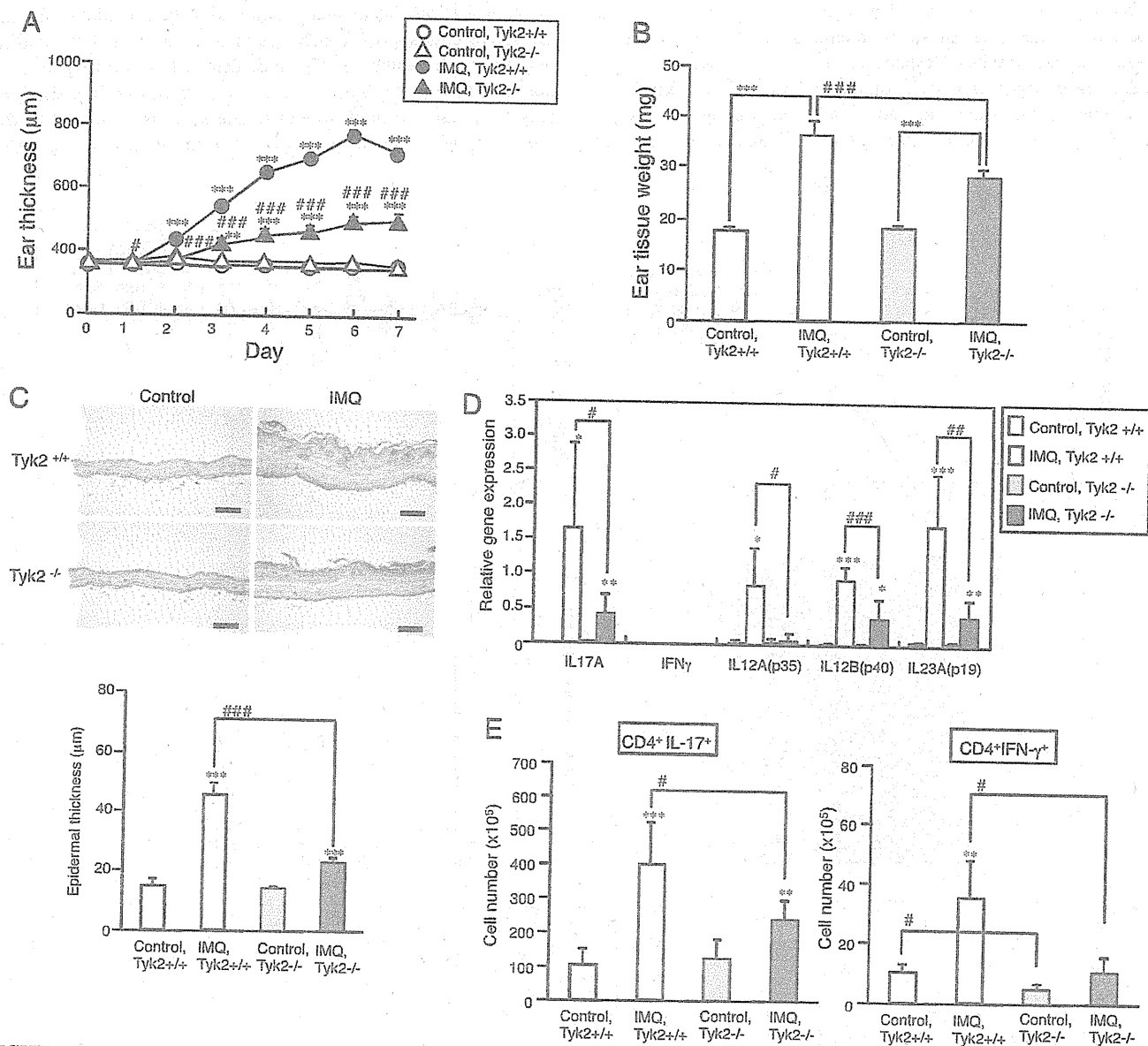


mm for  $Tyk2^{+/-}$  mice, and  $18.3 \pm 11.5 \times 10^{-2}$  mm for  $Tyk2^{-/-}$  mice). Therefore, *Tyk2* is crucial for DTH.

*IMQ-induced skin inflammation in Tyk2-deficient mice*

Recent studies suggested that IL-23 is functionally involved in the pathogenesis of psoriasis (33, 34). Expression of IL-23 is increased in psoriatic lesional skin, and intradermal injection of IL-23 into mouse skin results in erythema, a mixed inflammatory infiltrate, and epidermal hyperplasia (33). The IL-23/IL-17 axis also plays an important role in the development of IMQ-induced skin inflammation, as another model of psoriasis (29). Therefore, we investigated IMQ-induced skin inflammation in *Tyk2*-deficient mice. The ear swelling reaction was determined by the ear thickness and ear tissue weight. As shown in Fig. 4A, IMQ

treatment induced markedly enhanced ear thickness in  $Tyk2^{+/-}$  mice; however, a significant reduction in IMQ-induced ear thickness was observed in  $Tyk2^{-/-}$  mice. Similarly, the increase in ear tissue weight caused by IMQ treatment was also reduced in  $Tyk2^{-/-}$  mice compared with  $Tyk2^{+/-}$  mice (Fig. 4B). Histological analysis also revealed that ears injected with IMQ developed epidermal hyperplasia with inflammatory cellular infiltration (Fig. 4C). We next examined expression levels of the related cytokines in ear skin 24 h after IMQ was applied for 4 consecutive days. As shown in Fig. 4D, expression of IMQ-induced Th17-related cytokines was significantly reduced in  $Tyk2^{-/-}$  mice; interestingly, IL-12 (p35) expression was also reduced in these mice. We further investigated the number of  $CD4^+IL-17^+$  or  $CD4^+IFN-\gamma^+$  T cells in draining lymph nodes from  $Tyk2^{+/-}$  and  $Tyk2^{-/-}$



**FIGURE 4.** IMQ-induced skin inflammation in *Tyk2*-deficient mice. **A**, Ear skin of  $Tyk2^{+/-}$  and  $Tyk2^{-/-}$  mice was treated or not with IMQ for 6 consecutive days. Ear thickness was measured on the days indicated. Data represent mean ear thickness  $\pm$  SD for seven mice per group. **B**, The ear tissue weight on day 7 was measured. Data represent mean ear tissue weight  $\pm$  SD for seven mice per group. **C**, Representative histological features of IMQ-treated ear skin of  $Tyk2^{+/-}$  and  $Tyk2^{-/-}$  mice. H&E staining. Scale bar, 200  $\mu$ m. Epidermal hyperplasia, as quantified by imaging software from H&E-stained ear sections. Data represent mean epidermal thickness  $\pm$  SD for five mice per group. **D**, Effect of *Tyk2* deficiency on expression level of cytokines in ear skin at 24 h after application of IMQ for 4 consecutive days. Data represent mean gene expression  $\pm$  SD relative to GAPDH for five mice per group. **E**, The number of  $CD4^+IL-17^+$  and  $CD4^+IFN-\gamma^+$  T cells from draining lymph nodes of  $Tyk2^{+/-}$  and  $Tyk2^{-/-}$  mice that were treated or not with IMQ on the ear. After four consecutive days IMQ application, total draining lymph node cells were counted and stained CD4, IL-17, and IFN- $\gamma$  after PMA/ionomycin stimulation. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with control group; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  compared with  $Tyk2^{+/-}$  mice.

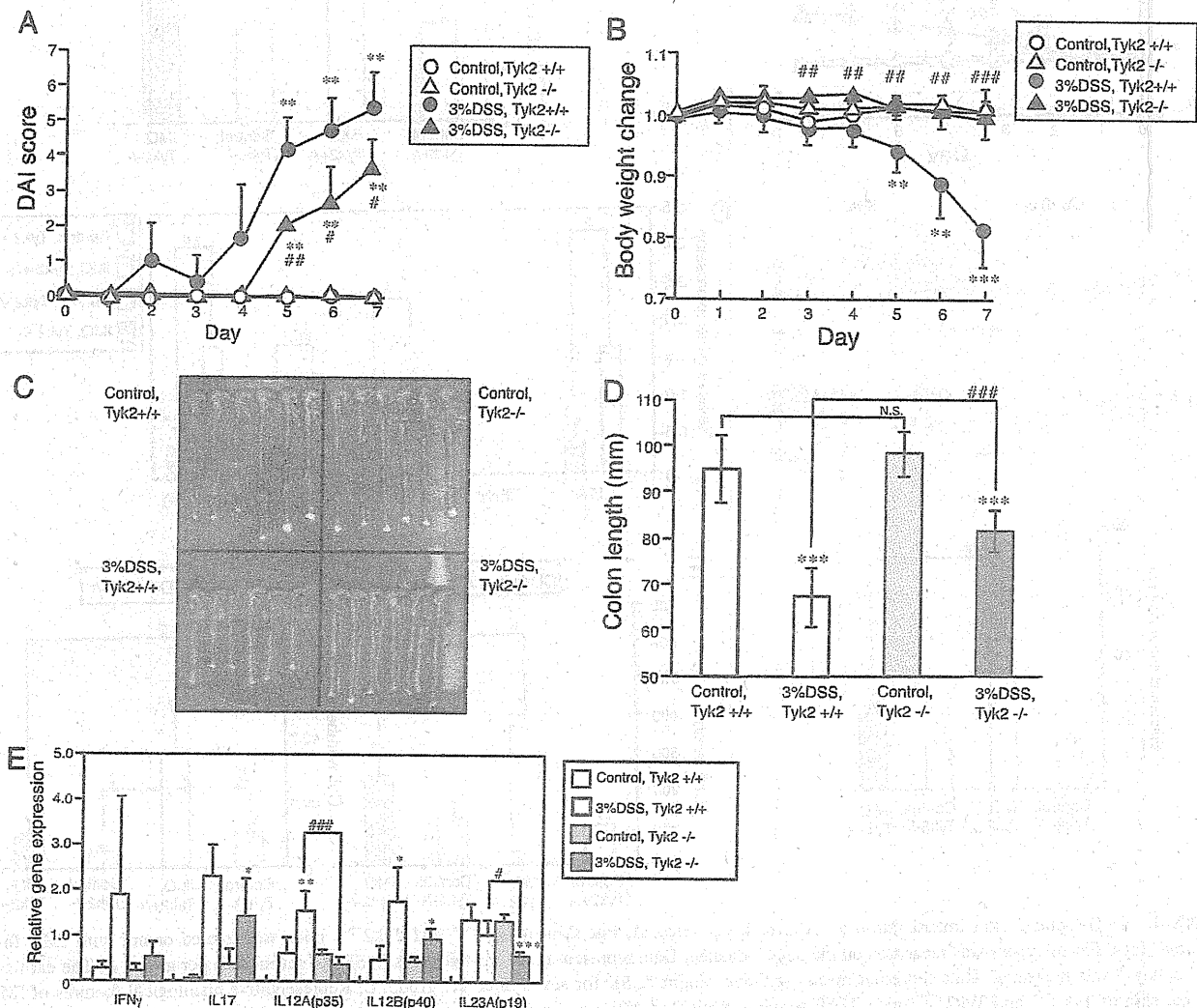
mice with or without IMQ treatment. As shown in Fig. 4E, the number of both CD4<sup>+</sup>IL-17<sup>+</sup> and CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells after IMQ treatment decreased markedly in Tyk2<sup>-/-</sup> mice. These results indicated that both Th1 and Th17 cells are involved in IMQ-induced skin inflammation through Tyk2. Therefore, Tyk2 is highly involved in the skin inflammation induced by IMQ treatment.

#### Experimental colitis in Tyk2-deficient mice

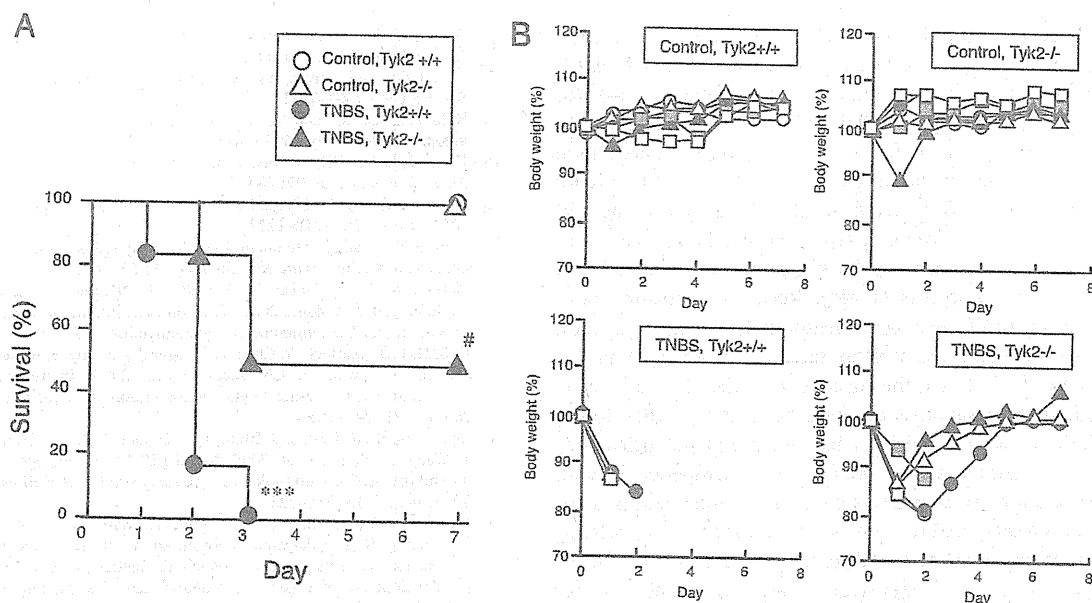
We further investigated whether Tyk2 plays a critical role in the pathogenesis of colitis, because two IL-12 family members (IL-12 and IL-23) were shown to play central roles in mediating intestinal inflammation (35–37). We first used DSS-induced colitis, a model of human Crohn's disease. All mice survived until sacrifice on day 7 (i.e., after 7 d of DSS treatment). Tyk2<sup>+/+</sup> and Tyk2<sup>-/-</sup> mice that received untreated water did not show any clinical signs (diarrhea, fecal occult blood, perianal bleeding, rectal prolapse, or weight loss) of spontaneous intestinal inflammation. In Tyk2<sup>+/+</sup> mice, DSS treatment produced experimental colitis, as assessed by the DAI, which began to increase on day 2. Tyk2<sup>-/-</sup> mice receiving DSS-containing water showed slower (beginning on day 5) and lower disease activity than did Tyk2<sup>+/+</sup> mice (Fig. 5A). The

significant reduction in the severity of DSS-induced colitis in Tyk2<sup>-/-</sup> mice was confirmed by evaluation of body weight loss and colon shortening. As shown in Fig. 5B, Tyk2<sup>+/+</sup> mice with DSS treatment started to exhibit decreased body weight on day 3 and lost ~20% of their body weight by day 7. In Tyk2<sup>-/-</sup> mice, DSS treatment had no effect on the body weight. Similarly, DSS treatment induced more severe shortening of the colon length in Tyk2<sup>+/+</sup> mice compared with Tyk2<sup>-/-</sup> mice (Fig. 5C, 5D). We next examined expression levels of the related cytokines in colon tissue. As shown in Fig. 5E, expression of DSS-induced Th1- or Th17-related cytokines was reduced in colon tissue from Tyk2<sup>-/-</sup> mice. Therefore, Tyk2 controls the disease activity in DSS-induced colitis through controlling the Th1/Th17 axis.

The involvement of Tyk2 in experimental colitis was also examined using the haptenating reagent TNBS. TNBS-induced colitis is dependent on T cells and is believed to be a model of human ulcerative colitis (31). TNBS treatment induced massive colitis, and all of the treated Tyk2<sup>+/+</sup> mice died within 3 d (Fig. 6A). In contrast, half of the Tyk2<sup>-/-</sup> mice treated with TNBS survived (Fig. 6A), and the body weight of the survivors returned to the normal range after they recovered from diarrhea (Fig. 6B).



**FIGURE 5.** DSS-induced experimental colitis in Tyk2-deficient mice. DAI (A) and body weight change (B) during the course of DSS treatment in Tyk2<sup>+/+</sup> and Tyk2<sup>-/-</sup> mice were monitored every day. C and D, Colon lengths in control and DSS-treated Tyk2<sup>+/+</sup> and Tyk2<sup>-/-</sup> mice were evaluated on day 7. Data represent the mean  $\pm$  SD for six mice per group. E, Effect of Tyk2 deficiency on expression level of cytokines in colon tissue at day 7 after DSS treatment. Data represent mean gene expression  $\pm$  SD relative to GAPDH for five mice per group. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 compared with control group; # $p$  < 0.05, ### $p$  < 0.001, #### $p$  < 0.001 compared with Tyk2<sup>+/+</sup> mice.



**FIGURE 6.** TNBS-induced experimental colitis in Tyk2-deficient mice. *A*, Mice were treated with 3.5 mg of TNBS in 40% ethanol by rectal instillation to induce colitis or with 40% ethanol alone to serve as colitis controls. TNBS-induced colitis-associated mortality in Tyk2<sup>+/+</sup> and Tyk2<sup>-/-</sup> mice was monitored every day. Data represent the survival ratio of mice. \*\*\**p* < 0.001 compared with control, #*p* < 0.05 compared with Tyk2<sup>+/+</sup> mice. *B*, Body weight changes during the course of TNBS treatment in each Tyk2<sup>+/+</sup> or Tyk2<sup>-/-</sup> mouse.

Therefore, Tyk2 seems to be a key molecule for controlling the development of experimental colitis in mice.

## Discussion

Tyk2 plays essential roles in IL-12- and/or IL-23-mediated signaling. Tyk2 deficiency resulted in impaired IFN- $\gamma$  secretion by IL-12-stimulated splenocytes, as well as IL-17 secretion by IL-23-stimulated splenocytes. However, Tyk2-deficient splenocytes showed normal responses to Con A and IL-2. In this study, we showed that both Th1 and Th17 cell differentiation from naive CD4<sup>+</sup> cells are affected in Tyk2-deficient mice. Furthermore, Tyk2 was not involved in Treg differentiation from naive CD4<sup>+</sup> cells by TGF- $\beta$ . We also found that Tyk2 participates in the development of a variety of experimental diseases, including DTH-induced footpad thickness, IMQ-induced psoriasis-like skin inflammation, and DSS- or TNBS-induced colitis. Taken together, our data strongly suggested that Tyk2 plays important roles in the development of various immune or inflammatory diseases by controlling the Th1/Th17 axis.

Recently, a patient homozygous for a Tyk2 mutation, which resulted in a premature termination codon and the absence of Tyk2 protein, was identified (24). The signaling defects in this Tyk2-deficient patient led to a complex clinical picture, including hyper-IgE syndrome and susceptibility to multiple infectious pathogens. Moreover, Tyk2 was recently identified as a strong multiple sclerosis-susceptibility gene by a genome-wide association study (38); in addition, Tyk2 polymorphisms were found to be associated with an increased risk for systemic lupus erythematosus (39). In the case of Tyk2-deficient mice, Tyk2 was reported to be a critical genetic regulator of EAE (21, 22), which is a model of multiple sclerosis. Although B10.Q/Ai mice, which express the Tyk2G allele, are susceptible to CIA, a Tyk2A mutation renders B10.D1 mice resistant to CIA (23, 40). These phenotypes caused by Tyk2 deficiency are likely to be mediated, in part, by alterations to the Th1/Th2 ratio and by impairment of Th17 cells. Our *in vivo* experiments further suggested the possible involvement of Tyk2 in immune diseases, which are mainly related to IL-12 and/or IL-23, in line with the above studies. DTH responses were

reported to be defective in IL-12p40- and IL-23p19-deficient mice (28, 32). Skin inflammation model induced by IMQ, TLR7/8 ligands, is a new model for human psoriasis, and IL-23p19- and IL-17A-deficient mice showed lower scores for erythema, scaling, and thickness after IMQ treatment (29). In fact, mRNA expression of IMQ-induced Th17-related cytokines was significantly reduced in Tyk2<sup>-/-</sup> mice (Fig. 4*D*). However, mRNA expression of Th1-related cytokines was also reduced in Tyk2<sup>-/-</sup> mice. Furthermore, the number of CD4<sup>+</sup>IL-17<sup>+</sup> or CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells in draining lymph nodes after IMQ treatment decreased markedly in Tyk2<sup>-/-</sup> mice (Fig. 4*E*). Therefore, both Th1 and Th17 cells are involved in IMQ-induced skin inflammation through Tyk2. Moreover, the DSS-induced colitis model is a little more complicated. Inflammatory bowel disease is characterized by sustained intestinal mucosa inflammation, which is mainly caused by excessive macrophage activation and Th1 and/or Th17 immune responses. However, it also occurs in SCID mice, which lack lymphocytes (41). Oral DSS activates intestinal macrophages, leading to massive production of inflammatory cytokines and chemokines. Subsequently, a number of lymphocytes are recruited to the inflamed sites, resulting in Th1 and Th17 responses. During this inflammatory process, Tyk2 could regulate the Th1 and Th17 responses, whereas the effects of Tyk2 on macrophages are unclear. Indeed, mRNA expression of DSS-induced Th1- or Th17-related cytokines was reduced in colon tissue from Tyk2<sup>-/-</sup> mice (Fig. 5*E*). One report described that Tyk2-deficient macrophages lack NO production upon stimulation with LPS (42), suggesting the possible involvement of Tyk2 in macrophage functions *in vivo*. In addition, Tyk2<sup>-/-</sup> dendritic cells were reported to be defective in IL-12 and IL-23 production upon stimulation with CpG oligodeoxynucleotide (43). Thus, dendritic cells/macrophages may also play an important role in the pathogenesis of these diseases, because our results suggested that Tyk2 deficiency may affect both pathogenic IFN- $\gamma$  and IL-17. Further experiments are still required in this regard.

The first *in vivo* evidence that Jaks are critical for cytokine signaling came from studies of a group of human disorders termed SCID. Jak3 selectively associates with the common  $\gamma$  cytokine

receptor chain, and mutations in its receptor are known to cause SCID. Therefore, mutations of Jak3 were sought and found to underlie some cases of autosomal recessive SCID (1, 44). Recently, a patient with Tyk2 deficiency was reported to exhibit impaired immune system functions (24). Regarding mutations in Jaks, the somatic Jak2 valine-to-phenylalanine (V617F) mutation has been identified; it is detected in up to 90% of patients with polycythemia and in a sizeable proportion of patients with other myeloproliferative disorders, such as essential thrombocythemia and idiopathic myelofibrosis (1, 45). Recently, activating point mutations in the *JAK1* gene were identified in patients with acute lymphoblastic leukemia, as well as rarely in acute myeloid leukemia patients (46). Since the discovery of the Bcr-Abl kinase inhibitor imatinib, great advances have been made in developing kinase inhibitors with exquisite selectivities and potencies (47). Therefore, Jak inhibitors may confer great therapeutic benefits through disease control in patients with autoimmune diseases and leukemia, which presumably result from high levels of circulating cytokines that signal through Jak enzymes. Our results further suggest that Tyk2 could represent a target molecule for the treatment of immune abnormalities.

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### Disclosures

The authors have no financial conflicts of interest.

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

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## Abstract

Tyrosine kinase-2 (Tyk2) participates in the signaling pathways of multiple cytokines in innate and acquired immunity. In the present study, we investigated 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, whereas 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 T<sub>H</sub>1/T<sub>H</sub>17-related cytokines, the other proinflammatory 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:** anti-type II collagen antibody-induced arthritis, rheumatoid arthritis, Tyk2

## 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<sup>+</sup> T cells infiltrating into the RA synovial membrane are predominantly T<sub>H</sub>1 phenotypes (5, 6). According to a current paradigm, a pathogenic role of T<sub>H</sub>1-type cellular immunity is supposed to prevail over a beneficial T<sub>H</sub>2 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 auto-antibodies, 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<sup>d</sup>) mice and CB8

in B10.RIII (H-2<sup>f</sup>) 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-type II collagen 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).

Tyrosine kinase-2 (Tyk2), a Jak family of kinases, is activated in response to various cytokines including 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- $\alpha/\beta$ -mediated signals to suppress hematopoietic cell growth, but not for those to induce antiviral activities (18, 19). Thus, the involvement of Tyk2 in IFN- $\alpha/\beta$  signaling is restricted. In the case of IL-12-mediated signaling, signals for IFN- $\gamma$  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<sup>+</sup> 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, USA; B10.Q/J), unlike B10.Q/Ai mice, their counterparts bred at Taconic Farms (Tarrytown, NY, USA) 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 non-conservative 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 auto-antibodies after collagen challenge and the inflammatory responses after reactions of auto-antibodies to joints. The CAIA model requires only inflammatory responses after a challenge with a cocktail of anti-CII 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 $\kappa$ B $\alpha$  antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-pSTAT3(Tyr705) and anti-pSTAT4(Tyr693) antibodies from Cell Signaling Technologies (Beverly, MA, USA); and anti-actin antibody from Millipore (Billerica, MA, USA). 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 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 intra-dermally immunized at the tail with an emulsion of 150  $\mu$ g of bovine

CII (Collagen Gijutsu Kensyukai, Tokyo, Japan) in 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, wild-type (WT) or Tyk2<sup>-/-</sup> mice were intravenously (i.v.) injected with a mixture of five anti-CII 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*

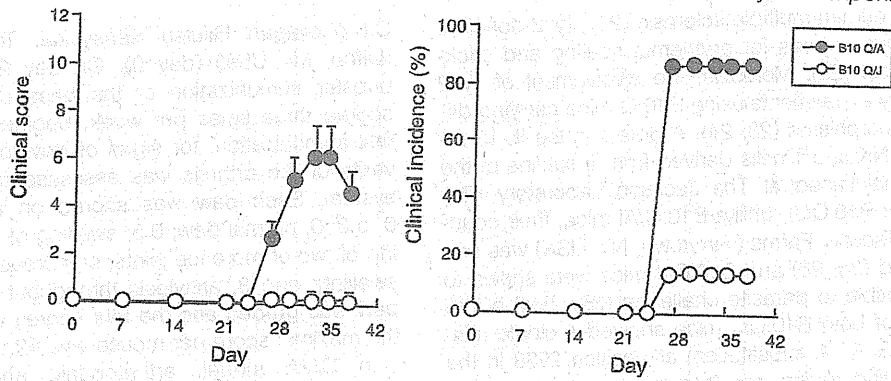
For histological processing, paws were fixed in phosphate buffer containing 10% formaldehyde and decalcified with EDTA. Paws were processed by routine methods to paraffin blocks. Specimens were sectioned at 6  $\mu$ m and stained with H&E. The sections were evaluated for the degree of synovial hyperplasia,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

### *Extraction of paw RNA and TaqMan analysis of gene expression*

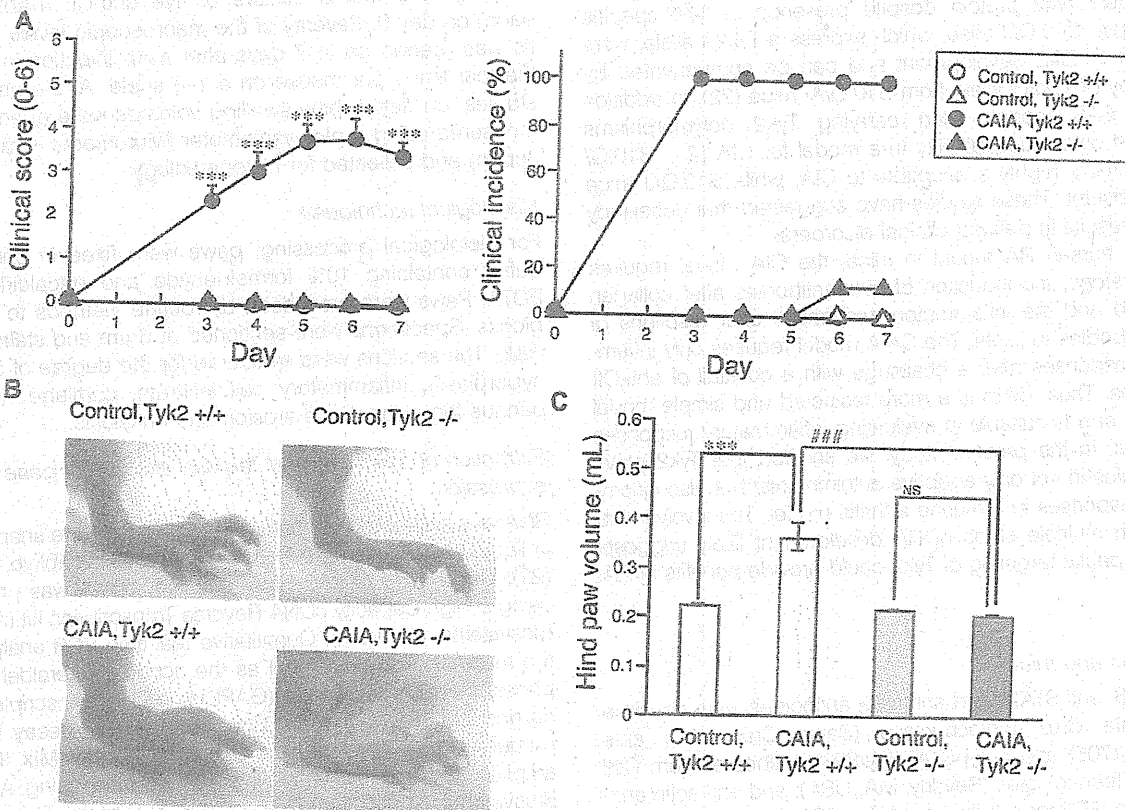
RNA was extracted from cells in paws, which were snap frozen in liquid nitrogen, using ISOGEN (Nippon Gene, Tokyo, Japan) (29). Using 5  $\mu$ g of total RNA template, cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). Quantitative real-time PCR analyses of the respective gene, as well as the control glyceraldehyde-3-phosphate dehydrogenase (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, 3 days after CAIA induction, popliteal lymph nodes were collected and 10<sup>6</sup> lymph node cells were lysed in 20  $\mu$ l of radio-immunoprecipitation assay buffer (Santa Cruz Biotechnology). The cell lysates were resolved on SDS-PAGE and transferred to Polyvinylidene fluoride transfer membrane (PerkinElmer, Boston, MA, USA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore).



**Fig. 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 CII on days 0 and 21, and arthritis was induced after the second 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 + SD.  $^{\#}P < 0.05$ ,  $^{\#\#}P < 0.01$ ,  $^{\#\#\#}P < 0.001$  (compared with the B10.Q/Ai mice group).



**Fig. 2.** CAIA model in Tyk2<sup>-/-</sup> mice. WT and Tyk2<sup>-/-</sup> mice were i.v. injected with an anti-collagen antibody cocktail on day 0. (A) Clinical arthritis score change and incidence 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<sup>-/-</sup> mice did not. Each value represents mean ± SD.  $^{\#\#\#}P < 0.001$  (compared with control group);  $^{\#\#\#}P < 0.001$  (compared with WT mice group).

**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 *t*-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

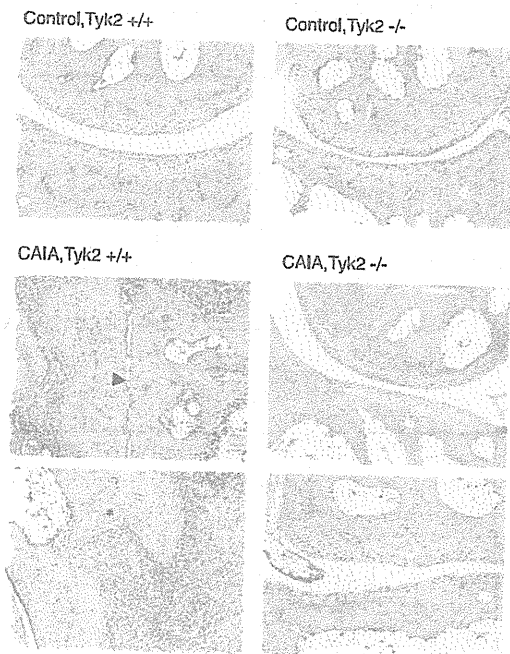
software (SAS System Release 8.2; SAS Institute Inc., Cary, NC, USA).

**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)





**Fig. 3.** Pathological appearance and pathological change in the CAIA model. Representative H&E-stained tarsal joint sections from CAIA-induced WT and *Tyk2*<sup>-/-</sup> mice on day 7 as described in Methods. WT mice joints showed inflammatory cells infiltration, erosion synovium (arrowhead) and bone destruction (asterisk), but *Tyk2*<sup>-/-</sup> mice had no critical change ( $\times 200$  magnification).

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  $\sim 2$  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*<sup>-/-</sup> mice. CIA is mediated by auto-antibodies 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*<sup>-/-</sup> 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 mAbs alone was i.v. administrated to *Tyk2*<sup>-/-</sup> and 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. 2(A), 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  $\sim 4$  on days 5–7. In contrast, *Tyk2*<sup>-/-</sup> mice were resistant to CAIA,

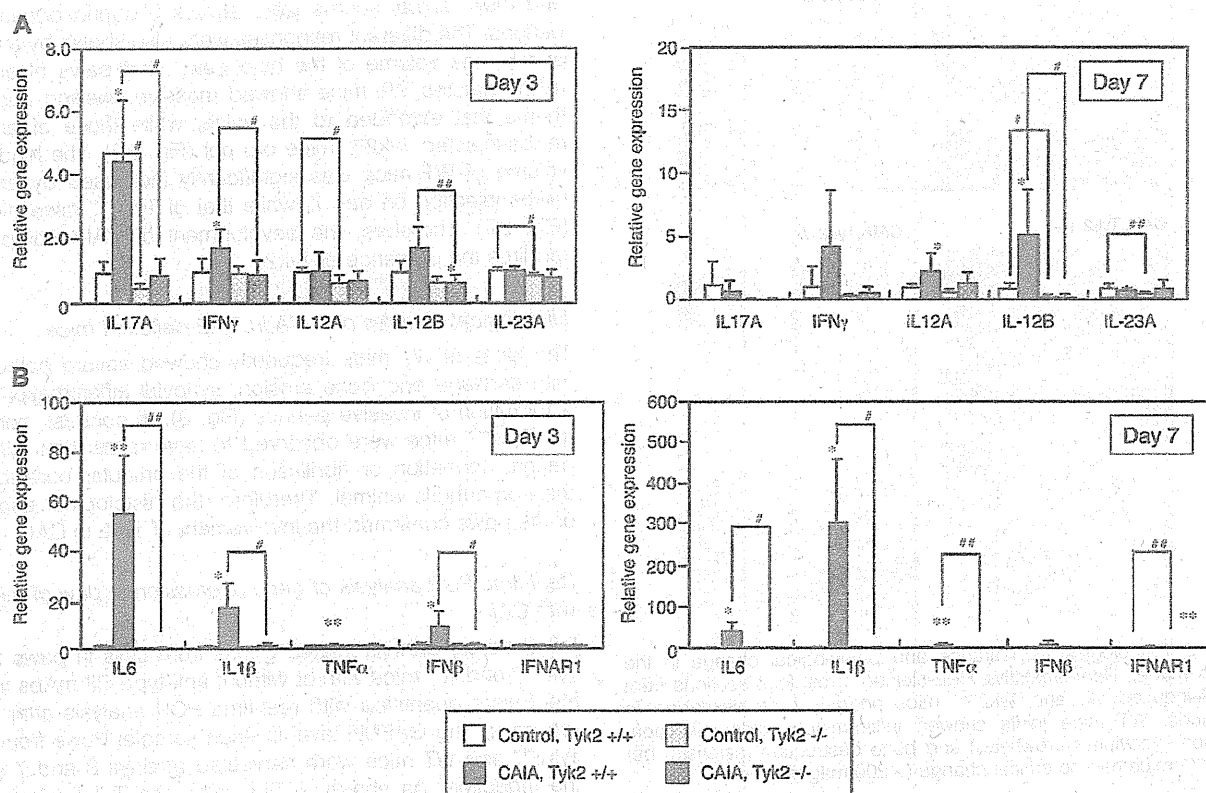
and their clinical scores were always 0 during observation periods. The different responses were also shown by a photo and by the volume of the hind paw. Hind paws of anti-CII mAbs-injected WT mice showed massive swelling and erythema that extended to the ankle, while those of anti-CII mAbs-injected *Tyk2*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice were observed to have more than minimal pannus formation or fibrillation of the articular cartilage in the non-arthritic 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*<sup>-/-</sup> 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*<sup>-/-</sup> and WT mice were harvested at days 3 and 7 after the induction. As shown in Fig. 4(A), the  $T_H1/T_H17$ -related cytokines such as IFN- $\gamma$  and IL-17 were significantly induced at day 3 by anti-CII mAbs injection in WT mice, whereas expression of these cytokines decreased at day 7, indicating that the  $T_H1/T_H17$ -related cytokines are involved in early stage of development of CAIA. Importantly, these cytokines were significantly reduced in *Tyk2*<sup>-/-</sup> mice. The inflammatory cytokines such as IL-6, IL-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$  and IFN- $\beta$  were also induced by anti-CII mAbs injection in WT mice at days 3 and 7 (Fig. 4B), and their induction was significantly impaired in *Tyk2*<sup>-/-</sup> 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 days 3 and 7 (Fig. 5). This macrophage accumulation was also impaired in *Tyk2*-deficient mice at day 7. Similarly, a neutrophil marker, 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*<sup>-/-</sup> mice compared with WT mice, indicating that decreased expression of CCL2 and CXCL1 may result in a reduced accumulation of macrophages and neutrophils. In addition, gene expression of matrix metalloproteinase 9 and MMP3, which are involved in matrix degradation, was strongly induced in WT mice, but not in *Tyk2*<sup>-/-</sup> 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  $T_H1/T_H17$ -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.



**Fig. 4.** Gene expression of cytokines in the CAIA model. Three or seven days after CAIA induction, gene expressions of  $T_H1/T_H17$ -related (A) and other pro-inflammatory cytokines (B) were evaluated in the hind paw of WT mice and  $Tyk2^{-/-}$  mice as described in Methods. IL-17A and IFN- $\gamma$  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 + SD. \* $P < 0.05$ , \*\* $P < 0.01$ , (compared with the control group); # $P < 0.05$ , ## $P < 0.01$  (compared with the WT mice group).

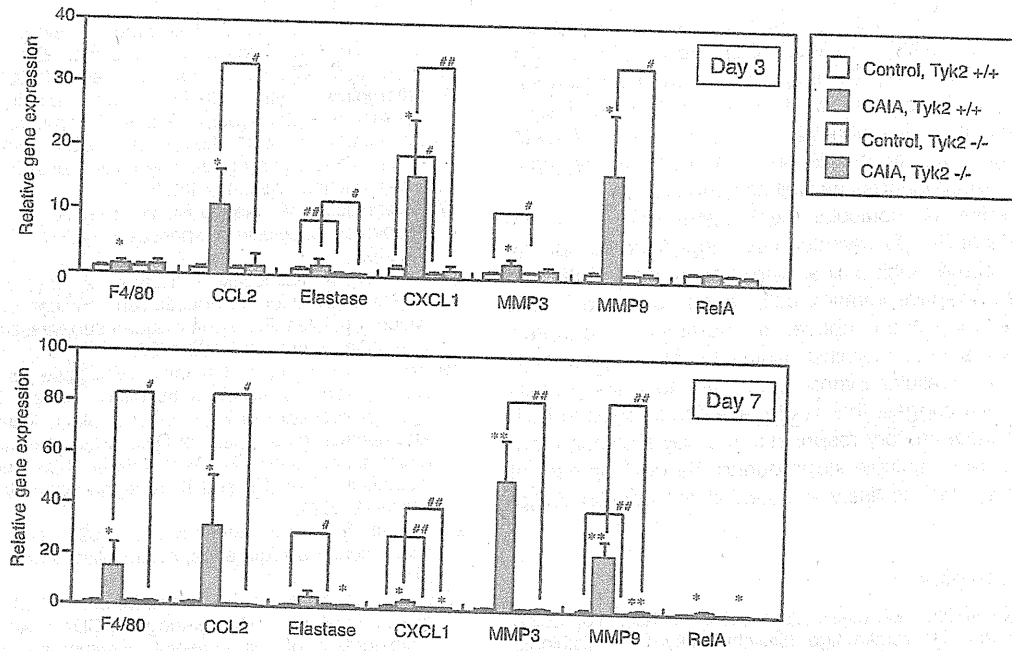
To see molecular mechanisms underlying the above findings, we finally investigated activation of STAT3 and STAT4 during development of CAIA. As shown in Fig. 6, anti-CII mAbs injection induced phosphorylation of STAT3 and STAT4 in cells from draining lymph nodes was observed in WT but not  $Tyk2^{-/-}$  mice. These results showed that the  $T_H1/T_H17$ -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 $\beta$ 1 subunit, which associates with  $Tyk2$ . IL-12 guides  $CD4^+$  T cells to  $T_H1$  cells, which produce signature cytokine IFN- $\gamma$  along with pro-inflammatory cytokines; and IL-23 is involved in the expansion, maintenance and functional maturation of  $T_H17$  cells, which play essential roles in the pathogenesis of chronic inflammatory disorders (31, 32). Thus,  $Tyk2$  seems to be indispensable not only for the  $T_H1$  axis but also immune responses mediated by IL-17-producing  $T_H17$  cells. Therefore, Shevach *et al.* who first reported the involvement of  $Tyk2$  in CIA suggested that the pathological effects of  $Tyk2$  polymorphisms in arthritis are defects of  $T_H1$ -mediated response through IL-12 signaling (25). Indeed, CII-specific T cells derived from B10.Q/J failed to produce IFN- $\gamma$ , whereas T cells from B10.Q/Ai mice could produce normal amounts of IFN- $\gamma$ . 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 auto-antibodies. 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

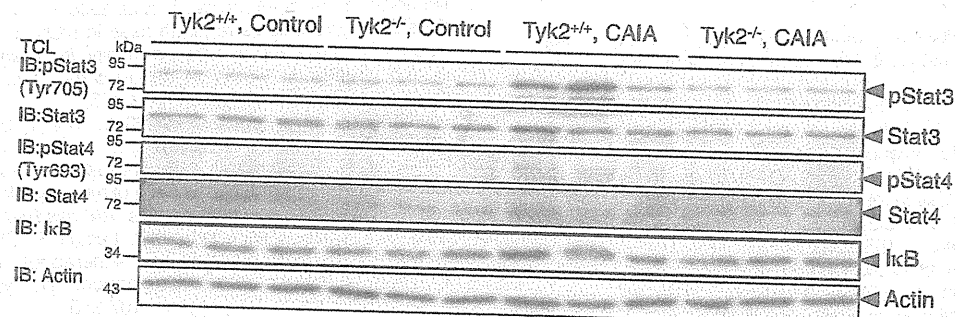


**Fig. 5.** Inflammation-related gene expression in the CAIA model. Gene expressions of inflammation were evaluated in the hind paw. Chemokines gene expression, CCL2 and CXCL1, were highly elevated at day 3, and F4/80, macrophage marker, was elevated at day 7. Elastase, neutrophil marker, did not significantly changed in this experiment. MMPs expression was significantly elevated during the experiments. Each value represents mean + SD. \* $P < 0.05$ , \*\* $P < 0.01$  (compared with the control group); # $P < 0.05$ , ## $P < 0.01$  (compared with the WT mice group).

clarified that Tyk2 also play 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<sup>-/-</sup> 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 auto-antibodies 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<sup>-/-</sup> mice did not develop severe CAIA (34). As we showed here, Tyk2<sup>-/-</sup> 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<sup>-/-</sup> mice severely impaired the production of IFN- $\gamma$ , TNF, IL-6 and MMPs. With regard to IFN- $\gamma$ , this cytokine seems to oppositely suppress the development of arthritis because IFN- $\gamma$ <sup>-/-</sup> 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 synovio-cytes, 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),



**Fig. 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 $\kappa$ B expression were analyzed as described in Methods. In cells from CAIA-induced WT group, STAT3 and STAT4 phosphorylation were observed, but completely diminished in Tyk2<sup>-/-</sup> mice. Data represent independent cell lysate from three mice of each group.

suggesting the possible involvement of Tyk2 in macrophage functions *in vivo*. Tyk2<sup>-/-</sup> 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 CIA, 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.

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