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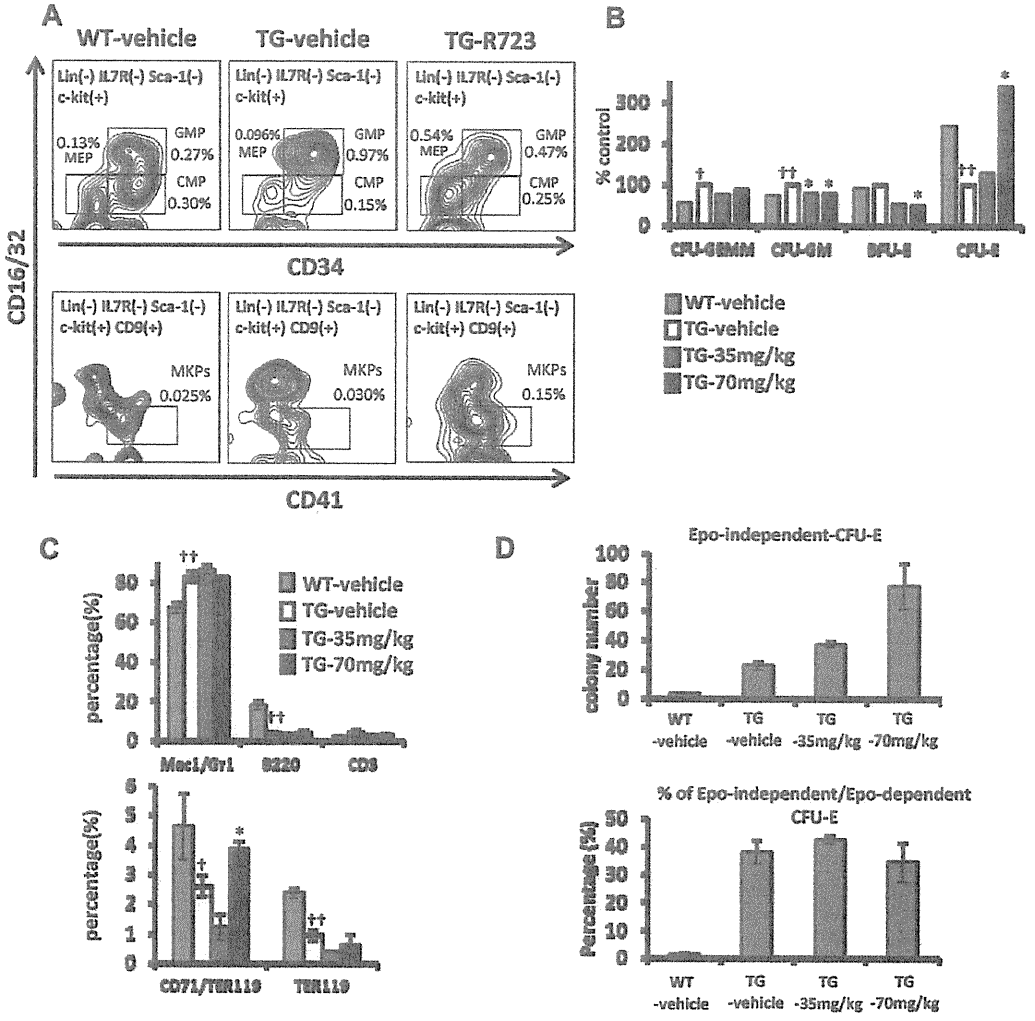

Figure 5. Effect of R723 on BM of V617F-TG mice. (A) BM progenitors in V617F-TG mice and effect of in vivo R723 treatment. V617F-TG mice were dosed orally with R723 at 70 mg/kg or vehicle for 4 weeks. BM cells were collected, and the proportion of CMPs ($Lin^-Sca\text{-}1^-cKit^-CD34^+ Fc\gamma R^{lo}$), GMPs ($Lin^-Sca\text{-}1^-cKit^-CD34^+Fc\gamma R^{hi}$), MEPs ($Lin^-Sca\text{-}1^-cKit^+CD34^-Fc\gamma R^{-/lo}$), and MKPs ($Lin^-cKit^+CD9^+Fc\gamma R^{lo}$ $CD41^+$) was determined by FACS. In V617F-TG mice, the proportion of GMPs increased, and those of CMPs, MEPs, and MKPs were comparable to those in WT mice. Four weeks of R723 treatment in V617F-TG mice normalized the proportion of GMPs. MEPs and MKPs were increased compared with WT mice or V617F-TG mice treated with vehicle. These results are representative of 6 independent experiments. (B) Changes in BM progenitor cells after R723 treatment. The numbers of CFU-GEMM and CFU-GM colonies increased and the numbers of CFU-E colonies decreased in the BM from 18-week-old TG-vehicle mice compared with those in the BM from the age-matched WT-vehicle mice (†$P < .05$; ‡$P < .01$). R723 treatment for 6 weeks suppressed CFU-GM and BFU-E colonies (*$P < .05$). In contrast, CFU-E colonies increased in V617F-TG mice after R723 treatment (**$P < .01$). Data are presented as means ± SE. (C) FACS analysis of BM cells. The percentage of Mac-1/Gr-1^+ myeloid cells increased and that of $B220^+$ B cells decreased in 18-week-old V617F-TG mice treated with vehicle (TG-vehicle) compared with the age-matched WT mice treated with vehicle (WT-vehicle) (‡$P < .01$). R723 treatment for 6 weeks had little effect on the proportion of BM myeloid or B cells in V617F-TG mice (top panel). The proportion of CD71/TER119 double-positive erythroblasts and TER119 single-positive erythroblasts decreased in TG-vehicle mice compared with that in WT-vehicle mice (†$P < .05$; ‡$P < .01$). Significant restoration of CD71/TER119-positive erythroblasts was observed in V617F-TG mice after R723 treatment for 6 weeks (*$P < .05$). TER119 single-positive erythroblasts were not restored by R723 treatment (bottom panel). Data are presented as means ± SE. (D) Changes in EPO-independent CFU-E colonies after in vivo R723 treatment. The number of EPO-independent CFU-E colonies increased in BM cells from 18-week-old TG-vehicle mice compared with that in BM cells from the age-matched WT-vehicle mice (†$P < .01$). In vivo R723 treatment for 6 weeks increased the number of EPO-independent CFU-E colonies (*$P < .01$). Data are presented as means ± SE. Whereas EPO-dependent CFU-E colonies increased in V617F-TG mice after in vivo R723 treatment (panel B), the proportion of EPO-independent/EPO-dependent CFU-E colonies remained the same.

and MKP cells were comparable to those of the WT mice. Four weeks of R723 treatment in V617F-TG mice normalized the proportion of GMP cells ($P < .05$). MEP and MKP cells were increased compared with the WT or V617F-TG mice treated with vehicle ($P < .01$; Figure 5A and supplemental Table 2). Conversely, the LSK cell population was not changed by 4 weeks of R723 treatment. The proportion of BM progenitors was also analyzed by colony formation assay. CFU-GEMM and CFU-GM colonies were increased in number in the BM from the TG-vehicle group compared with that from the WT-vehicle group. The suppression of CFU-GM was observed in V617F-TG by R723 treatment (Figure 5B).

Mac-1/Gr-1^+ myeloid cells in the BM of the TG-vehicle group were increased, and $B220^+$ B cells were decreased compared with that of the WT-vehicle group (Figure 5C). R723 treatment had little effect on the proportion of BM myeloid and B cells in V617F-TG mice.

We then extended our analysis to erythroid-lineage cells. The proportions of CD71/TER119 double-positive early erythroblasts and TER119 single-positive late erythroblasts were decreased in

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the TG-vehicle group compared with those in the WT-vehicle group (Figure 5C and supplemental Table 2). Significant restoration of CD71/TER119 double-positive early erythroblasts was observed in the TG-70mg/kg group, but TER119 single-positive late erythroblasts were not restored by R723 treatment. CFU-E colonies were decreased in number in the BM from the TG-vehicle group compared with that in the WT-vehicle group. The suppression of BFU-E was observed in V617F-TG mice by R723 treatment; however, the number of CFU-E colonies increased in the TG-70mg/kg group compared with that in the TG-vehicle group (Figure 5B). The number of EPO-independent CFU-E colonies was increased in the V617F-TG group by in vivo R723 treatment compared with that in the TG-vehicle group (Figure 5D). Whereas the number of EPO-dependent CFU-E colonies was increased in V617F-TG mice by in vivo R723 treatment, the proportion of EPO-independent/EPO-dependent CFU-E colonies remained the same.

Discussion

In the present study, R723, a potent, orally bioavailable JAK2-selective inhibitor, was shown to have significant in vitro activity against JAK2V617F. In addition, R723 demonstrated good efficacy and low toxicity in vivo in 3 animal models. Our data indicate that R723 has the potential for effective treatment of V617F-positive MPNs.

R723 was equipotent against both the WT and the V617F mutant-type of JAK2 in biochemical assays and, correspondingly, R723 equally inhibited colony formation of both V617F-TG and WT BM cells. In the PHZ-induced anemia model, R723 was able to significantly delay EPO-driven hematocrit recovery from chemical insult, indicating its in vivo ability to inhibit WT JAK2 kinase in the context of EPO receptor pathway overstimulation. R723 was also effective in 2 JAK2V617F-driven animal models, the Ba/F3-JAK2V617F leukemia model, and in V617F-TG mice. In the aggressive murine leukemia model using Ba/F3-JAK2V617F cells, R723 had clear beneficial effects on disease progression by suppressing the proliferation of tumor cells, which led to an incremental improvement in survival and a decrease in the overall tumor burden in the spleen and BM, although the blood of the R723-treated mice did not inhibit phosphorylation of STAT5 in Ba/F3-JAK2V617F cells completely, only by 30%-40% (supplemental Figure 4 and 1C). The ultimate confirmation of R723 efficacy came from testing it in V617F-TG mice with features closely resembling those seen with human PMF,[9] which is often poorly controlled with conventional therapy and the most appropriate target of a new therapeutic approach using a JAK2 inhibitor. V617F-TG mice have shorter lives than WT mice. In our study, the animals died suddenly, precluding detailed histopathologic analysis. Among a small cohort of V617F-TG mice in which limited necropsy was performed, subcutaneous and intestinal hemorrhage and gangrenous bowels were observed, which might have been caused by abdominal vessel obstruction. In addition, the bleeding time of V617F-TG mice was extremely prolonged compared with that of WT mice (data not shown). We therefore speculated that the cause of death was thrombosis and/or hemorrhage. Mullally et al[32] and Akada et al[33] also reported that thrombotic events might be the cause of V617F-expressing mice. Oral administration of R723 in V617F-TG mice led to the prolongation of survival and profound improvements in leukocytosis, thrombocytosis, and hepatosplenomegaly. These results raise hope for improvements in the treatment of PMF patients. Conversely, anemia, megakaryocyte hyperplasia, and fibrosis in BM were little affected. The effect of R723 on BM fibrosis varied greatly between models. It was not dissolved in V617F-TG mice during R723 treatment, and instead became just as advanced as it was in V617F-TG mice treated with vehicle. R723 treatment caused a significant reduction of myelofibrosis in recipient mice transplanted with V617F-TG BM cells together with WT BM cells (supplemental Figure 5), but the reason for this is not yet clear. Severe progression of myelofibrosis might be resistant to JAK2 inhibition, because the degree of myelofibrosis severity in recipient mice was much milder than that in V617F-TG mice. Interestingly, this outcome closely resembled the results of human clinical trials of other JAK2 inhibitors, in which the main improvements were limited to rapid decreases in spleen size and normalization of leukocyte count without resolution of fibrosis and only marginal improvements in transfusion dependence.[25,26] R723 also has effects in recipient mice transplanted with BM cells from V617F-TG mice. In this transplantation model, R723 seemed to prefer inhibiting the growth of cells harboring V617F to WT cells, although R723 had a nearly identical inhibition profile for both WT and V617F mutant JAK2 in the in vitro kinase assay and equally inhibited colony formation of both WT and V617F-TG BM cells.

The *JAK2V617F* mutation is observed in the majority of PV patients and in approximately half of PMF patients. The effect of the JAK2 mutation on erythropoiesis differs from disease to disease. PV is characterized by erythrocytosis, whereas the main feature of PMF is progressive anemia. The situation was similar in our mouse model. We previously reported 2 lines of V617F-transgenic mice: line 1 transgenic mice exhibiting PV- or ET-resembling features and line 2 transgenic mice (this line was used to evaluate the effect of R723) exhibiting PMF-like diseases such as BM fibrosis, leukoerythroblastosis, and anemia. *JAK2V617F* knock-in mice resembling human PV were described recently.[32] The reason that erythrocytosis or anemia resulted from the same JAK2 mutation both in human diseases and mice models is not clear. We found that some genes were specifically expressed in line 2 transgenic mice (data not shown), and now characterize their roles. These proteins may cooperate with JAK2V617F to influence erythropoiesis.

The effect of R723 on cell components seems to differ by cell lineage. As mentioned previously, R723 did not improve anemia but did effectively improve leukocytosis and thrombocytosis in V617F-TG mice. The frequency of CFU-E and the CD71/TER119 double-positive cells, which represent late erythroid progenitors and erythroblasts,[34] respectively, decreased in V617F-TG mice compared with WT mice, and R723 treatment elevated these erythroid progenitors to levels nearly equal to that of WT mice. This improvement was probably due to the ability of R723 to inhibit the abnormal JAK2V617F signal. However, TER119 single-positive cells, which represent late erythroblasts, were still low in V617F-TG mice after R723 treatment. The generation of committed erythroid progenitors did not require EPO, but the terminal maturation from CD71/TER119 double-positive cells to TER119 single-positive cells did require EPO,[35] indicating that differentiation depends on JAK2 activation. Because R723 inhibits WT JAK2 as well as JAK2V617F, the inhibition of WT JAK2 by R723 might abrogate the terminal differentiation of erythroblasts to erythrocytes, and this might be the reason that anemia was not improved in V617F-TG mice treated with R723. JAK2V617F-selective inhibitors, which do not inhibit WT JAK2, might overcome this problem.

There are JAK2V617F-positive disease-initiating cells that have long-term, multipotent, and self-renewing activity in the

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hematopoietic stem cell compartment.[36,37] Mullually et al[32] reported that the *JAK2V617F* mutation had nominal effects on the size or function of the LSK compartment critical for MPN initiation and that the JAK2 inhibitor TG101348 failed to eliminate the MPN-initiating population in *JAK2V617F* knock-in mice, even though the treatment demonstrated histopathological improvement of the erythroid hyperplasia and statistically significant reductions in spleen size. As we found in the present study, the compartment of LSK cells increased in V617F-TG mice and R723 treatment did not suppress them, showing again that the effect of R723 on cell components augmented by JAK2V617F seems to differ by cell lineage. R723 effectively suppresses granulopoiesis induced by JAK2V617F, does not suppress LSK cells expanded by JAK2V617F, and suppresses the abnormal JAK2V617F signals in late erythroid progenitors and erythroblasts and the normal EPO signals in late erythrocytes.

To use a JAK2 inhibitor for the treatment of MPNs, long-term therapy is likely to be required for maintenance of remission, such as in the case of imatinib mesylate for BCR-ABL–positive chronic myeloid leukemia.[36] In addition to the standard toxicities, emphasis should be placed on minimization of activity against other kinases, especially JAK1 or JAK3, to prevent immunosuppression associated with prolonged inhibition of JAK3 and possibly JAK1.[37,38] R723 demonstrated reasonable selectivity in both in vitro kinase- and cell-based assays, although the selectivity was somewhat different between the 2 assay systems. In cell-based assays, a few fold selectivity was observed when using mouse CTLL-2 or human primary T cells compared with related JAK2 assays. A single oral dose of R723 demonstrated a preference for targeting the JAK2-dependent GM-CSF pathway in granulocytes rather than the JAK1- or JAK3-dependent IL-15 pathway in T cells. Six weeks of oral administration of R723 to V617F-TG mice showed that the inhibitory effect of R723 on T or B lymphocytes was much less than that on myeloid cells. These data demonstrate that R723 has a limited immunosuppressive effect and a potentially favorable clinical safety profile. All JAK2 inhibitors currently used in clinical trials inhibit JAK2 potently, but their off-target profiles are variable.[21-24] The clinical implications of the distinct profiles remain to be determined.

In conclusion, we identified a potent inhibitor of JAK2, R723, which showed some selectivity in vitro in biochemical and cell-based assays. R723 demonstrated efficacy in vivo in 3 animal models addressing different aspects of disease progression. In V617F-TG mice, which closely mimic human PMF, R723 significantly improved survival, hepatosplenomegaly, leukocytosis, and thrombocytosis, thus confirming the viability of a targeted therapy approach in managing *JAK2V617F*-positive MPNs. We conclude that R723 could become a viable option available to PMF, PV, and ET patients who develop resistance to conventional therapies.

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Authorship

Contribution: K. Shide and V.M. performed research; V.M., E.T., S.F, C.L., M.G., W.L., J.R., J.M., S.B., J.C., C.L., A.R., S.S., P.P., G.P., A.T., M.D., R.S., D.G.P., and Y.H. contributed to screening and initial characterization of the inhibitor; V.M. and Y.H. guided the screening and initial characterization and wrote the manuscript; T.K., H.K.S., and T.M. analyzed results; and K. Shide and K. Shimoda conceived the research, guided its design, analysis, and interpretation, and wrote the manuscript.

Conflict-of-interest disclosure: V.M., E.T., S.F., C.L., M.G., W.L., J.R., J.M., S.B., J.C., C.L., A.R, S.S., P.P., G.P., A.T., M.D., R.S., D.G.P., and Y.H. are or have been employees of Rigel Pharmaceuticals, Inc. The remaining authors declare no competing financial interests.

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Involvement of Tyrosine Kinase-2 in Both the IL-12/Th1 and IL-23/Th17 Axes In Vivo

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Tyrosine kinase-2 (Tyk2), a member of the Jak family of kinases, mediates the signals triggered by various cytokines, including type I IFNs, IL-12, and IL-23. In the current study, we investigated the in vivo involvement of Tyk2 in several IL-12/Th1– and IL-23/Th17–mediated models of experimental diseases, including methylated BSA injection-induced footpad thickness, imiquimod-induced psoriasis-like skin inflammation, and dextran sulfate sodium- or 2,4,6-trinitrobenzene sulfonic acid-induced colitis. In these disease models, Tyk2 deficiency influenced the phenotypes in immunity and/or inflammation. Our findings demonstrate a somewhat broader contribution of Tyk2 to immune systems than previously expected and suggest that Tyk2 may represent an important candidate for drug development by targeting both the IL-12/Th1 and IL-23/Th17 axes. ***The Journal of Immunology*****, 2011, 187: 181–189.**

Various combinations of Jak family members selectively associate with cytokine receptors to transmit signals that are involved in various cellular events (1). In the case of tyrosine kinase-2 (Tyk2), it is activated in response to various cytokines, including IFNs, IL-6, IL-10, IL-12, IL-13, and IL-23 (2–7). However, Tyk2 is dispensable for IL-6– and IL-10–mediated signaling in mice (8, 9). We and other investigators reported that Tyk2 is required for IFN-α/β–mediated signals to suppress hematopoietic cell growth but not for the signals that induce antiviral activities (10, 11). Thus, the involvement of Tyk2 in IFN-α/β–mediated signaling is restricted. In contrast, IL-12–mediated signals, especially those for IFN-γ production by T cells, are highly dependent on Tyk2 (8, 9, 12). Because Tyk2 is recruited to IL-12Rβ1, IL-23–mediated signaling also probably involves Tyk2 (7). Consequently, experiments using Tyk2-deficient cells revealed that different levels of dependence on Tyk2 are evident among several cytokines.

IL-12, whose receptor is associated with Tyk2 and Jak2 and mainly activates the transcription factor STAT4, is the lineage-specific cytokine responsible for Th1 generation (5, 13). Phosphorylation of STAT4, in conjunction with signals derived from TCR-mediated stimuli, induces the expression of T-bet, a master transcriptional regulator of IFN-γ–producing Th1 cells (14). Although IL-23 was initially shown to induce the differentiation of Th17 cells, it is now generally accepted that the differentiation of Th17 cells is dependent on TGF-β and IL-6 and that IL-23 is instead involved in the expansion, maintenance, and functional maturation of Th17 cells (15). IL-17 is now believed to play essential roles in the pathogenesis of chronic inflammatory disorders, as well as in the host defenses against various pathogens (15, 16). It is noteworthy that IL-12 and IL-23 have common features. As heterodimeric cytokines, they share the p40 subunit, and their receptors share the IL-12Rβ1 subunit, which associates with Tyk2. Thus, Tyk2 seems to be indispensable for the IFN-γ/Th1 axis but is also involved in the immune responses mediated by IL-17–producing Th17 cells. Because Th1 and Th17 are both mainly involved in the proinflammatory immune responses that are controlled by Tyk2, mutations leading to loss of function of Tyk2 can be expected to lead to striking immunological phenotypes.

Experimental allergic encephalomyelitis (EAE), which is induced by immunization with myelin Ags or by adoptive transfer of myelin-specific CD4 effector cells, is an animal model of multiple sclerosis (17). Notably, recent studies demonstrated that Th17 cells are responsible for the development of EAE (18). Indeed, IL-23p19– and IL-12/IL-23 p40-deficient mice are resistant to EAE (19, 20). Tyk2-deficient mice also show lower clinical scores and inflamed CNS areas in this model (21). Moreover, the involvement of Tyk2 was confirmed by experiments using mice carrying different Tyk2 polymorphisms (22). B10.D1 mice, which express the Tyk2A allele, are resistant to EAE development and can be compensated by one copy of the Tyk2G allele from B10.Q/Ai mice. In addition to the EAE model, mice carrying Tyk2 polymorphisms exhibit susceptibility in a model for collagen-induced arthritis (CIA) (23). B10.Q/Ai mice are highly susceptible to CIA, whereas B10.D1 mice are resistant. These studies suggested that deficiency of Tyk2 results in defined clinical disorders. Recently, a patient with Tyk2 deficiency was reported (24). The patient experienced increased susceptibility to viral and mycobacterial infections, atopic dermatitis, and elevated IgE levels, thereby exhibiting broader and more profound immunological defects than expected from studies of Tyk2-deficient mice. The different

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Abbreviations used in this article: CIA, collagen-induced arthritis; DAI, disease activity index; DSS, dextran sulfate sodium; DTH, delayed-type hypersensitivity; EAE, experimental allergic encephalomyelitis; IMQ, imiquimod; mBSA, methylated BSA; TNBS, 2,4,6-trinitrobenzene sulfonic acid; Treg, regulatory T cell; Tyk2, tyrosine kinase-2.

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dependencies on Tyk2 between human and mice could arise from species specificity. Alternatively, precise analyses of Tyk2-deficient mice may reveal new aspects of Tyk2 functions in vivo.

In the current study, we investigated the in vivo involvement of Tyk2 in several IL-12/IL-23–dependent models of experimental diseases, namely delayed-type hypersensitivity (DTH), imiquimod (IMQ)-induced psoriasis-like skin inflammation, and dextran sulfate sodium (DSS)- or 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. Our results indicated a somewhat broader contribution of Tyk2 to immune systems than previously expected and suggested that Tyk2 may represent an important candidate for drug development by targeting both the IL-12/Th1 and IL-23/Th17 axes.

Materials and Methods

Mice

B10.D1-H2q/SgJ (B10.D1) mice bearing the Tyk2A allele and B10.Q/Ai mice with the Tyk2G allele were purchased from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms (Germantown, NY), respectively. Gene-targeted Tyk2-deficient mice were backcrossed for at least eight generations onto BALB/c mice (8, 25). 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.

Cell proliferation assay and ELISA

The proliferation of viable splenocytes after Con A treatment was measured using a WST-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) (26). Briefly, 10 μl WST-8 solution was added to the cells in each well and incubated for 3 h. Absorbance was measured at a test wavelength of 450 nm, and IL-2 in culture supernatants was measured by specific ELISA (Abcam, Cambridge, U.K.). To quantify IFN-γ and IL-17 production, splenocytes were stimulated with murine IL-2, IL-12, or IL-23 for 48 h or 72 h, and each culture supernatant was measured by specific ELISA (R&D Systems, Minneapolis, MN), according to the manufacturers' instructions.

Analysis of Th cell differentiation

Wild-type and Tyk2-deficient splenocytes were positively selected based on CD4 expression with Dynabeads CD4$^+$ T cell positive selection (Invitrogen, Carlsbad, CA), and the bead-bound cells were detached using DETACHaBEAD (Invitrogen, Carlsbad, CA). The CD4$^+$ T cells were subsequently separated based on CD62L expression with MACS CD62L MicroBeads and MACS separation columns (Miltenyi Biotec, Auburn, CA). The separated naive CD4$^+$CD62L$^+$ T cells were activated by plate-bound anti-CD3 and soluble anti-CD28 (BD Pharmingen, San Diego, CA) and cultured in the presence of murine IL-6 (50 ng/ml; PeproTech, Rocky Hill, NJ) and human TGF-β (5 ng/ml; R&D Systems) with murine IL-23 (10 ng/ml; R&D Systems) for Th17 cell, IL-12 (1 ng/ml; PeproTech) for Th1 cell, and TGF-β (5 ng/ml) for regulatory T cell (Treg) differentiation (27). After 3 d in culture, cells were restimulated with PMA and ionomycin for 4 h, followed by addition of GolgiPlug (BD Pharmingen). Cells were permeabilized and fixed with Cytofix/Cytoperm (BD Pharmingen), according to the manufacturer's instructions. Detection of IFN-γ– and IL-17–producing cells was determined by intracellular cytokine staining with anti–IFN-γ–FITC or anti–IL-17–PE (BD Pharmingen). Cells were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using CellQuest (BD Biosciences) and FlowJo (TreeStar, Ashland, OR) software.

DTH responses

Six mice per group were injected s.c. with 250 μg methylated BSA (mBSA) (Sigma-Aldrich, St. Louis, MO) at two sites in the abdomen in a combined total of 100 μl a 1:1 emulsion of CFA (BD Biosciences, San Diego, CA) and saline (28). On day 7 following immunization, the mice were challenged by injection of 50 μl 0.5 mg/ml mBSA in saline into one rear footpad, whereas the other rear footpad received 50 μl PBS. Measurements

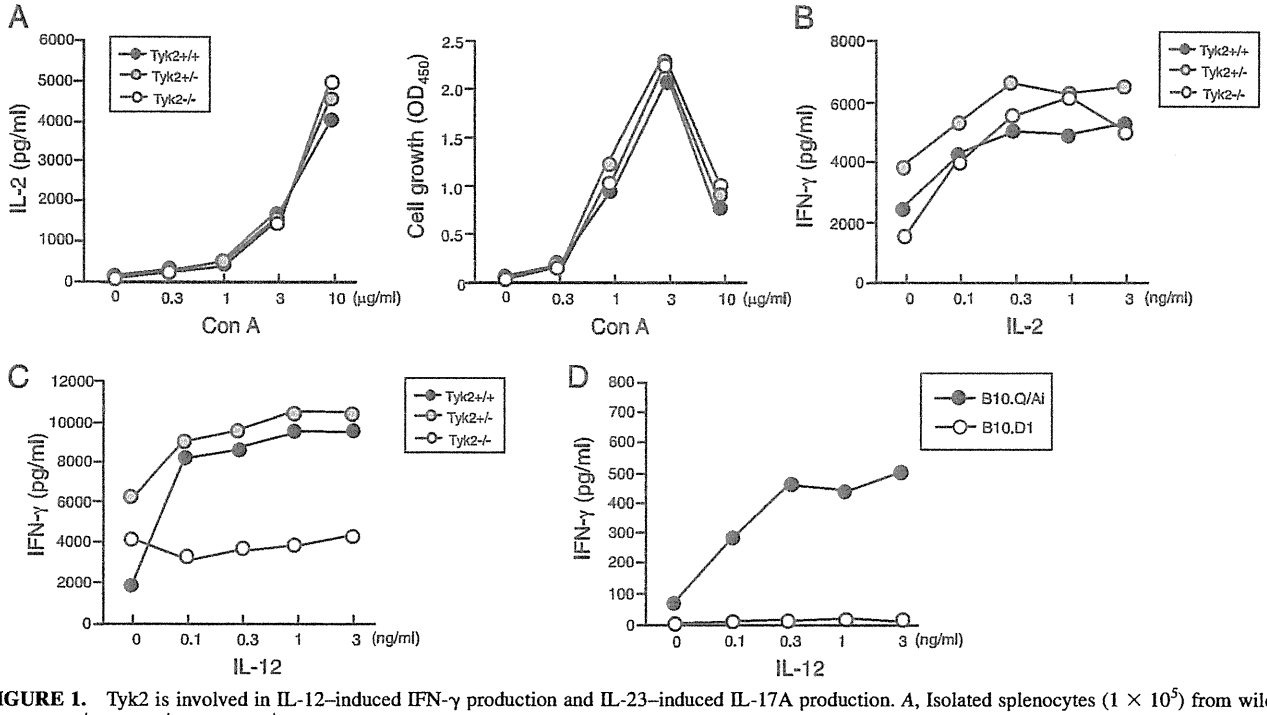

FIGURE 1. Tyk2 is involved in IL-12–induced IFN-γ production and IL-23–induced IL-17A production. *A*, Isolated splenocytes (1×10^5) from wild-type (Tyk2$^{+/+}$), Tyk2$^{+/-}$, or Tyk2$^{-/-}$ mice were analyzed for IL-2 production and proliferative responses after stimulation with or without Con A (0–10 μg/ml) for 48 h. Results are representative of two independent experiments. *B*, Isolated splenocytes (5×10^4) from Tyk2$^{+/+}$, Tyk2$^{+/-}$, or Tyk2$^{-/-}$ mice were analyzed for IFN-γ production after stimulation with or without IL-2 (0–3 ng/ml) in the presence of anti-CD3 mAb for 48 h. Results are representative of two independent experiments. *C*, Isolated splenocytes (5×10^4) from Tyk2$^{+/+}$, Tyk2$^{+/-}$, or Tyk2$^{-/-}$ mice were analyzed for IFN-γ production after stimulation with or without IL-12 (0–3 ng/ml) in the presence of anti-CD3 mAb for 48 h. Results are representative of two independent experiments. *D*, Isolated splenocytes (5×10^4) from B10.Q/Ai or B10.D1 mice were analyzed for IFN-γ production after stimulation with or without IL-12 (0–3 ng/ml) in the presence of anti-CD3 mAb for 48 h. Results are representative of two independent experiments.

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of footpad swelling were taken at 24 h after challenge, using a thickness gauge (Mitutoyo, Kanagawa, Japan). The magnitude of the DTH responses was determined from differences in footpad thickness between the Ag-and saline-injected footpads.

IMQ treatment and scoring severity of skin inflammation

At 8–11 wk of age, mice received a daily topical dose of 5 mg commercially available IMQ cream (5%) (Beselna Cream; Mochida Pharmaceuticals, Tokyo, Japan) on a side of both ears for 6 consecutive days (29). To score the severity of inflammation of the ear skin, both affected ear thickness and ear tissue weight were measured. At the days indicated, the ear thickness of both ears was measured using the thickness gauge (Mitutoyo) and averaged. Also, after application on 4 consecutive days, ears were collected for quantitative PCR analysis, and ear-draining lymph node cells were collected for FACS analysis. In FACS analysis, cells were stimulated for 4 h by PMA/ionomycin with GolgiPlug and stained with anti-CD4 Ab conjugated with Alexa Fluor 647 (BD Pharmingen) and anti–IFN-γ or anti–IL-17 Ab, as indicated.

DSS-induced colitis

Colitis was induced by means of drinking water supplemented with 3% DSS (45,000–50,000 m.w.; MP Biomedicals, Santa Ana, CA), as described previously (30). Control mice were treated in a similar manner with drinking water without DSS. The disease activity index (DAI) and histological score were assessed in accordance with established criteria (30), which combined scores of weight loss, consistency, and bleeding divided by 3, and acute clinical symptoms with diarrhea and/or extremely bloody stools.

Hapten-induced colitis

TNBS-induced colitis has been described (31). Mice were immunized with 3.5 mg TNBS (Sigma-Aldrich) in 40% ethanol by intrarectal

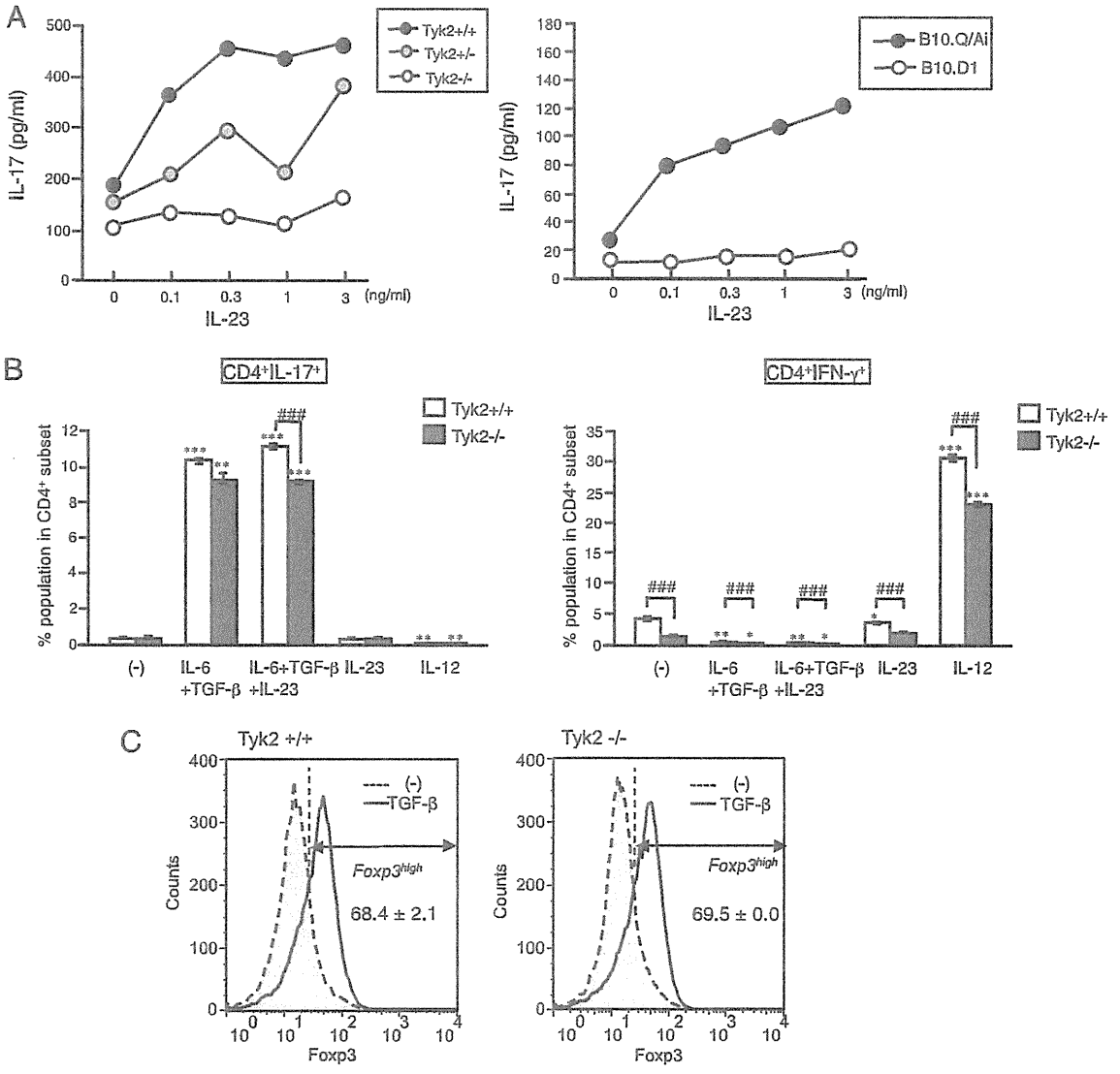

FIGURE 2. Tyk2 is involved in IL-23–induced IL-17A production and Th17 differentiation. *A*, Isolated splenocytes (2×10^5) from $Tyk2^{+/+}$, $Tyk2^{+/-}$, or $Tyk2^{-/-}$ mice were analyzed for IL-17 production after stimulation with or without IL-23 (0–3 ng/ml) in the presence of anti-CD3 mAb for 72 h. Results are representative of two independent experiments. Also, the same analysis was done using isolated splenocytes (5×10^4) from B10.Q/Ai or B10.D1 mice, and comparable results were observed. *B*, Isolated $CD4^+CD62L^+$ T cells (2.5×10^6) from $Tyk2^{+/+}$ or $Tyk2^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb (5 μg/ml) and soluble anti-CD28 mAb (1 μg/ml) in Th1/Th17 cell-inducing conditions. Seventy-two hours poststimulation, cells were harvested and immediately subjected to intracellular cytokine staining for IL-17 and IFN-γ. $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cell populations were gated, and their ratios were compared. The results are mean ± SD of three independent experiments. *C*, Isolated $CD4^+CD62L^+$ T cells (2.5×10^6) from $Tyk2^{+/+}$ or $Tyk2^{-/-}$ mice were stimulated in the Treg-inducing conditions. Seventy-two hours poststimulation, cells were analyzed by Foxp3 staining. Representative graphs of three independent experiments are shown. Dashed line indicates the staining of naive T cells without TGF-β stimulation, and solid line indicates staining with TGF-β stimulation. Results were analyzed based on the percentage of $Foxp3^{high}$ population in $Tyk2^{+/+}$ or $Tyk2^{-/-}$ cells. The results represent mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group; $^{\#\#\#}p < 0.001$ compared with $Tyk2^{+/+}$ group.

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administration. Each experimental group contained six 6–9-wk-old female mice.

Extraction of tissue RNA and TaqMan analysis of gene expression

RNA was extracted from ears and colons using ISOGEN (Nippon Gene, Tokyo, Japan). Using 5 μg total RNA template, cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA). 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 Gene Expression Master Mix. PCR amplification and evaluation were performed using 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.

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). Using this software, the epidermal thickness was measured at six positions per section and averaged.

Statistical analyses

All unpaired data were analyzed by an F test to evaluate the homogeneity of variance. If the variance was homogeneous, the Student t test was applied. If the variance was heterogeneous, the Welch t test was performed. For paired comparisons, the Student paired t test was performed. In other cases, the Wilcoxon rank-sum test, for scoring data, and the Tukey test, for the comparison of $Tyk2^{+/+}$, $Tyk2^{+/-}$, and $Tyk2^{-/-}$ mice, were performed. The log-rank test was performed in survival experiments. A 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

In vitro experiments reveal the involvement of Tyk2 in IL-12–induced IFN-γ production and IL-23–induced IL-17A production

To clarify and confirm the roles of Tyk2 in effector T cell functions, we used splenocytes derived from wild-type ($Tyk2^{+/+}$), Tyk2-heterodeficient ($Tyk2^{+/-}$), and Tyk2-deficient ($Tyk2^{-/-}$) mice. These three types of splenocytes exhibited similar responses to the T cell mitogen Con A, resulting in the production of IL-2 and cell growth (Fig. 1*A*). These cells also showed no significant differences in their IFN-γ production levels after IL-2 stimulation (Fig. 1*B*). However, the splenocytes from $Tyk2^{-/-}$ mice did not produce IFN-γ in response to IL-12, whereas those from $Tyk2^{+/+}$ and $Tyk2^{+/-}$ mice did (Fig. 1*C*). The impaired response to IL-12 was also observed in splenocytes from Tyk2-mutant mice (B10.D1) (Fig. 1*D*). Notably, the Tyk2-deficient mice used in this study have a BALB/c background, whereas B10.D1 mice have a C57BL/10SnSg background. In a previous report, we demonstrated unresponsiveness to IL-12 using Tyk2-deficient mice with a mixed background of 129/SV and C57BL/6 (8). Thus, the involvement of Tyk2 in IL-12–induced IFN-γ production is general and independent of the genetic background.

IL-12 and IL-23 are heterodimeric cytokines composed of a common p40 subunit and a p35 or p19 subunit, respectively. Unlike IL-12, IL-23 promotes a distinct $CD4^+$ T cell phenotype characterized by the production of IL-17, denoted Th17 cells (15). IL-23 enhances Th17 function and survival by acting on differentiated Th17 cells that express the IL-23R (15). Because the IL-12Rβ1 chain is also part of IL-23R and associates with Tyk2, we examined the effect of Tyk2 on IL-23–induced IL-17A production. As shown in Fig. 2*A*, IL-23–mediated stimuli induced IL-17 production in the splenocytes from $Tyk2^{+/+}$ and B10.Q/Ai control mice in dose-dependent manners. The splenocytes from $Tyk2^{-/-}$ and B10.D1 mice completely failed to produce IL-17 in response to IL-23–mediated stimuli. Notably, the splenocytes from $Tyk2^{+/-}$ mice exhibited a moderate decrease in IL-17 production after stimulation with all of the tested concentrations of IL-23. Therefore, IL-23–induced IL-17 production is strongly dependent on the Tyk2 protein context and is distinct from the effects of Tyk2 on IL-12–induced IFN-γ production. To further examine the involvement of Tyk2 in Th17 differentiation, we cultured $CD4^+CD62L^+$ naive T cells in the presence of TGF-β and IL-6. The cultures of $Tyk2^{+/+}$ cells contained significantly higher proportions of IL-17–producing cells than did those of $Tyk2^{-/-}$ cells, although both cultures induced IL-17–producing cells (Fig. 2*B*). The subsequent addition of IL-23 increased the numbers of IL-17–producing cells in the $Tyk2^{+/+}$ cell cultures but not in the $Tyk2^{-/-}$ cell cultures. Therefore, Tyk2 influences Th17 induction or maintenance by interacting with IL-23 signaling. The number of IFN-γ–producing cells by IL-12 stimulation was also affected in $Tyk2^{-/-}$ cell culture, as we described previously (8). We also examined IL-10–producing cells by intracellular staining in the same situation (data not shown). However, we could not detect any IL-10–producing cells as a result of the lower expression level of IL-10, indicating that IL-10 is not involved in Th17 differentiation in this situation. We further examined whether Tyk2 regulates Treg differentiation by TGF-β–mediated Foxp3 expression. As shown in Fig. 2*C*, TGF-β–induced differentiation into $Foxp3^+$ Tregs was normal in $Tyk2^{-/-}$ cells, suggesting that Tyk2 is not involved in TGF-β–mediated signaling for Treg differentiation. Taken together, Tyk2 is related to pathogenic Th1 and Th17 but not Treg differentiation and maintenance in vivo.

DTH responses in Tyk2-deficient mice

The data obtained in the above-described in vitro experiments encouraged us to examine $Tyk2^{-/-}$ mice using IL-12– and/or IL-23–related experimental models. DTH responses are strongly T cell dependent and were reported to be defective in IL-12p40– and IL-23p19–deficient mice (28, 32). To evaluate DTH responses in the absence of Tyk2, we sensitized groups of $Tyk2^{+/+}$, $Tyk2^{+/-}$, and $Tyk2^{-/-}$ mice with mBSA in CFA and then elicited DTH responses after 7 d by injection of mBSA or saline (negative control) into the footpad. As shown in Fig. 3, specific footpad swelling was observed in the three types of mBSA-injected mice, but the degrees of swelling paralleled the levels of Tyk2 protein ($83.9 \pm 9.1 \times 10^{-2}$ mm for $Tyk2^{+/+}$ mice, $46.0 \pm 30.5 \times 10^{-2}$

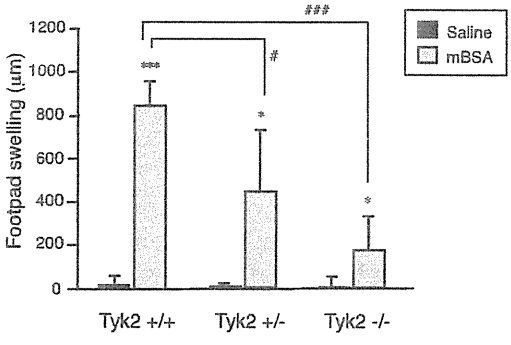

FIGURE 3. DTH responses in Tyk2-deficient mice. Mice immunized with mBSA were induced DTH reaction by the Ag injection into left heel. Ag-specific swelling 24 h after challenge was calculated as footpad thickness over the value measured just before the challenge. The results were averaged over all five mice in each group; error bars represent SDs. $^*p < 0.05$, $^{***}p < 0.001$ compared with saline-treated group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with $Tyk2^{+/+}$ mice.

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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. 4*A*, 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. 4*B*). Histological analysis also revealed that ears injected with IMQ developed epidermal hyperplasia with inflammatory cellular infiltration (Fig. 4*C*). 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. 4*D*, 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 $CD4^+IL\text{-}17^+$ or $CD4^+IFN\text{-}\gamma^+$ T cells in draining lymph nodes from $Tyk2^{+/+}$ and $Tyk2^{-/-}$

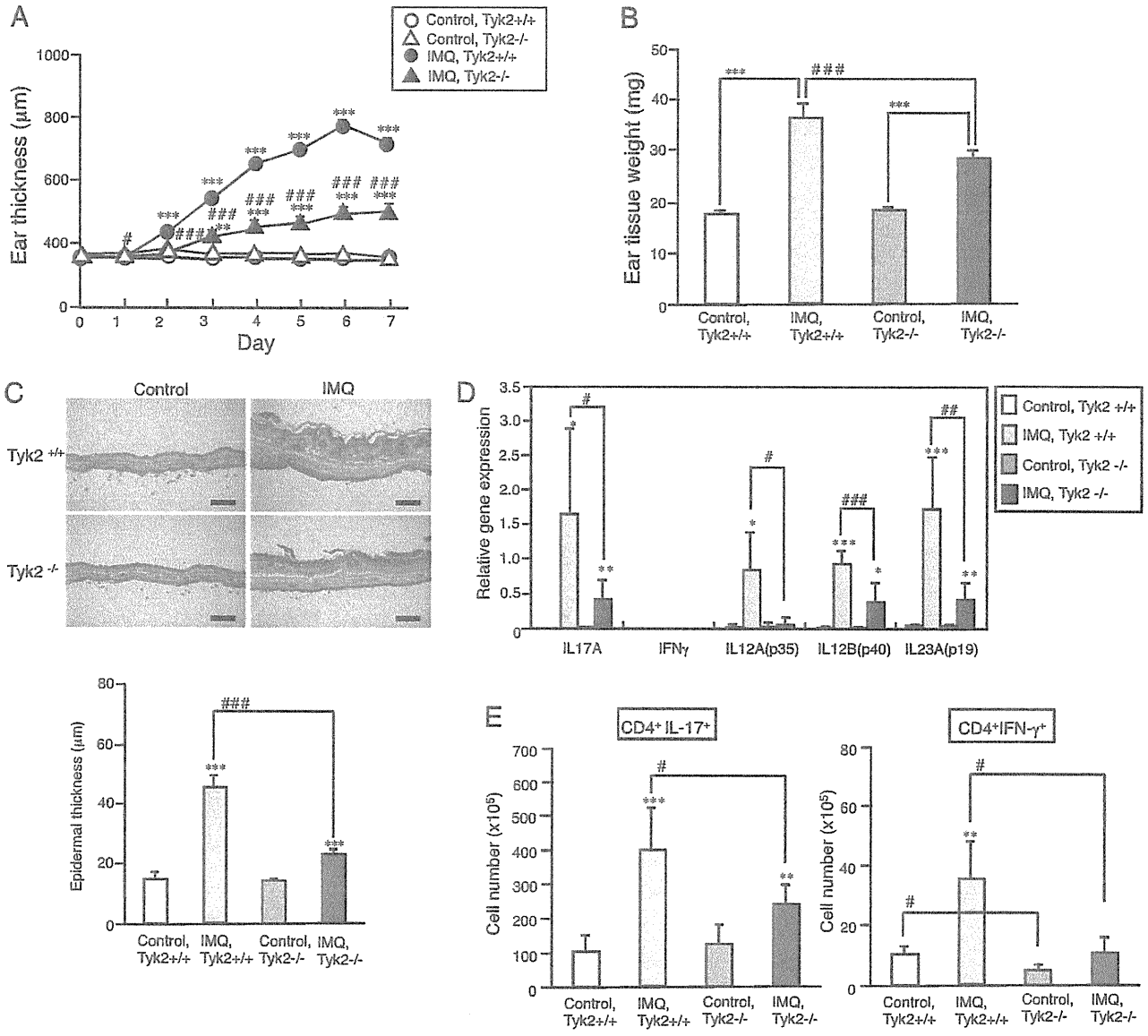

FIGURE 4. IMQ-induced skin inflammation in Tyk2-deficient mice. *A*, Ear skin of $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice was treated or not with IMQ for 6 consecutive days. Ear thickness was measured on the days indicated. Data represent mean ear thickness ± SD for seven mice per group. *B*, The ear tissue weight on day 7 was measured. Data represent mean ear tissue weight ± 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 μm. Epidermal hyperplasia, as quantified by imaging software from H&E-stained ear sections. Data represent mean epidermal thickness ± 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 ± SD relative to GAPDH for five mice per group. *E*, The number of $CD4^+IL\text{-}17^+$ and $CD4^+IFN\text{-}\gamma^+$ 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-γ 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.

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mice with or without IMQ treatment. As shown in Fig. 4*E*, the number of both $CD4^+IL\text{-}17^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells after IMQ treatment decreased markedly in $Tyk2^{-/-}$ 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). $Tyk2^{+/+}$ and $Tyk2^{-/-}$ 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 $Tyk2^{+/+}$ mice, DSS treatment produced experimental colitis, as assessed by the DAI, which began to increase on day 2. $Tyk2^{-/-}$ mice receiving DSS-containing water showed slower (beginning on day 5) and lower disease activity than did $Tyk2^{+/+}$ mice (Fig. 5*A*). The significant reduction in the severity of DSS-induced colitis in $Tyk2^{-/-}$ mice was confirmed by evaluation of body weight loss and colon shortening. As shown in Fig. 5*B*, $Tyk2^{+/+}$ mice with DSS treatment started to exhibit decreased body weight on day 3 and lost ~20% of their body weight by day 7. In $Tyk2^{-/-}$ mice, DSS treatment had no effect on the body weight. Similarly, DSS treatment induced more severe shortening of the colon length in $Tyk2^{+/+}$ mice compared with $Tyk2^{-/-}$ mice (Fig. 5*C*, 5*D*). We next examined expression levels of the related cytokines in colon tissue. As shown in Fig. 5*E*, expression of DSS-induced Th1- or Th17-related cytokines was reduced in colon tissue from $Tyk2^{-/-}$ 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 $Tyk2^{+/+}$ mice died within 3 d (Fig. 6*A*). In contrast, half of the $Tyk2^{-/-}$ mice treated with TNBS survived (Fig. 6*A*), and the body weight of the survivors returned to the normal range after they recovered from diarrhea (Fig. 6*B*).

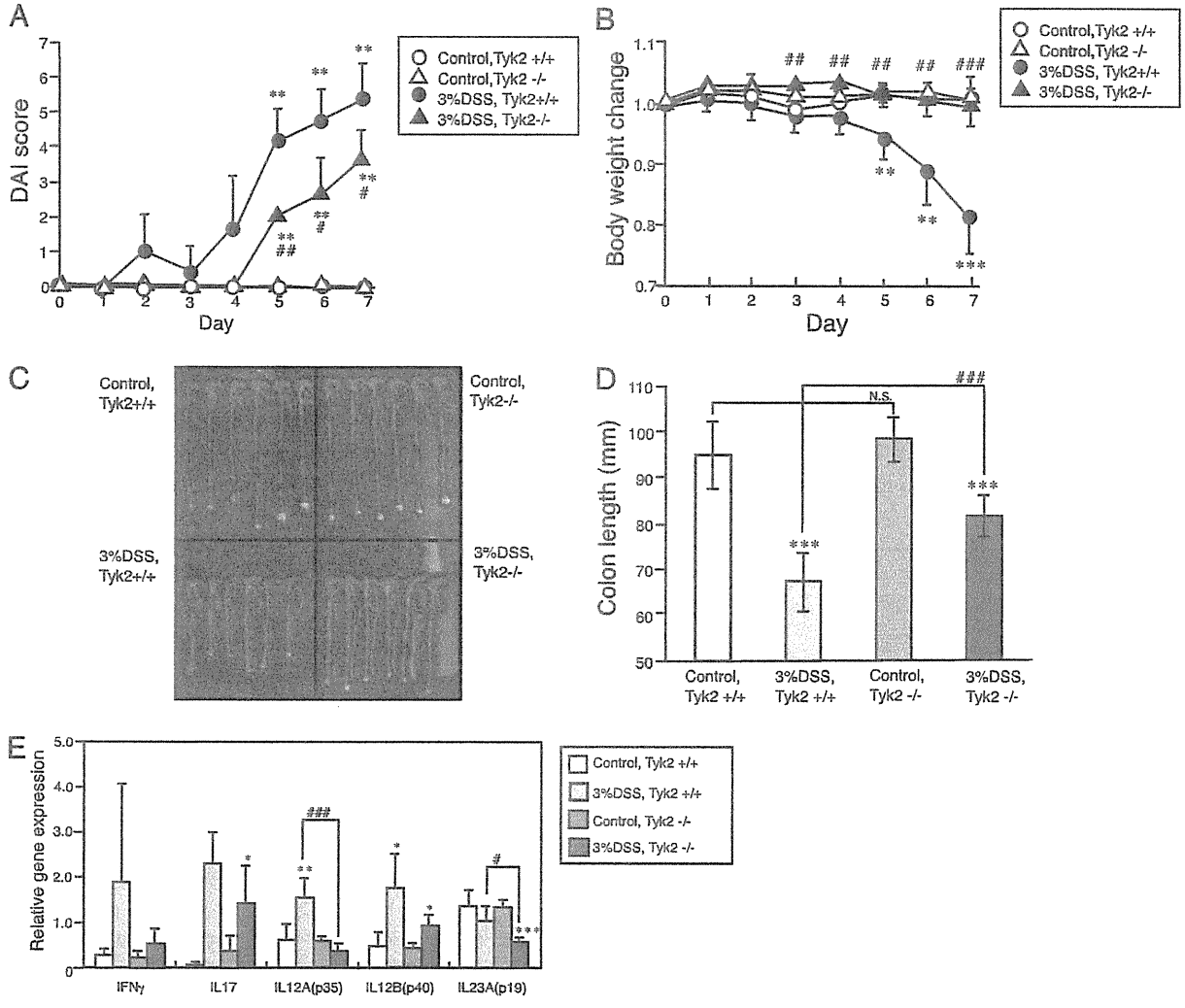

FIGURE 5. DSS-induced experimental colitis in Tyk2-deficient mice. DAI (*A*) and body weight change (*B*) during the course of DSS treatment in $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice were monitored every day. *C* and *D*, Colon lengths in control and DSS-treated $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice were evaluated on day 7. Data represent the mean ± 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 ± 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 $Tyk2^{+/+}$ mice.

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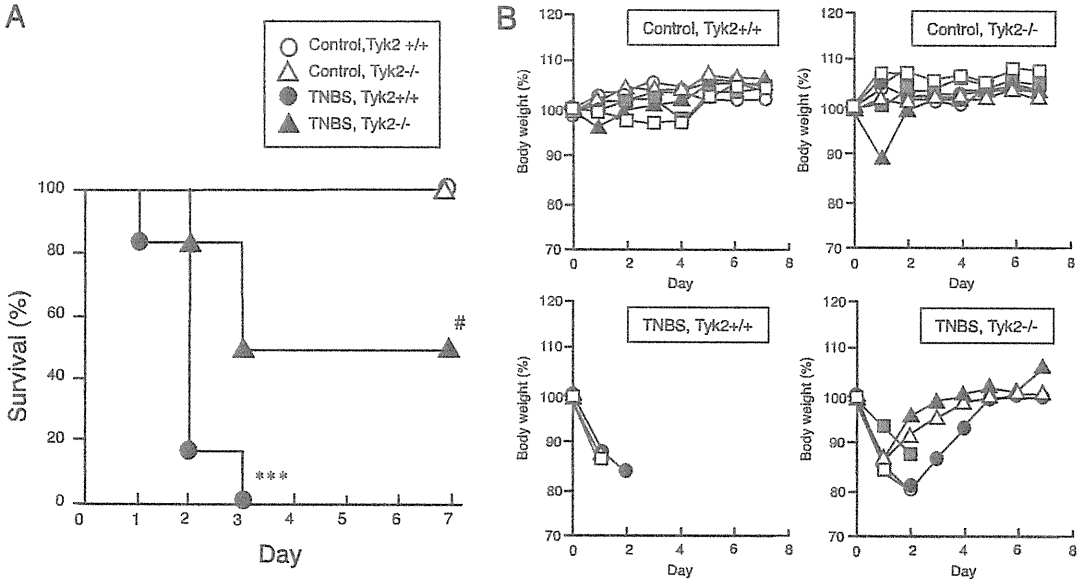

FIGURE 6. TNBS-induced experimental colitis in Tyk2-deficient mice. *A*, Mice were treated with 3.5 mg of TNBS in 40% ethanol by rectal instillation to induce colitis or with 40% ethanol alone to serve as colitis controls. TNBS-induced colitis-associated mortality in $Tyk2^{+/+}$ and $Tyk2^{-/-}$ mice was monitored every day. Data represent the survival ratio of mice. $^{***}p < 0.001$ compared with control, $^{\#}p < 0.05$ compared with $Tyk2^{+/+}$ mice. *B*, Body weight changes during the course of TNBS treatment in each $Tyk2^{+/+}$ or $Tyk2^{-/-}$ 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-γ 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 $CD4^+$ cells are affected in Tyk2-deficient mice. Furthermore, Tyk2 was not involved in Treg differentiation from naive $CD4^+$ cells by TGF-β. 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 $Tyk2^{-/-}$ mice (Fig. 4*D*). However, mRNA expression of Th1-related cytokines was also reduced in $Tyk2^{-/-}$ mice. Furthermore, the number of $CD4^+IL\text{-}17^+$ or $CD4^+IFN\text{-}\gamma^+$ T cells in draining lymph nodes after IMQ treatment decreased markedly in $Tyk2^{-/-}$ 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 $Tyk2^{-/-}$ 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, $Tyk2^{-/-}$ 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-γ 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 γ cytokine

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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_h1/T_h17-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, $CD4^+$ T cells infiltrating into the RA synovial membrane are predominantly T_h1 phenotypes (5, 6). According to a current paradigm, a pathogenic role of T_h1-type cellular immunity is supposed to prevail over a beneficial T_h2 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 ($H\text{-}2^q$) mice and CB8 in B10.RIII ($H\text{-}2^r$) 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-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-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

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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-IκBα 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 μg 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 $Tyk2^{-/-}$ 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 μm 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 μg 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 10^6 lymph node cells were lysed in 20 μl 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).

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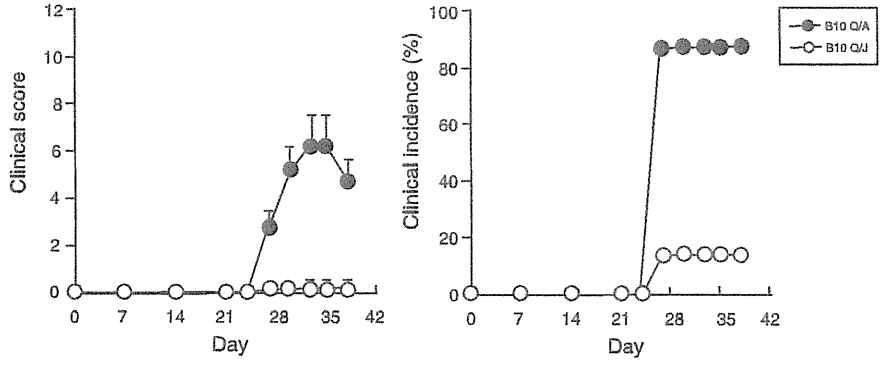

Fig. 1. CIA model in Tyk2 mutant B10.Q/J mice. B10.Q/Ai mice and Tyk2 mutant B10.Q/J mice were immunized with bovine CII on days 0 and 21, and arthritis was induced after the second immunization. Clinical arthritis score (A) and incidence (B) during the course of the experiment were strongly reduced 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).

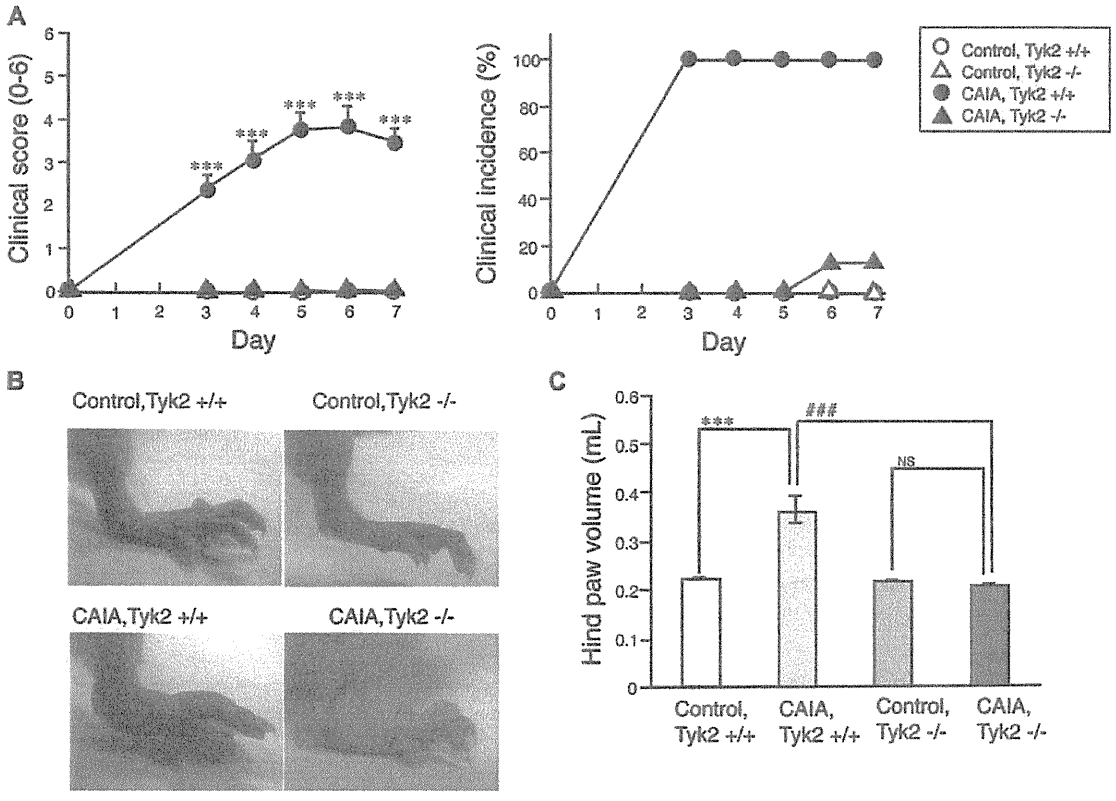

Fig. 2. CAIA model in $Tyk2^{-/-}$ mice. WT and $Tyk2^{-/-}$ mice were i.v. injected with an anti-collagen antibody cocktail on day 0. (A) Clinical arthritis score change and incidence during the experiment. (B) Representative appearance of the hind limb on day 7 after CAIA induction. WT mice limbs showed obvious swelling. (C) Hind paw volume on day 7. WT mice showed a significant increase in volume, but $Tyk2^{-/-}$ 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 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)

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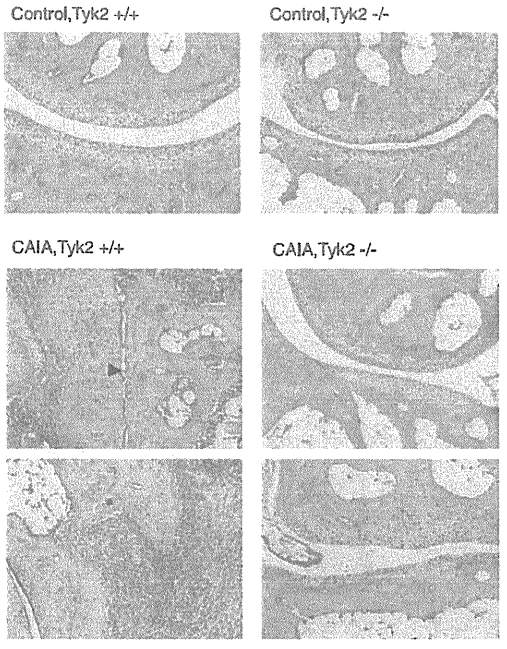

Fig. 3. Pathological appearance and pathological change in the CAIA model. Representative H&E-stained tarsal joint sections from CAIA-induced WT and Tyk2$^{-/-}$ mice on day 7 as described in Methods. WT mice joints showed inflammatory cells infiltration, erosion synovium (arrowhead) and bone destruction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

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 ~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 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$^{-/-}$ 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$^{-/-}$ 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 ~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 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$^{-/-}$ 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 days 3 and 7 after the induction. As shown in Fig. 4(A), the T_h1/T_h17-related cytokines such as IFN-γ 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$^{-/-}$ mice. The inflammatory cytokines such as IL-6, IL-1β, tumor necrosis factor (TNF)-α and IFN-β 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$^{-/-}$ 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$^{-/-}$ 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 matrix metalloproteinase 9 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 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.

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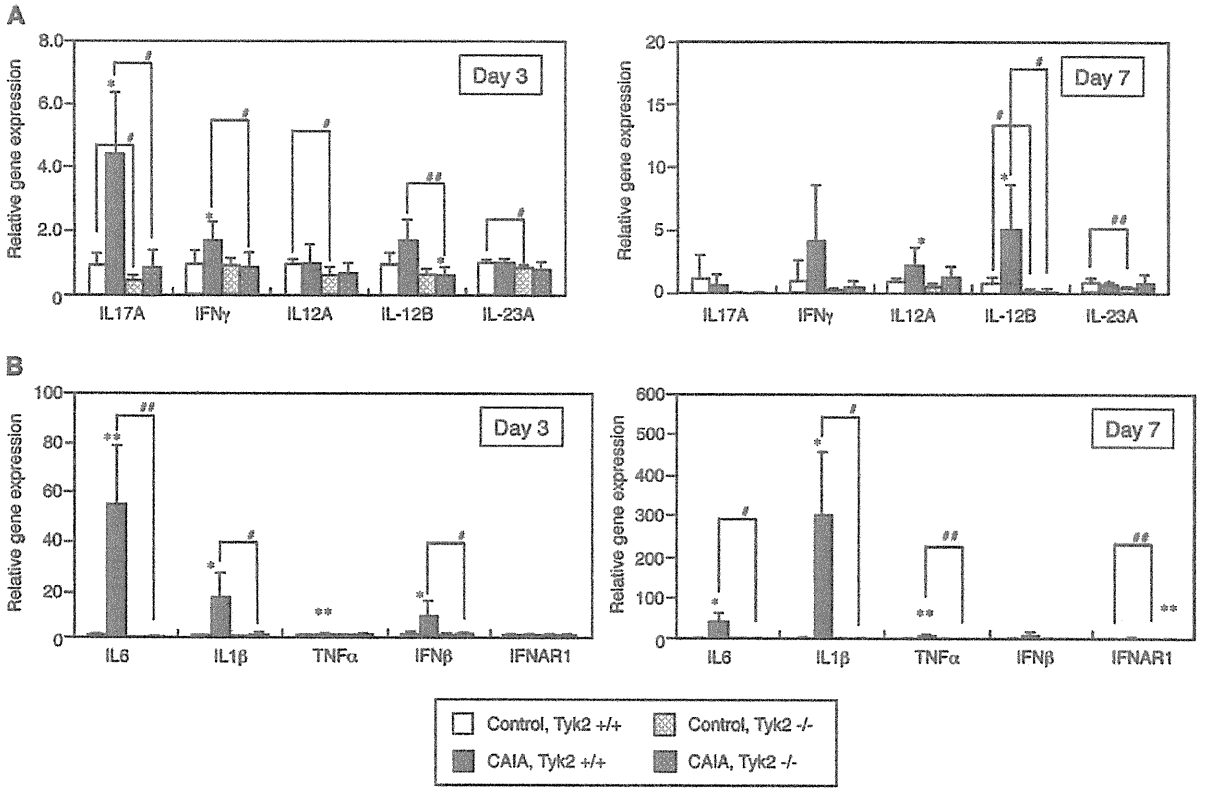

Fig. 4. Gene expression of cytokines in the CAIA model. Three or seven days after CAIA induction, gene expressions of T_h1/T_h17-related (A) and other pro-inflammatory cytokines (B) were evaluated in the hind paw of WT mice and $Tyk2^{-/-}$ mice as described in Methods. IL-17A and IFN-γ was significantly 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 + 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-12Rβ1 subunit, which associates with Tyk2. IL-12 guides $CD4^+$ T cells to T_h1 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 T_h17 cells, which play essential roles in the pathogenesis of chronic inflammatory disorders (31, 32). Thus, Tyk2 seems to be indispensible 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-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 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

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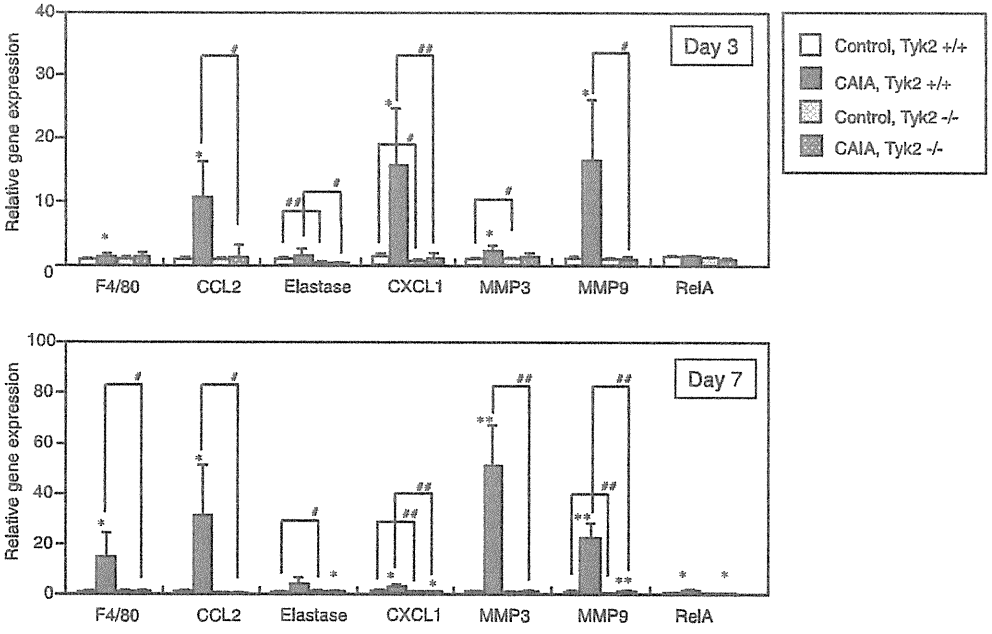

Fig. 5. Inflammation-related gene expression in the CAIA model. Gene expressions of inflammation were evaluated in the hind paw. Chemokines gene expression, CCL2 and CXCL1, were highly elevated at day 3, and F4/80, macrophage marker, was elevated at day 7. Elastase, neutrophil marker, did not significantly 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$^{-/-}$ 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$^{-/-}$ 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),

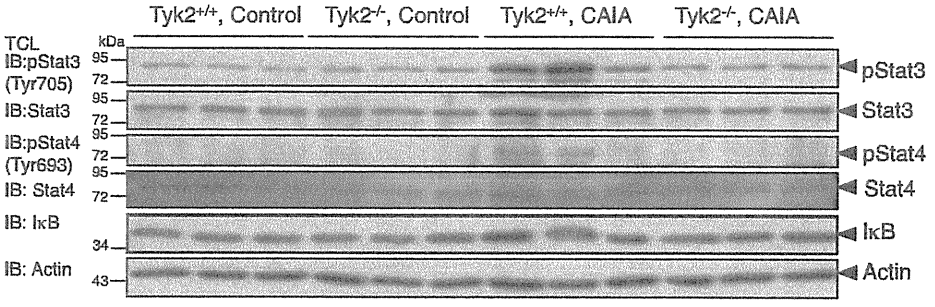

Fig. 6. Phosphorylation of STAT proteins in the lymph node cells from the CAIA model. Popliteal lymph node cells were collected at day 3 and phosphorylation of STATs and IκB expression were analyzed as described in Methods. In cells from CAIA-induced WT group, STAT3 and STAT4 phosphorylation were observed,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

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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.

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