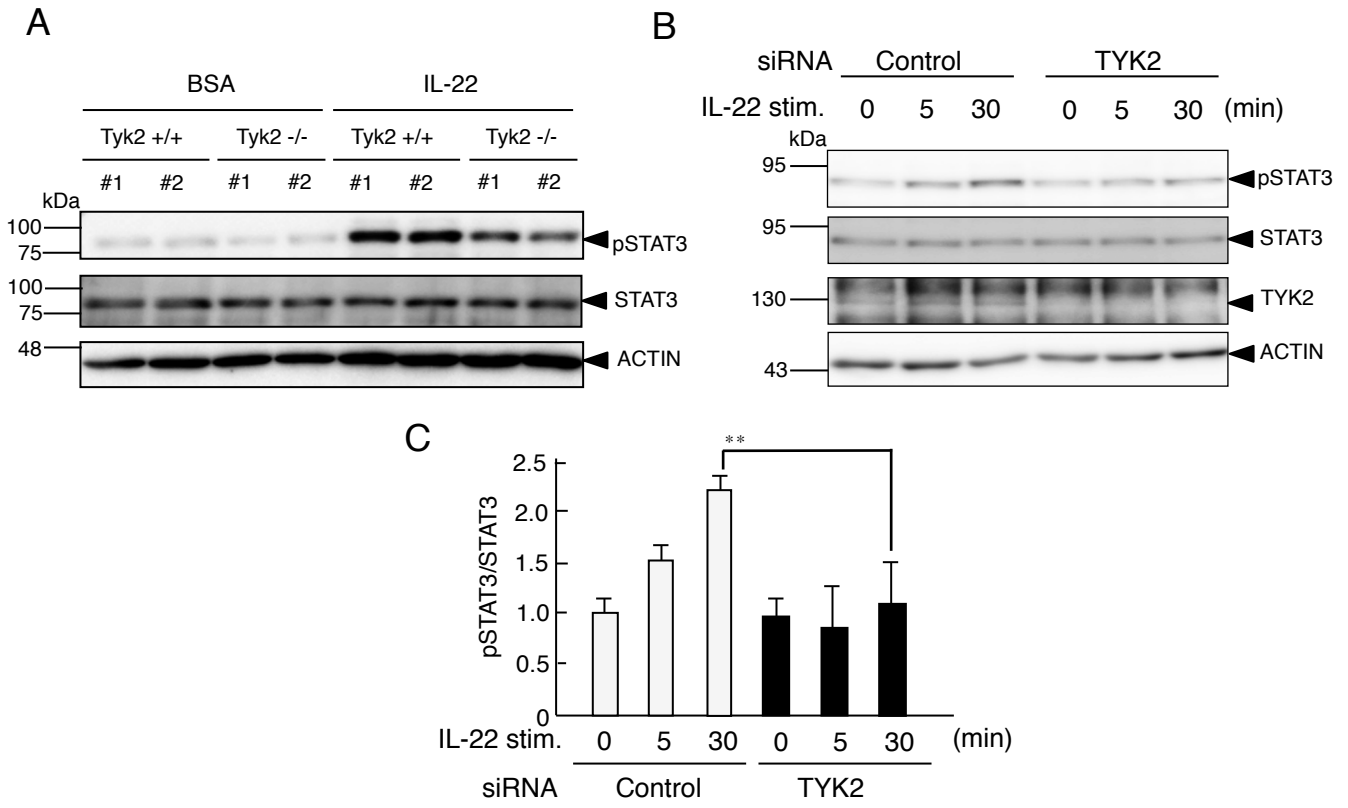


Figure 6

