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Tyk2-Signaling Plays an Important Role in Host Defense against *Escherichia coli* through IL-23-Induced IL-17 Production by γδ T Cells

Risa Nakamura,* Kenesuke Shibata,* Hisakata Yamada,* Kazuya Shimoda,‡ Keiichi Nakayama,† and Yasunobu Yoshikai‡*

Tyrosine kinase 2 (Tyk2), a member of the JAK-signal transducer family, is involved in intracellular signaling triggered by various cytokines, including IL-23. We have recently reported that resident γδ T cells in the peritoneal cavity of naïve mice produced IL-17 in response to IL-23. In this study, we examined importance of Tyk2-mediated signaling in the IL-17 production by γδ T cells using Tyk2 deficient (−/−) mice. γδ T cells in the peritoneal cavity of Tyk2−/− mice displayed effector/memory phenotypes and TCR V repertoire similar to those in Tyk2+/+ mice and produced comparable level of IL-17 to those in Tyk2−/− mice in response to PMA and ionomycin, indicating normal differentiation to IL-17-producing effectors in the absence of Tyk2-signaling. However, γδ T cells in Tyk2−/− mice produced less amount of IL-17 in response to IL-23 in vitro than those in Tyk2+/+ mice. Similarly, γδ T cells in the peritoneal cavity of Tyk2−/− mice showed severely impaired IL-17 production after an i.p. infection with *E. coli* despite comparable level of IL-23 production to Tyk2+/+ mice. As a consequence, Tyk2−/− mice showed a reduced infiltration of neutrophils and severely impaired bacterial clearance after *Escherichia coli* infection. These results indicate that Tyk2-signaling is critical for IL-23-induced IL-17 production by γδ T cells, which is involved in the first line of host defense by controlling neutrophil-mediated immune responses. *The Journal of Immunology*, 2008, 181: 2071–2075.

Tyrosine kinase 2 (Tyk2) is a member of the JAK family and is activated in response to various cytokines including type I IFN, IL-6, IL-10, IL-12, and IL-13 (10). However, studies using Tyk2-deficient mice revealed different levels of dependence on Tyk2 between different cytokines (11, 12). Tyk2 was dispensable for IL-6- and IL-10-mediated signaling and was only partly required for signaling from type I IFN. In contrast, IL-12-mediated signaling, especially those for IFN-γ production by T cells, was highly dependent on Tyk2. As Tyk2 is recruited to IL-12Rβ1 (9), IL-23-mediated signaling also likely involves Tyk2. In fact, an impaired IL-23-induced phosphorylation of STAT3 was shown in Tyk2-deficient T cells (13), suggesting an importance of Tyk2 for IL-23-mediated signaling for IL-17 production.

γδ T cells are widely distributed in nonlymphoid tissues and have been shown to be involved in host defense mechanism at an early phase of infection (14–16). We recently reported that an i.p. infection of *Escherichia coli* induced IL-17 production from resident peritoneal γδ T cells expressing Vδ1 TCR (17). IL-17 production by the γδ T cells was mediated by IL-23 and was important for local neutrophil influx and protection against *E. coli*. In the present study, we examined the role of Tyk2 in IL-23-mediated signaling by using peritoneal γδ T cells in Tyk2-deficient mice. Our results demonstrate that Tyk2 is not required for differentiation of γδ T cells to produce IL-17 but is important for IL-17 production by γδ T cells in response to IL-23 in vitro as well as in vivo.

**Materials and Methods**

**Mice**

Tyk2−/− mice were generated as has been previously described (11) and have been backcrossed onto C57BL/6 mice more than eight times. The

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*Abbreviations used in this paper: Tyk2, tyrosine kinase 2; PEC, peritoneal exudate cell; LPL, lamina propria lymphocyte; IEL, intraepithelial lymphocyte.*
mice were bred under specific pathogen-free conditions in our institute. Six- to eight-week-old male mice were used for the experiments. This study was approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine, Kyushu University. Experiments were conducted under the control of the Guidelines for Animal Experiment.

**Microorganisms**

*E. coli* (no. 26: American Type Culture Collection (ATCC), Rockville, MD) grown in tryptose-soya broth (Nissui Pharmaceutical) was washed repeatedly, resuspended in 50% glycerol-containing PBS, and small aliquots were stored at −80°C until used. For all the experiments, mice were i.p. infected with 1 × 10^9 CFU of *E. coli*.

**Assessment of bacterial growth**

At the indicated time after infection, the peritoneal contents were washed with 1 ml of HBSS and harvested after gentle massage. Samples were serially diluted with HBSS. The livers and spleens were removed and placed in homogenizers containing 3 ml of HBSS. Samples were spread on trypto-soya agar (Nissui Pharmaceutical) plates, and colonies were counted after incubation for 24 h at 37°C.

**Measurement of IL-17 and IL-23 in peritoneal exudates**

Supernatants of peritoneal exudates at indicated time points were obtained by centrifugation at 440 g for 15 min at 65°C. The radioactivity of each band of PCR product transferred to a Gene Screen Plus filter (New England Nuclear). The following primers were used: Forward primers: Vβ6, 5'-CTCTGTGAACTTCC-3', Vβ7, 5'-TCACTTGG-3'; Reverse primers: Cβ1, 5'-AGTTCCCTGCAGATCCA-3'; Cβ2, 5'-TGAAAGAGACCCTACATCCCTTGA-3'.

**RNA isolation, cDNA synthesis for RT-PCR, and TCR repertoire analysis**

*γδ* T cells in the peritoneal cavity were sorted using FACS aria (BD Biosciences) at the purity of >95%. mRNA of the purified *γδ* T cells was extracted using TRIzol reagent (Invitrogen Life Technologies), and cDNA was synthesized using Superscript II (Invitrogen) according to the manufacturer’s instruction. For the Vγ and Vδ repertoire analysis, combinations of following primers were used: Forward primers: Vγ1, 5'-ACACAGCTATAACCTTGG-3'; Vγ2, 5'-CGGCAAAAAAACAAATCACAG-3'; Vγ4, 5'-TGTCTCTTGGACACCCTTACC-3'; Vγ5, 5'-TGCTCTGACAAACCCCTACC-3'; Vγ6, 5'-GGAATTCGAAAGAACATATGGTCTCCCT-3'; Vδ7, 5'-AAAGCTAGGAGGCTCTCTTCG-3'; Vδ8, 5'-ATTCCAGAACGCAAATGAAAGG-3'; Vδ2, 5'-AGTCTCTGACATCGAC-3'; Vδ3, 5'-TCTCTGCGTATGCGTCTGAC-3'; Vδ4, 5'-ACCCTGTTCCGTCATCTGC-3'; Vδ6, 5'-TCAAGTCCTACAGCTTGC-3'; Vδ7, 5'-CGCAGAGCTGCGTAAGTACT-3'; Vδ8, 5'-AAGGAAGAGGAGGACCTC-3'. Reverse primers: Cy5, 5'-CTTATGGGATTGTGTGTCAGC-3'; Cβ1, 5'-CGAATTCCCAACATCTTCC-3'; PCR was performed on a PCR thermal cycler (Takara Corporation). For the southern blot analysis, PCR products were transferred to a Gene Screen Plus filter (New England Nuclear). The following pairs of specific primers were used: IL-23R, 5'-AGTTCCCTGCAGATCCA-3'; GAPDH, 5'-TGGTGAAGGTCGTCCTATTG-3'. Each sample of known cell concentration and then ran the test samples at a same number of *γδ* T cells (GL-3 positive) in PEC, spleen, lung, liver, and IEL was measured by Duoset ELISA development system (R&D systems) or 10 ng/ml PMA (Sigma-Aldrich) and PE-conjugated streptavidin were purchased from eBioscience. Biotin-conjugated anti-TCR-γδ T cells (GL3) was purchased from Caltag Laboratories. Stained cells were run on a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using CellQuest software (BD Biosciences). To calculate cell numbers in each organ by using flow cytometer, we first applied a standard sample of known cell concentration and then ran the test samples at a same flow rate for a constant acquisition time.

**Intracellular cytokine staining**

For ex vivo intracellular cytokine staining, PEC were collected 6 h after infection with *E. coli* and then were incubated with 10 μg/ml brefeldin A (Sigma-Aldrich) for 1 h. For intracellular cytokine staining of in vitro stimulated cells, PEC from naive mice were used for the experiments. Cells were stimulated with or without 2 ng/ml or 20 ng/ml rL23 (R&D Systems) or 10 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) for 7 h in a CO2 incubator at 37°C. Ten μg/ml brefeldin A was added for the last 5 h incubation. Cells were stained with allophycocyanin-conjugated CD3ε, FITC-conjugated anti-TCR-γδ T cells for 30 min at 4°C. Thereafter, intracellular staining was performed according to the manufacturer’s instruction (BD Biosciences). In brief, 100 μl of BD Cytofix/Cytoperm solution (BD Biosciences) was added to the cell suspension with mild mixing and placed for 20 min at 4°C. Fixed cells were washed with 250 μl of BD Perm/Wash solution (BD Biosciences) twice and then stained intracellularly with PE-conjugated anti-CD3ε mAb for 30 min at 4°C. Stained cells were run on a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using CellQuest software (BD Biosciences).
Expression of IL-23R in purified T cells from the peritoneal cavity of naive Tyk2+/+ mice was also no difference in the expression of other surface markers, including CD62L, CD122, CD25, and CD69 (data not shown).

We next examined the ability of the γδ T cells in the peritoneal cavity to produce IL-17 by intracellular staining. As shown in Fig. 2B, γδ T cells in naive Tyk2−/− mice expressed IL-23R comparable to those in control mice. As we had found γδ T cells bearing V61 were responsible for IL-17 production (17), TCR Vγ repertoire of the TCR γδ T cells in naive Tyk2−/− mice exclusively expressed Vγ6 gene and V61 gene rearranged to the Jβ2 gene, and those in Tyk2+/+ mice preferentially expressed Vγ6 and V61 in addition to Vγ1, Vγ2, Vγ4, and Vγ6. Thus, although the number of γδ T cells in the peritoneal cavity was decreased in Tyk2−/− mice, the γδ T cells in Tyk2−/− mice were virtually indistinguishable from those in control mice in regard to the expression of surface markers, IL-23R, and TCR Vγ6 and V61 genes.

In vitro IL-17 production by γδ T cells from the peritoneal cavity of naive Tyk2−/− mice
We next examined the ability of the γδ T cells in the peritoneal cavity to produce IL-17 by intracellular staining. As shown in Fig.

![Figure 2](image-url)

**FIGURE 2.** Characterization of γδ T cells in the peritoneal cavity of naive Tyk2−/− mice. A, Expression of CD44 and Ly6c on γδ T cells in the peritoneal cavity of naive mice were examined by flow cytometry. B, γδ T cells were FACS sorted and expression of IL-23R was analyzed by real-time RT-PCR. C, TCR Vγ (upper panels) and Vδ gene (lower panels) expression in the purified γδ T cells was analyzed by Southern blotting. Data are representatives of three independent experiments.

![Figure 3](image-url)

**FIGURE 3.** IL-17 production by peritoneal γδ T cells in Tyk2−/− mice in vitro. A, PEC from Tyk2+/+ (left) or Tyk2−/− mice (right) was stimulated with rIL-23 (2 ng/ml or 20 ng/ml) for 7 h. B, PEC, splenocytes, liver mononuclear cells, LPL, and IEL were stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin for 7 h. Brefeldin A was added to all the cultures for the last 5 h, and cells were tested for intracellular staining of IL-17. The numbers in the upper right quadrants indicate the percentage of IL-17-positive cells in γδ T cells. Data are representatives of three independent experiments.

**Statistics**
Statistical significance was calculated by the Student’s t test using Prism software (GraphPad). Differences with p values of <0.05 were considered statistically significant.

**Results**

Development of γδ T cells in Tyk2−/− mice
We first examined the number of TCRγδ T cells in various organs in naive Tyk2−/− mice. As shown in Fig. 1, the number of γδ T cells in the peritoneal cavity and intestinal lamina propria was significantly lower in Tyk2−/− mice than Tyk2+/+ mice (p < 0.05). In contrast, the numbers of γδ T cells in the spleen, thymus, lung, liver, and IEL in Tyk2−/− mice were not different from those in Tyk2+/+ mice (Fig. 1, data not shown). Most of the γδ T cells in the peritoneal cavity of naive Tyk2−/− mice showed CD44+ memory phenotype similar to those in Tyk2+/+ mice (Fig. 2A). Percentage of Ly-6C positive cells in the peritoneal γδ T cells in Tyk2−/− mice was much the same as that in Tyk2+/+ mice. There was also no difference in the expression of other surface markers including CD62L, CD122, CD25, and CD69 (data not shown). Expression of IL-23R in purified γδ T cells from the peritoneal cavity was quantitatively examined by real-time RT-PCR. As shown in Fig. 2B, γδ T cells in naive Tyk2−/− mice expressed IL-23R comparable to those in control mice. As we had found γδ T cells bearing V61 was responsible for IL-17 production (17), TCR Vγ repertoire of the TCRγδ T cells was also analyzed by RT-PCR followed by Southern blotting. As shown in Fig. 2C, γδ T cells in the peritoneal cavity of Tyk2−/− mice exclusively expressed Vγ6 gene and V61 gene rearranged to the Jβ2 gene, and those in Tyk2+/+ mice preferentially expressed Vγ6 and V61 in addition to Vγ1, Vγ2, Vγ4, and Vγ6. Thus, although the number of γδ T cells in the peritoneal cavity was decreased in Tyk2−/− mice, the γδ T cells in Tyk2−/− mice were virtually indistinguishable from those in control mice in regard to the expression of surface markers, IL-23R, and TCR Vγ6 and V61 genes.

In vitro IL-17 production by γδ T cells from the peritoneal cavity of naive Tyk2−/− mice
We next examined the ability of the γδ T cells in the peritoneal cavity to produce IL-17 by intracellular staining. As shown in Fig.
In vivo IL-17 production by the γδ T cells in Tyk2−/− mice following E. coli infection

Tyk2 IN IL-17-PRODUCING γδ T CELLS

To determine in vivo significance of Tyk2-signaling for IL-17 production by γδ T cells, we injected E. coli i.p. into Tyk2−/− mice. IL-17-producing γδ T cells were detected after a brief ex vivo culture with brefeldin A, which were clearly reduced in Tyk2−/− mice. As reported previously, IL-17-producing cells were hardly found other than γδ T cells (Ref. 17 and data not shown). We also measured the amount of cytokines in the peritoneal cavity of E. coli infected mice. IL-17 production was severely impaired in Tyk2−/− mice, although IL-23 production in the peritoneal cavity of Tyk2−/− mice was comparable to those in Tyk2+/+ mice (Fig. 4B). Consistent with the previous report (17), infiltration of neutrophils in the peritoneal cavity, which reached the peak at 24 h after infection, was observed in both Tyk2+/+ and Tyk2−/− mice, but the number of neutrophils was significantly reduced in Tyk2−/− mice (Fig. 4C, left). Furthermore, Tyk2−/− mice showed significantly impaired bacterial clearance in peritoneal cavity (p < 0.01) (Fig. 4C, right). We also found significantly increased bacterial burden in the spleen (p < 0.05) and the liver (p < 0.05) of Tyk2−/− mice (data not shown). These results clearly demonstrated in vivo significance of Tyk2-signaling for the host defense against E. coli through IL-23-induced IL-17 production by γδ T cells.

Discussion

We and others have found that Tyk2 is important for IL-12-induced signaling and contributes to IFN-γ production by NK cells and CD4+ and CD8+ T cells (12, 21, 22). In this study, we showed that Tyk2 also plays an important role in IL-23-induced IL-17 production by the γδ T cells in vitro. We further found that Tyk2 is critical for IL-17 production by γδ T cells and protection against E. coli infection in vivo. There have been reports showing involvement of Tyk2 in IL-23-mediated signaling using Tyk2-deficient mice, including an impaired STAT3 phosphorylation of ConA-stimulated T cells (13) and an impaired IFN-γ production of dendritic cells (23). However, to our knowledge, this is the first report demonstrating an importance of Tyk2 in IL-23-mediated signaling for IL-17 production.

The lack of IL-17 production in response to IL-23 by γδ T cells in Tyk2−/− was not attributed to a defect in differentiation to IL-17-producing effector cells, because normal IL-17 production by γδ T cells in Tyk2−/− mice was induced by stimulation with PMA and ionomycin. Furthermore, γδ T cells in Tyk2−/− mice were indistinguishable from those in Tyk2 mice in effector/memory phenotype and the expression of IL-23R. They expressed Vγ6 and Vδ1, which we have demonstrated to be the major IL-17-producing γδ T cell repertoire in the PEC of wild-type mice (17). These results suggest that the peritoneal γδ T cells differentiate into IL-17-producing effector cells in situ in the absence of Tyk2. At present, it is unclear what factors are required for differentiation of γδ T cells to produce IL-17. In the case of CD4 T cells, it is generally accepted that TGF-β in combination with IL-6 induces differentiation of Th17 cells (5). Interestingly, it was shown that an absence of Tyk2 did not affect the phosphorylation of STAT3 by IL-6 (12). Thus, it is speculated that the factors involved in the differentiation of IL-17-producing γδ T cells are not affected by the absence of Tyk2.

IL-23 was initially demonstrated to induce differentiation of Th17 cells, but is currently thought to be involved in maintenance (expansion or survival) of Th17 cells (5). In this regard, it is of note that the number of γδ T cells in the peritoneal cavity and LPL of naive Tyk2−/− mice were significantly lower than Tyk2+/+ mice, the level of IL-17 production was significantly lower in γδ T cells in Tyk2−/− mice in response to exogenous IL-23, although the difference in the percentage of IL-17-producing γδ T cells between Tyk2−/− mice and Tyk2+/+ mice was lesser in response to higher amounts of exogenous IL-23. However, most of peritoneal γδ T cells of not only Tyk2+/+ mice but also Tyk2−/− mice produced IL-17 in response to PMA and ionomycin (Fig. 3B). These results suggest that Tyk2-signaling is dispensable for differentiation of peritoneal γδ T cells to IL-17-producing effectors but is important for IL-17 production by γδ T cells in response to IL-23. In contrast to the γδ T cells in PEC, only a limited percentage of γδ T cells in the spleen, thymus, lung, and liver produced IL-17 in response to PMA and ionomycin in both Tyk2+/+ mice and Tyk2−/− mice (Fig. 3B, data not shown). IL-17-producing γδ T cells were nearly absent in IEL, whereas they were abundantly found in LPL.
mice, whereas the number of γδ T cells in spleen, thymus, lung, liver, and IEL, which contain relatively a small number of IL-17-producing cells, was not significantly different between Tyk2−/− and Tyk2−/− mice. This supports an involvement of Tyk2 in IL-23-mediated signaling for selective maintenance of IL-17-producing γδ T cells. However, we have not detected mitogenic or antiapoptotic effect of IL-23 on γδ T cells in vitro as yet. It is also possible that Tyk2 is directly involved in the development of peritoneal γδ T cells by other mechanisms. Further studies are needed to clarify these issues. IL-23 was also shown to augment IL-17 production from activated/memory CD4 T cells (7, 8, 24). Therefore, resident γδ T cells are similar to activated/memory CD4 T cells not only in the expression pattern of surface molecules but also in the way to exert their effector functions in response to IL-23, although stimulation with IL-23 alone is insufficient to induce IL-17 production in the case of CD4 T cells (our unpublished data).

Although γδ T cells from Tyk2−/− mice displayed severely reduced responsiveness to a small amount of IL-23, a high concentration of IL-23 partly restored their ability to produce IL-17. It was demonstrated that Tyk2 was partially involved in type I IFN-mediated signaling, whereas IL-12-mediated cellular responses were highly dependent on Tyk2. Defective phosphorylation of Stat4 or Stat3 in response to IL-23 was also demonstrated. However, it was also shown that a higher dose of IL-23 overcame the effect of Tyk2 deficiency on the differentiation of Th1 cells (11), similar to the case of IL-23 in this study. Leaky expression of neither Tyk2 protein nor Tyk2 mRNA was detected in Tyk2−/− mice (Ref. 11 and data not shown). Therefore, it is possible that other JAKs, likely JAK2, compensate for the defect of Tyk2 to respond to a high dose of the cytokines. Nevertheless, our in vivo data showing severely reduced IL-17 production during E. coli infection indicate that IL-23-signaling is highly dependent on Tyk2 in physiological conditions.

Recently, a patient with homozygous mutation of Tyk2, which causes a loss of Tyk2 protein, was reported (25). The patient was clinically diagnosed as hyper IgE syndrome and showed susceptibility to various types of pathogens including virus, fungi, and intracellular and extracellular bacteria. Interestingly, Tyk2 deficiency in human showed severely impaired responses not only to IL-12 but also to type I IFN, IL-6, and IL-10, in contrast to Tyk2-deficient mice. An abolished phosphorylation of Stat4 and IFN-γ production in response to IL-23 was also shown using T cells from the patient. In this study, we speculate that an impaired IL-17 production in response to IL-23 might also be involved in the immunodeficient phenotype of the patient, as we demonstrated in this study by using Tyk2−/− mice. Further studies will uncover in vivo roles of Tyk2 in different aspects of immune responses.

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Disclosures
The authors have no financial conflict of interest.

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