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Title	Tyk2 deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice
Author(s)	Ishizaki, Masayuki; Muromoto, Ryuta; Akimoto, Toshihiko et al.
Description	2012年7月(出版12ヶ月後)公開
Citation	International Immunology, 23(9), 575-582 https://doi.org/10.1093/intimm/dxr057
Issue Date	2011-07
Doc URL	https://hdl.handle.net/2115/49521
Rights	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in International Immunology following peer review. The definitive publisher-authenticated version "International Immunology 2011 23(9):575-582" is available online at: http://intimm.oxfordjournals.org/content/23/9/575 .
Type	journal article
File Information	Tyk2-II.pdf



**Title: Tyk2 deficiency protects joints against destruction in anti-type II collagen
antibody-induced arthritis in mice**

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Running title: Involvement of Tyk2 in experimental arthritis

32 pages and 6 figures

Abstract

Tyrosine kinase-2 (Tyk2) participates in the signaling pathways of multiple cytokines in innate and acquired immunity. In the present study, we investigated in the *in vivo* involvement of Tyk2 in anti-type II collagen antibody-induced arthritis (CAIA) using Tyk2-deficient mice. Hind paws of wild-type mice showed massive swelling and erythema by arthritogenic antibody injection, while Tyk2 deficient mice did not show any signs of arthritis. Indeed, neither the infiltration of inflammatory cells nor the fibrillation of articular cartilages was observed in Tyk2-deficient mice. Tyk2 deficiency also reduced the production of Th1/Th17-related cytokines, the other pro-inflammatory cytokines and matrix metalloproteases, which are induced in the CAIA paw. Our results demonstrate a critical contribution of Tyk2 in the development of arthritis, and we propose that Tyk2 might be an important candidate for drug development.

Keywords: Tyk2, anti-type II collagen antibody-induced arthritis, rheumatoid arthritis

Introduction

Several disorders affect the joints in humans and give rise to chronic arthritis. Among these diseases, rheumatoid arthritis (RA) is one of the most disabling and carefully studied diseases with regards to the tissue-specific attack of diarthrodial joints leading to the destruction of cartilage and bone (1, 2). However, RA is probably not a single disease, but rather a clinical syndrome caused by a variety of different pathological processes (3). Disease susceptibility is associated with antigen presentation to T lymphocytes by particular HLA-DR haplotypes (4). Also, CD4⁺ T cells infiltrating into the RA synovial membrane are predominantly Th1 phenotypes (5, 6). According to a current paradigm, a pathogenic role of Th1-type cellular immunity is supposed to prevail over a beneficial Th2 response. Therefore, animal models of arthritis provide important tools for the dissection of the various cellular and molecular mechanisms leading to the arthritis of RA. Collagen-induced arthritis (CIA) in mice is widely used as an experimental model for human RA (7). Treatment of mice with collagens induces autoantibodies, which bind to a particular region of type II collagen (CII). However, arthritogenic epitopes are apparently clustered within a certain region of CII depending upon the MHC types in mice, such as CB11 in DBA/1 (H-2^d) mice and CB8 in B10.RIII (H-2^r)

mice (8, 9). Thus, CIA susceptibility is low in C57BL/6 mice and is resistant in BALB/c mice.

The ability to induce arthritis using this arthritogenic antibody cocktail provides an efficient protocol for the induction of anti-CII antibody-induced arthritis (CAIA) that can be applicable for C57BL/6 and BALB/c mice and used as a shorter, more synchronized alternative to the CIA model (9).

Tyk2, a Jak family of kinases, is activated in response to various cytokines including interferons (IFNs), IL-6, IL-10, IL-12, IL-13, and IL-23 (10-15). However, Tyk2 was dispensable for IL-6- and IL-10-mediated signaling in mice (16, 17). We have reported that Tyk2 is required for IFN- α/β -mediated signals to suppress hematopoietic cell growth, but not for those to induce antiviral activities (18, 19). Thus, the involvement of Tyk2 in IFN- α/β -signaling is restricted. In the case of IL-12-mediated signaling, signals for IFN- γ production by T cells were highly dependent on Tyk2 (16, 17, 20). Thus, experiments using Tyk2-deficient cells have revealed that a different level of dependence on Tyk2 is evident among several cytokines.

Experimental allergic encephalomyelitis (EAE), which is induced by immunization with myelin antigens or by an adoptive transfer of myelin-specific CD4⁺ effector cells, is an animal model of human multiple sclerosis (21). Tyk2-deficient mice showed lower scores for

erythema, scaling, and thickness in this model (22). Moreover, the involvement of Tyk2 was confirmed by experiments using B10.Q mice carrying different Tyk2 polymorphisms (23, 24). A defect in the IL-12 responsiveness of NK and T cells derived from a subline of the B10.Q mouse maintained at The Jackson Laboratory (Bar Harbor, ME; B10.Q/J), unlike B10.Q/Ai mice, their counterparts bred at Taconic Farms (Tarrytown, NY), was serendipitously found (25, 26) and B10.Q/J mice were shown to be highly susceptible to parasite challenge (26). Tyk2 cDNA from the spleen of both B10.Q/J mice showed a single missense mutation (G→A substitution) at position 2538 in the B10.Q/J Tyk2 coding region, resulting in a nonconservative amino acid substitution (E775K) in an invariant motif of the pseudokinase (Janus kinase homology 2) domain (23). This mutation appeared to result in the absence of the B10.Q/J-encoded Tyk2 protein, despite presence of Tyk2-specific transcripts. B10.Q/J mice, which express a Tyk2A allele, were resistant to EAE development and can be compensated by one copy of Tyk2G allele from B10.Q/Ai mice (22). In addition to the EAE model, mice carrying Tyk2 polymorphisms exhibited other susceptibility in a model for CIA (24). B10.Q/Ai mice were highly susceptible to CIA, while B10.Q/J mice were resistant. These studies have suggested that deficiency of Tyk2 results in defined clinical disorders.

In the human RA model in mice, the CIA model requires multiple steps; the induction of autoantibodies after collagen-challenge and the inflammatory responses after reactions of autoantibodies to joints. The CAIA model requires only inflammatory responses after a challenge with a cocktail of anti-type II collagen antibodies. Thus, CAIA is a more restricted and simple model than CIA, and is suitable in evaluating inflammatory responses in arthritis.

In the present study, we showed that Tyk2 plays central roles in not only adaptive autoimmunity, but also inflammatory responses in a murine arthritis model. The involvement of Tyk2 in multiple steps of RA development likely suggests that therapeutic targeting of Tyk2 could provide benefits in RA.

Methods

Antibodies and mice

Anti-STAT3, anti-STAT4 and anti-I κ B α , antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-pSTAT3(Tyr705), and anti-pSTAT4(Tyr693) antibodies from Cell Signaling Technologies (Beverly, MA); anti-actin antibody from Millipore (Billerica, MA). B10.D1-H2q/SgJ (B10.Q/J) mice bearing the Tyk2A allele and B10.Q/Ai mice with the Tyk2G allele were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and Taconic Farms (Germantown, NY, USA), respectively. Tyk2-deficient mice were backcrossed for >8 generations onto BALB/c mice (27). 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 Co., Ltd.

Induction and assessment of arthritis

In CIA model, B10.Q/Ai or B10.Q/J mice were intradermally immunized at the tail with an emulsion of 150 μ g of bovine type II collagen (CII) (Collagen Gijutsu Kensyukai, Tokyo, JAPAN) in complete Freund's adjuvant (CFA) (Difco, MI, USA) (Day 0). On day 21, the

mice received booster immunization at the base of the tail. Mice were scored three times per week, beginning 3 weeks after the first immunization, for signs of developing arthritis. The severity of the arthritis was assessed using a visual scoring system. Each paw was scored on a graded scale from 0 to 3: 0, normal paw; 0.5, swelling of one toe joint; 1, swelling of two or more toe joints, or increased swelling; 2, severe swelling; and 3, ankylosis throughout the entire paw. Each paw was graded and the four scores were added such that the maximal score per mouse was 12.

In CAIA model, arthritogenic antibody cocktail was obtained from Chondrex (WA, USA), and arthritis was induced according to the manufacturer's instructions (28). Briefly, WT or Tyk2^{-/-} mice were intravenously injected with a mixture of five anti-type II collagen mAbs (6 mg each) on day 0. Severity of the macroscopic levels of arthritis was graded up to 7 days after mAb injection in each of the four limbs per mouse on a 1-4 scale. At the end of the studies, on day 7, paw swelling volumes were quantitatively measured using a plethysmometer (Muromachi Kikai, Tokyo, JAPAN) and collected for histopathology.

Histological techniques

For histological processing, paws were fixed in phosphate buffer containing 10% formaldehyde and decalcified with EDTA. Paws were processed by routine methods to paraffin blocks. Specimens were sectioned at 6 μ m and stained with H&E. The sections were evaluated for the degree of synovial hyperplasia, inflammatory cell infiltrate, cartilage damage, pannus formation, bone erosion, and ankylosis.

Extraction of paw RNA and TaqMan analysis of gene expression

RNA was extracted from cells in paws, which were snap frozen in liquid nitrogen, using ISOGEN (Nippon Gene, Tokyo, Japan)(29). Using 5 μ g of total RNA template, cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). Quantitative real-time PCR analyses of the respective gene, as well as the control GAPDH mRNA transcripts were carried out using TaqMan Gene Expression assay probe/primer mixture and TaqMan Universal Master Mix II. PCR amplification and evaluation were performed using Applied Biosystems 7900HT Fast Real-Time PCR System. The reverse transcription and PCR conditions were according to the manufacturer's instructions, and PCR was carried up to 40 cycles.

Western blotting

The western blotting assays were performed as described previously (29). Briefly, three days after CAIA induction, popliteal lymph nodes were collected and 10^6 lymph node cells were lysed in 20 μ l of RIPA buffer (Santa Cruz). The cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore).

Statistical analyses

All data were analyzed by an F test to evaluate the homogeneity of variance. If the variance was homogeneous, a Student's t test was applied. If the variance was heterogeneous, a Welch's test was performed. In other cases, Wilcoxon rank sum test was performed in scoring data. 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 Inc., Cary, NC).

Results

B10.Q/J mice show a deficient CIA response.

To first reconfirm the pathophysiological significance of Tyk2 in arthritis, we analyzed CIA using B10.Q/Ai (Tyk2G alleles) and B10.Q/J (Tyk2A alleles) mice. Both mice were immunized with CII in CFA and boosted 3 weeks later. B10.Q/Ai mice controls started to have swelling in their joints within 1 week and showed severe arthritis around 2 weeks after boost. Approximately 90% of CII-treated B10.Q/Ai mice showed arthritis and their mean clinical scores were estimated over 6 points (Fig. 1). In contrast, only one of seven CII-treated B10.Q/J mice developed mild swelling, and their clinical scores were much lower than B10.Q/Ai mice. Therefore, mice carrying Tyk2G alleles showed high susceptibility to CIA, while mice carrying Tyk2A alleles were resistant.

Tyk2-deficient mice show reduced severity and an incidence of CAIA.

To more directly explore the pathophysiological role of Tyk2 in arthritis, we employed CAIA using BALB/c background Tyk2^{-/-} mice. CIA is mediated by autoantibodies against CII,

and although CAIA shows similar arthritis, it requires treatment with a high amount of cocktail with anti-type CII mAbs alone or with a cocktail boosted with LPS. Because $Tyk2^{-/-}$ mice showed resistance to the LPS responses (data not shown), we attempted to treat these mice with a cocktail of anti-CII mAbs alone. A cocktail of anti-CII monoclonal antibodies alone was i.v. administrated to $Tyk2^{-/-}$ and wild-type (WT) BALB/c mice. An observer unaware of their genotypes monitored the visual scoring system for signs of developing arthritis every day. As shown in Fig. 2A, WT mice treated with a cocktail of anti-CII mAbs started to develop arthritis within 3 days of injection, and the clinical scores were evaluated as approximately 4 on days 5-7. In contrast, $Tyk2^{-/-}$ mice were resistant to CAIA, and their clinical scores were always 0 during observation periods. The different responses were also shown by a photo and by the volume of the hind paw. Hind paws of anti-CII mAbs-injected WT mice showed massive swelling and erythema that extended to the ankle, while those of anti-CII mAbs-injected $Tyk2^{-/-}$ mice did not (Fig. 2B). The hind paw volume of WT mice was significantly increased by anti-CII mAbs-injection on day 7, while that of $Tyk2^{-/-}$ mice did not (Fig. 2C). Therefore, the development of CAIA completely requires the presence of $Tyk2$.

Histological features of CAIA in Tyk2-deficient mice

The joints of WT mice frequently showed severe pathology with cartilage and bone erosion, synovial inflammation, and a formation of invasive pannus (Fig. 3). In contrast, none of the Tyk2^{-/-} mice were observed to have more than minimal pannus formation or fibrillation of the articular cartilage in the nonarthritic animal. Therefore, the histological analysis of the paws confirmed the involvement of Tyk2 in CAIA.

Real-time PCR analysis of gene expression in paw of mice with CAIA.

Inflammatory arthritis-related genes from cells in paws from Tyk2^{-/-} and WT mice with or without anti-type CII mAbs-treatment were quantified with real-time PCR analysis after correcting for the GAPDH level in each sample. Paws from the Tyk2^{-/-} and WT mice were harvested at day 3 and 7 after the induction. As shown in Fig. 4A, the Th1/Th17-related cytokines such as IFN- γ and IL-17, were significantly induced at day3 by anti-CII mAbs-injection in WT mice, whereas expression of these cytokines decreased at day7, indicating that the Th1/Th17-related cytokines are involved in early stage of development of CAIA. Importantly, these cytokines were significantly reduced in Tyk2^{-/-} mice. The inflammatory cytokines such as IL-6, IL-1 β , TNF- α and IFN- β were also induced by anti-CII

mAbs-injection in WT mice at day 3 and 7 (Fig. 4B), and their induction was significantly impaired in $Tyk2^{-/-}$ mice compared with WT mice. Furthermore, a macrophage marker, F4/80 showed macrophage accumulation was enhanced by anti-CII mAbs-injection in WT mice at day3 and 7 (Fig. 5). This macrophage accumulation was also impaired in $tyk2$ -deficient mice at day 7. Similarly, a neutrophil maker, elastase showed a reduced accumulation in $Tyk2$ -deficient mice (Fig. 5). Notably, macrophage/neutrophil-attracting chemokines such as CCL2 and CXCL1 were up-regulated by anti-CII mAbs-injection in WT, and their induction was significantly impaired in $Tyk2^{-/-}$ mice compared with WT mice, indicating that decreased expression of CCL2 and CXCL1 may resulted in a reduced accumulation of macrophages and neutrophils. In addition, gene expression of MMP9 and MMP3, which are involved in matrix degradation, was strongly induced in WT mice, but not in $Tyk2^{-/-}$ mice (Fig. 5). Therefore, anti-CII mAbs-injection induced an accumulation of macrophages and neutrophils and a number of inflammatory arthritis-related genes including the Th1/Th17-related cytokines, and $Tyk2$ is involved in an accumulation of macrophages and neutrophils and the induction of gene expression of pro-inflammatory cytokines and MMPs.

To see molecular mechanisms underlying the above findings, we finally investigated activation of STAT3 and STAT4 during development of CAIA. As shown in Fig. 6, anti-CII

mAbs-injection induced phosphorylation of STAT3 and STAT4 in cells from draining lymph nodes was observed in WT but not Tyk2 mice. These results showed that the Th1/Th17-related cytokines functionally act in cells of paws from WT mice, but not in Tyk2^{-/-} mice

Discussion

Recently, a patient with Tyk2 deficiency was reported (30). The patient experienced high susceptibility to viral and mycobacterial infections, atopic dermatitis, and an elevated level of IgE, thereby indicating that Tyk2 plays essential roles in the regulation of human immune systems. As generally accepted, RA is an autoimmune and inflammatory disease whose murine model experiments are available (7). In the present study, we demonstrated the central role of Tyk2 in the pathogenesis of RA in both innate and acquired immune systems. Tyk2 deficiency markedly decreased susceptibility to the development of arthritis in the CIA and CAIA murine models.

Experiments using Tyk2^{-/-} cells have revealed that Tyk2 functions primarily in IL-12 and IL-23 signaling (16, 17, 30). Both IL-12 and IL-23 have common features. As heterodimeric cytokines, they share the p40 subunit and their receptors share the IL-12R β 1 subunit, which associates with Tyk2. IL-12 guides CD4⁺ T cells to Th1 cells, which produce signature cytokine IFN- γ along with pro-inflammatory cytokines; and IL-23 is involved in the expansion, maintenance, and functional maturation of Th17 cells, which play essential roles in the pathogenesis of chronic inflammatory disorders (31, 32). Thus, Tyk2 seems to be

indispensable for the Th1 axis, but also immune responses mediated by IL-17-producing Th17 cells. Therefore, Shevach and his colleagues who first reported the involvement of Tyk2 in CIA suggested that the pathological effects of Tyk2 polymorphisms in arthritis are defects of Th1-mediated response through IL-12 signaling (25). Indeed, CII-specific T cells derived from B10.Q/J failed to produce IFN- γ ; whereas, T cells from B10.Q/Ai mice could produce normal amounts of IFN- γ . We could reproduce their data (Fig. 1 and data not shown), and their suggestion is likely to be true. However, the CIA model requires multiple steps to develop arthritis (8). CIA is dependent on T and B cell responses against collagens, leading to the production of autoantibodies. Sequentially, the immune complex formation and complement activation triggers inflammatory responses, resulting in clinical arthritis. Although the early immune responses are surely dependent on Tyk2, its involvement in the latter inflammatory responses remains to be solved. Our main data using the CAIA model clarified that Tyk2 also plays important roles in the inflammatory stages. Indeed, paws from mice received anti-type II mAbs treatment had fewer macrophage/neutrophil infiltration and less pro-inflammatory cytokines and MMPs. This might be in part involved in the impaired expression of CCL2 and CXCL1 chemokines in Tyk2^{-/-} mice.

CAIA, which is an antibody transfer model, bypasses the T and B cell-dependent events in

CIA (9). Thus, we can analyze innate immune, as well as inflammatory responses after autoantibodies are produced. Indeed, CAIA has been utilized to screen a number of molecules for the treatment of RA. For example, a small molecule (GW2580), which is a low molecular weight inhibitor for c-Fms, was shown to reduce arthritis severity in this model (33). MMP-9^{-/-} mice did not develop severe CAIA (34). As we showed here, Tyk2^{-/-} mice showed great resistance to developing arthritis in CAIA. Histological analysis indicated that Tyk2 deficiency reduced infiltration of leukocytes and inflammatory cells into the synovium. In addition, Tyk2^{-/-} mice severely impaired the production of IFN- γ , TNF, IL-6, and MMPs. With regard to IFN- γ , this cytokine seems to oppositely suppress the development of arthritis because IFN- γ ^{-/-} mice were reported to show resistance to antigen-induced arthritis. As generally believed, TNF and IL-6 are pro-inflammatory cytokines, and MMPs are implicated in the degradation and damage of articular cartilage in RA. In CAIA, MMPs are produced by chondrocytes and synoviocytes, as well as macrophages (35). Specific c-Fms inhibition was reported to potently block TNF release in CAIA (31). In addition, one report described that Tyk2-deficient macrophages lack NO production upon stimulation with LPS (36), suggesting the possible involvement of Tyk2 in macrophage functions in vivo. Tyk2^{-/-} dendritic cells were reported to be defective in IL-12 and IL-23 production upon stimulation with CpG

oligodeoxynucleotide (37). Thus, our results are likely to suggest essential roles of Tyk2 in multiple steps of CAIA, depending on a variety of cells, such as chondrocytes, synoviocytes, and macrophages as well as lymphocytes.

Understanding of molecular mechanisms concerning the pathogenesis of RA has revealed new targets for therapeutic intervention; some block critical cytokines, such as TNF and others target adaptive immune cells, such as B and T cells (38-40). As mentioned above, in addition to adaptive autoimmune responses against synovial joint antigens, non-antigen-specific cellular events contribute to pathogenesis of RA. Our data suggest that Tyk2 plays central roles in both immune and inflammatory responses, thereby indicating that Tyk2 is involved in multiple steps during the development of RA. Therefore, Tyk2 is likely a potential therapeutic target for RA.

Acknowledgements

We thank Dr. Kotaro Ishibashi (Daiichi-Sankyo) for his help in breeding $Tyk2^{-/-}$ mice, and Dr. Yutaka Iigo (Daiichi-Sankyo) for histologic assessment of arthritic paws.

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Figure legends

Figure 1. CIA model in Tyk2 mutant B10.Q/J mice. B10.Q/Ai mice and Tyk2 mutant B10.Q/J mice were immunized with bovine type II collagen on days 0 and 21, and arthritis was induced after the 2nd immunization. Clinical arthritis score (a) and incidence (b) during the course of the experiment were strongly reduced in B10.Q/J mice. Each value represents mean + S.D. [#]p<0.05, ^{##}p<0.01, ^{###}p<0.001 (compared with the B10.Q/Ai mice group)

Figure 2. CAIA model in Tyk2^{-/-} mice. WT and Tyk2^{-/-} mice were intravenously injected with an anti-collagen antibody cocktail on day 0. a) Clinical arthritis score change during the experiment. b) Representative appearance of the hind limb on day 7 after CAIA induction. WT mice limbs showed obvious swelling. c) Hind paw volume on day 7. WT mice showed a significant increase in volume, but Tyk2^{-/-} mice didn't. Each value represents mean +/- S.D. ^{**}p<0.01, ^{***}p<0.001 (compared with control group); ^{##}p<0.01, ^{###}p<0.001 (compared with WT mice group)

Figure 3. Pathological appearance and pathological change in the CAIA model.

Representative H.E.-stained tarsal joint sections from CAIA-induced WT and Tyk2^{-/-} mice on day 7 as described in Methods. WT mice joints showed inflammatory cells infiltration, erosion synovium (arrowhead) and bone destruction (asterisk), but Tyk2^{-/-} mice had no critical change. (200× magnification)

Figure 4. Gene expression of cytokines in the CAIA model. Three or seven days after CAIA induction, gene expressions of Th1/Th17-related (A) and other pro-inflammatory cytokines (B) were evaluated in the hind paw of WT mice and Tyk2^{-/-} mice as described in Methods. IL17A and IFN- γ was significantly increased in WT mice at day 3, but not at day 7. Results are given as fold expression, compared with reference GAPDH expression, and then normalized with averaged WT control expression. Each value represents mean + S.D. *p<0.05, **p<0.01, (compared with the control group); #p<0.05, ##p<0.01 (compared with the WT mice group)

Figure 5. Inflammation-related gene expression in the CAIA model. Gene expressions of inflammation were evaluated in the hind paw. Chemokines gene expression, CCL2 and CXCL1, were highly elevated at day 3, and F4/80, macrophage marker, was elevated at day 7.

Elastase, neutrophil marker, didn't significantly changed in this experiment. MMPs expression were significantly elevated during the experiments. Each value represents mean + S.D. * $p < 0.05$, ** $p < 0.01$, (compared with the control group); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (compared with the WT mice group))

Figure 6. Phosphorylation of STAT proteins in the lymph node cells from the CAIA model. Popliteal lymph node cells were collected at day 3 and phosphorylation of STATs and I κ B expression were analysed as described in Methods. In cells from CAIA-induced WT group, STAT3 and STAT4 phosphorylation were observed, but completely diminished in Tyk2^{-/-} mice. Data represent independent cell lysate from 3 mice of each group.

Figure 1

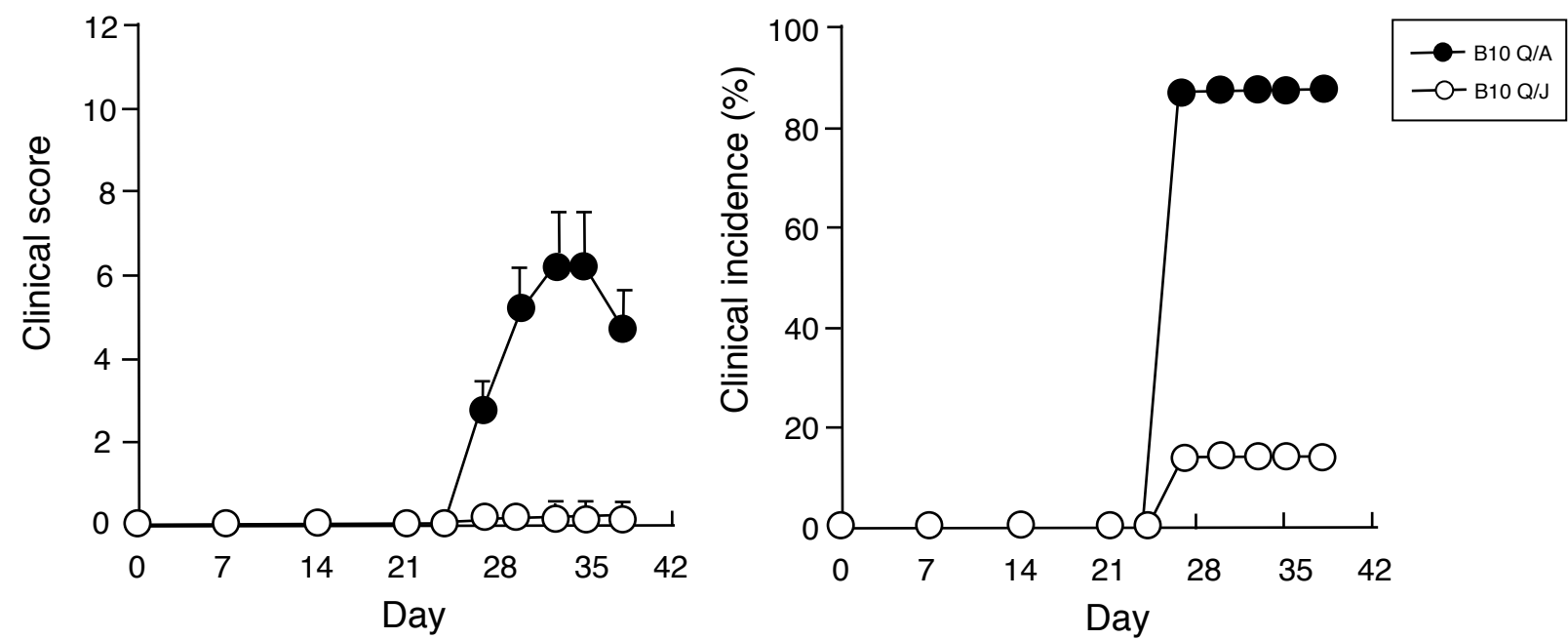


Figure 2

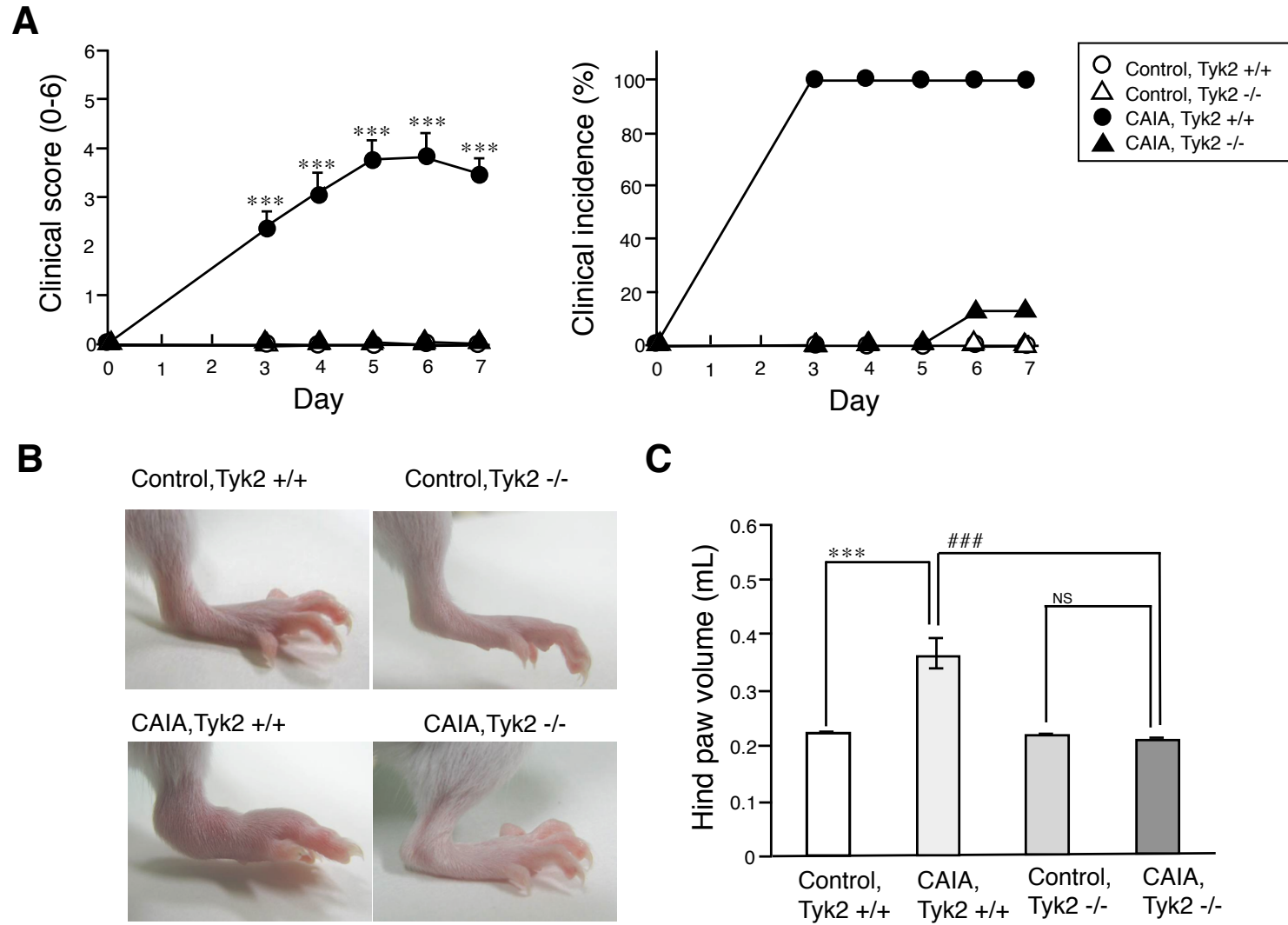
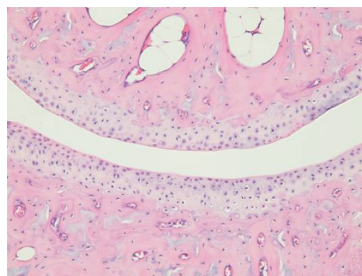
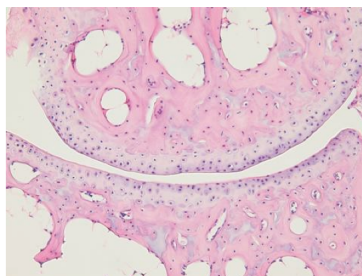


Figure 3

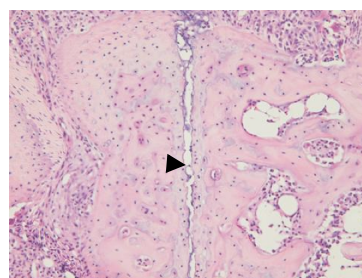
Control, Tyk2 +/+



Control, Tyk2 -/-



CAIA, Tyk2 +/+



CAIA, Tyk2 -/-

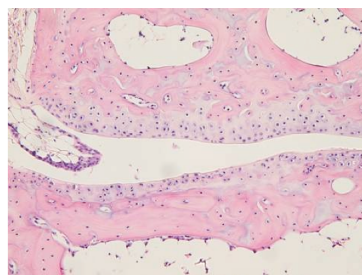
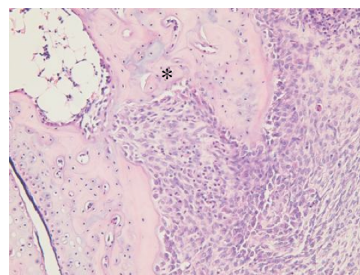
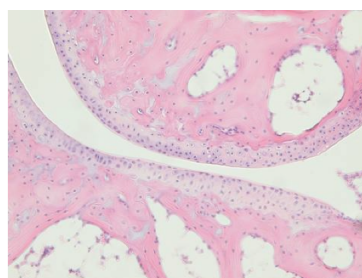


Figure 4

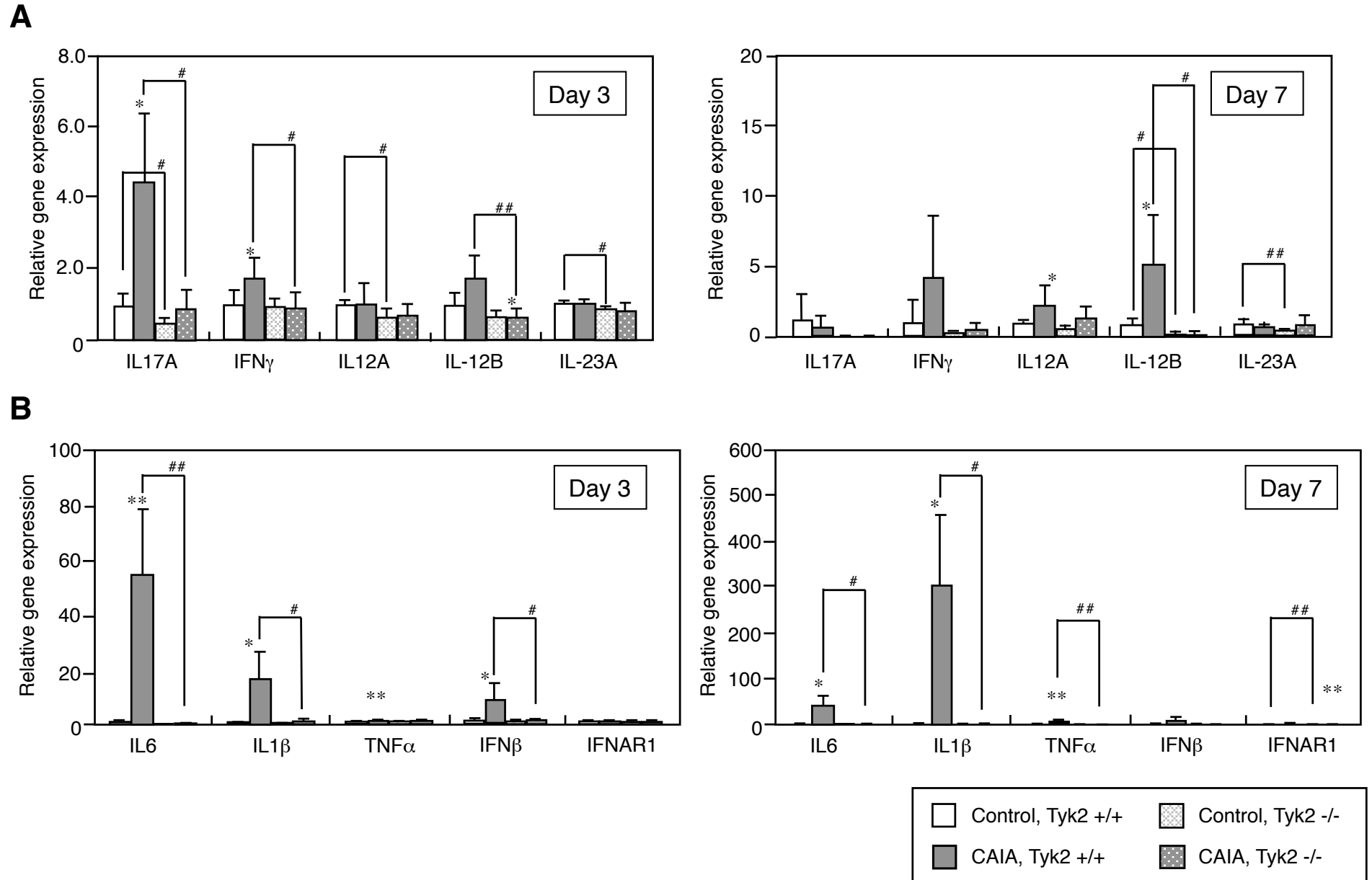


Figure 5

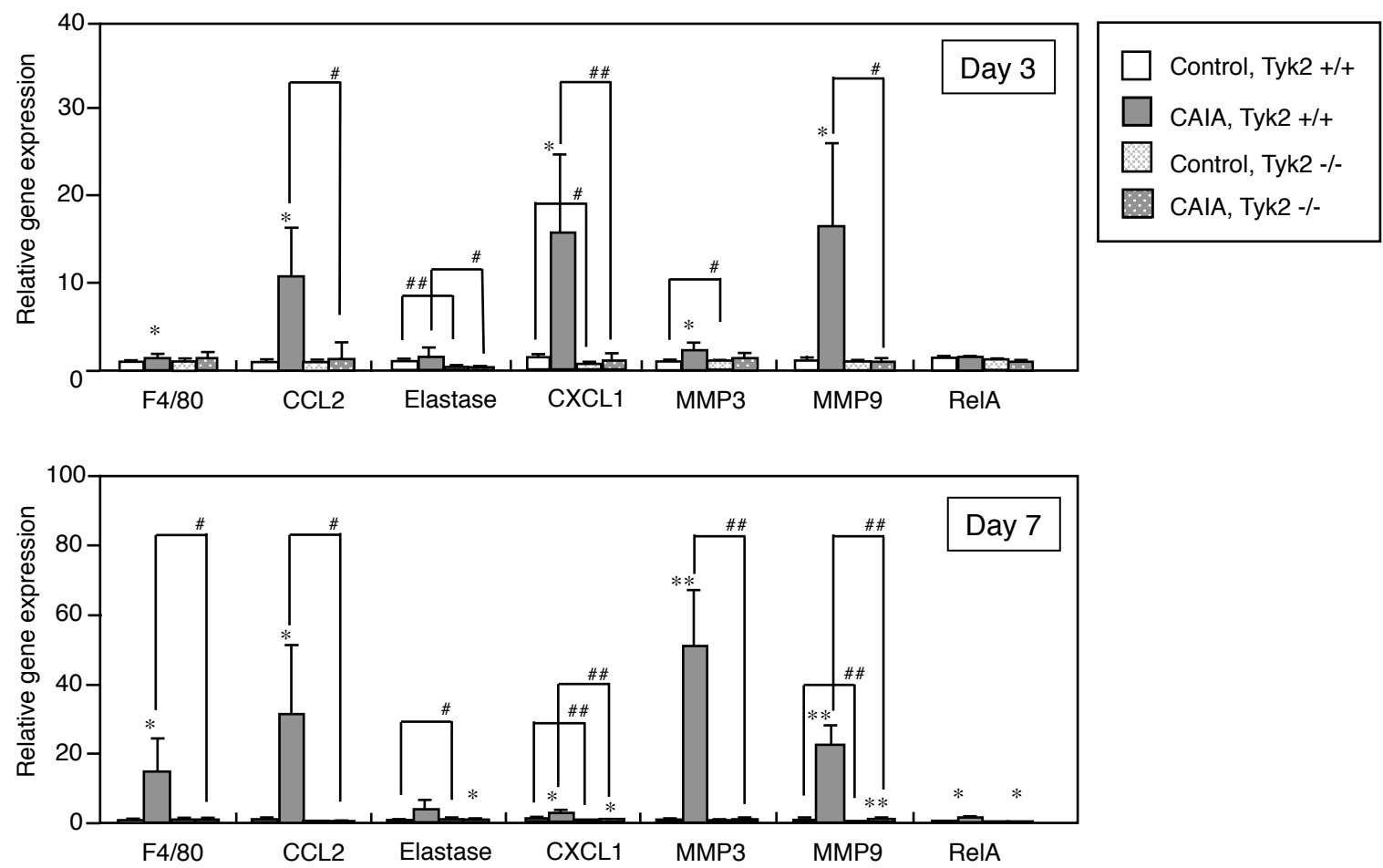


Figure 6

