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Tyk2 Signaling in Host Environment Plays an Important Role in Contraction of Antigen-Specific CD8⁺ T Cells following a Microbial Infection¹

Wei Li,* Hisakata Yamada,* Toshiki Yajima,* Ryusuke Nakagawa,* Kazuya Shimoda,‡ Keiichi Nakayama,† and Yasunobu Yoshikai²*

Tyrosine kinase 2 (Tyk2), a member of Jak signal transducer family contributes to the signals triggered by IL-12 for IFN-γ production. To elucidate potential roles of Tyk2 in generation and maintenance of Ag-specific CD8⁺ T cells, we followed the fate of OVA-specific CD8⁺ T cells in Tyk2-deficient (−/−) mice after infection with recombinant Listeria monocytogenes expressing OVA (rLM-OVA). Results showed that the numbers of OVA257–264/Kb tetramer-positive CD8⁺ T cells in Tyk2−/− mice were almost the same as those in Tyk2+/+ mice at the expansion phase on day 7 but were significantly larger in Tyk2−/− mice than those in Tyk2+/+ mice at the contraction phase on day 10 and at the memory phase on day 60 after infection. The intracellular expression level of active caspase-3 was significantly decreased in the OVA-specific CD8⁺ T cells of Tyk2−/− mice on day 7 compared with those of Tyk2+/+ mice. Adaptive transfer experiments revealed that Tyk2 signaling in other factors rather than CD8⁺ T cells played a regulatory role in CD8⁺ T cell contraction following infection. Administration of exogenous IFN-γ from day 6 to day 9 restored the CD8⁺ T cell contraction in Tyk2−/− mice after infection with rLM-OVA. These results suggest that Tyk2 signaling for IFN-γ production in host environment plays an important role in contraction of effector CD8⁺ T cells following a microbial infection. The Journal of Immunology, 2007, 178: 4482–4488.

Tyrosine kinase 2 (Tyk2)³ is a member of Jak family, which has been identified as an essential molecule for signal transduction via cytokine receptors for IFN-α/β, IL-6, IL-10, IL-12, and IL-13 (1). Tyk2-deficient (−/−) mice display a lack of responsiveness to a small amount of IFN-α, but they respond normally to a high concentration of IFN-α/β (2, 3). Furthermore, these mice normally respond to IL-6 and IL-10, suggesting that Tyk2 is dispensable for mediating for IL-6 and IL-10 signaling and does not play major roles in IFN-α signaling. We and others have recently shown that IL-12-induced functions of NK cells and CD4⁺ T cells are defective in Tyk2−/− mice (2–5). Tyk2−/− mice showed a reduced CD8⁺ CTL responses following infection with lymphocytic choriomeningitis virus (3). IL-12-induced IFN-γ production by CD8⁺ T cells was strongly suppressed in Tyk2−/− mice infected with Leishmania major (5). Thus, Tyk2 plays an important role in signal transduction via receptors for IL-12 and contributes to the signals triggered by IL-12 in NK cells, CD4⁺ T cells and CD8⁺ T cells.

Upon encounter with a pathogenic microbe, naive Ag-specific CD8⁺ T cells proliferate and differentiate into effector CD8⁺ T cells during the expansion phase. Most of the effector T cells die by apoptosis during the contraction phase, but the few that survive become memory T cells and persist for a long period of time (6–8). Because the size of memory CD8⁺ T cell pool depends at least partly on the amounts of surviving T cells from T cell contraction, identification of molecular mechanisms responsible for regulating T cell contraction may provide an insight into vaccine development for microbial infection. There are several lines of evidence that T cell autonomous cell death (ACAD), also called growth factor withdrawal-induced apoptosis (9–11), is responsible for the death of the majority of activated T cells responding to a foreign Ag (12–15). We have recently found that endogenous IL-15 plays an important role in protection of effector CD8⁺ T cells from apoptosis during contraction phase after Listeria monocytogenes infection (16). In contrast, it has been recently shown that the death of the majority of activated T cells responding to a foreign Ag in vivo can be enhanced by IFN-γ produced in early inflammation after Listeria infection (17–19). Thus, cytokine milieu may play a critical role in regulating CD8⁺ T cell death during the contraction phase following bacterial infection.

In the present study, we examined the roles of Tyk2 signaling in CD8⁺ T cell contraction using Tyk2−/− mice that were subsequently infected with recombinant Listeria monocytogenes expressing OVA (rLM-OVA) (17). Our results demonstrate that the size of effector CD8⁺ T cells during the contraction phase is partly dependent on Tyk2 signaling for IFN-γ production in host environment.

Materials and Methods
Mice
Generation of Tyk2−/− mice has been previously described (2). Tyk2−/− mice with a C57BL/6 background were backcrossed into C57BL/6 mice more than five times. All mice were used in the experiments at 6–10 weeks.

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of age. Age- and sex-matched littermates were used as control mice. OT-I mice expressing the OVA257–264/Kb-specific TCR and C57BL/6 Ly5.1-congenic mice were obtained from The Jackson Laboratory.

**Microorganism**

Recombinant OVA expressing *L. monocytogenes* (rLM-OVA) was provided from Dr. H. Shen (Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, PA) (20). Bacterial virulence was maintained by serial passages in C57BL/6 mice. Fresh isolates were obtained from infected spleens, grown in trypto-soya broth (Nissui Pharmaceutical), washed repeatedly, resuspended in PBS, and stored at −70°C in small aliquots.

**Challenge with bacteria**

For bacterial growth, mice were inoculated i.p. with rLM-OVA in 0.2 ml of PBS on day 0 at a sublethal dose 5 × 10⁵ CFU, which corresponds to one-tenth of the LD₉₀ for C57BL/6 mice. On days 3 and 6 after inoculation, mice were killed by cutting the cervical artery. The peritoneal exudate cells (PEC) were lavaged with 5 ml of ice-cold HBSS and harvested after gentle massage. The spleens and livers were removed and separately placed in homogenizers containing 2 ml of HBSS. The organs were completely homogenized, and the homogenates were serially diluted with cold HBSS. These samples were spread on trypto-soya agar plates, and colonies were counted after incubation for 24 h at 37°C.

**Antibodies and reagents**

FITC-conjugated IFN-γ (XMG1.2) mAb and anti-CD44 (IM7) mAb; PE-conjugated anti-Vα2 (B20.1) mAb and anti-CD44 (IM7) mAb; CyChrome-conjugated anti-CD8α (53-6.7) mAb; allophycocyanin-conjugated streptavidin; and biotin-conjugated anti-Ly5.1 (A20) and Ly5.2 (104) mAbs were purchased from The Jackson Laboratory.

**Flow cytometric analysis**

The cells were incubated with saturating amounts of FITC-, PE-, CyChrome-, and biotin-conjugated Abs for 30 min at 4°C. To detect biotin-conjugated mAbs, cells were stained with allophycocyanin-conjugated streptavidin after incubation with a primary mAb. Nonspecific Fc receptor-mediated binding of mAb was blocked by isotype rat anti-mouse IgG (clone 2.4G2). Cells were analyzed with a FACs flow cytometer. The data were analyzed with FACs Research software (BD Biosciences).

**Intracellular cytokine synthesis analysis**

Spleen cells and PEC from infected mice were harvested, washed, and suspended at 10⁵ cells/ml in complete culture medium, and then incubated for 4 h at 37°C in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) and 5 μg/ml OVA257-264 peptide. These cells were harvested, washed, and incubated for 30 min at 4°C with PE-conjugated anti-CD44 mAb and CyChrome-conjugated anti-CD8 mAb. After surface staining, cells were subjected to intracellular cytokine staining using the Fast Immune Cytokine System according to the manufacturer’s instructions (BD Biosciences). After washing, the cells were stained with FITC-conjugated anti-IFN-γ mAb for 30 min at room temperature, and the fluorescence of the cells was analyzed using a flow cytometer.

**Adoptive transfer**

Spleen cells of naïve Tyk2⁻/⁻ or Tyk2⁺/⁺ mice (Ly5.2⁺) or those of Tyk2⁺/⁻ or Tyk2⁻/⁻ mice that had been infected with 5 × 10⁵ rLM-OVA, were obtained and passed though nylon-wool columns. CD8⁺ T cells (1 × 10⁶) that had been negatively purified using MACS by depletion of the cells expressing CD4, CD11c, DX-5, y8TcR, or I-Aa were adoptively transferred i.v. into naïve C57BL/6 mice (Ly5.1⁺) that were infected with 5 × 10⁵ or 5 × 10⁶ rLM-OVA 12 h later. In some experiments, 2 × 10⁵ purified naive OT-I cells (Ly5.1⁺) were adoptively transferred i.v. into naïve Tyk2⁺/⁺ and Tyk2⁻/⁻ mice (Ly5.2⁺) that were infected with 5 × 10⁵
CD8+ T cell contraction in Tyk2-deficient mice

Tyk2+/− mice on days 3 and 6 after Listeria infection (p < 0.01 or 0.05). However, the bacteria were eliminated completely by day 14 in both groups of mice. Thus, although Tyk2−/− mice showed susceptible to infection at the early stage, the bacteria did not persist long past infection.

Kinetic of OVA257−264-specific CD8+ T cells in Tyk2−/− mice after infection with rLM-OVA

To directly follow Ag-specific CD8+ T cells in Tyk2−/− mice after infection with rLM-OVA, we stained CD8+ T cells with tetrameric H-2Kb molecule folding with OVA257−264 peptide in the spleen and PEC after rLM-OVA infection. Percentages of OVA257−264/H-2Kb tetramer-positive CD8+ T cells in the spleen and PEC of Tyk2−/− mice were almost the same as those in Tyk2+/− mice on day 7 at the expansion phase after infection with rLM-OVA (Fig. 2A). In contrast, the percentages were higher in Tyk2−/− mice at the contraction phase on day 14 and later at the memory phase after infection (Fig. 2A). Absolute numbers of OVA257−264-specific CD8+ T cells were calculated by multiplying percentage of CD8+ T cells positive for OVA257−264 peptide in the spleen and PEC (Fig. 2B). The absolute numbers of OVA257−264-specific CD8+ T cells were significantly larger in Tyk2−/− mice later on day 14 after infection with rLM-OVA (p < 0.01 or 0.05).

To determine the frequency of CD8+ Tc1 cells, we next examined intracellular IFN-γ production by CD8+ T cells in response to OVA257−264 peptide by cytokine FACS analysis. The results showed that the relative numbers of OVA257−264-specific CD8+ T cells positive for IFN-γ were fewer in the spleen and PEC of Tyk2−/− mice than in Tyk2+/− mice on day 7 after infection (Fig. 3A, p < 0.05). These results indicated that generation of the Ag-specific CD8+ Tc1 cells was impaired in Tyk2−/− mice although the Ag-specific CD8+ T cells normally expanded in Tyk2−/− mice following rLM-OVA infection (Fig. 2). In contrast, the numbers of

In vivo treatment with recombinant IFN-γ

Mice were injected i.v. with IFN-γ (PeproTech) at 200 μg/week daily for 4 days (total 800 μg) or with PBS after rLM-OVA infection.

Statistical analysis

Data were analyzed by Student’s t test and the value of p < 0.05 was considered as the level of statistical significance. Analysis was completed using Stat-View 4.5 software (Abacus Concepts).

Results

Kinetics of bacterial growth in organs after infection with rLM-OVA in Tyk2−/− mice

To elucidate the roles of Tyk2 in rLM-OVA infection, we examined kinetics of bacterial growth in the peritoneal cavity, liver and spleen of Tyk2−/− mice after i.p. infection with rLM-OVA. As shown in Fig. 1, the numbers of bacteria in the peritoneal cavity and spleen in Tyk2−/− mice were significantly larger than those in

rLM-OVA 12 h later. Transferred OT-I cells were identified by staining with mAbs to Ly5.1, CD8, and Vα2.

FIGURE 3. Kinetic of OVA257−264-specific CD8+ Tc1 cells in Tyk2−/− mice infected with rLM-OVA assessed by intracellular cytokine staining of IFN-γ. A, Staining profile of OVA257−264-specific CD8+ T cells positive for IFN-γ. Tyk2+/− or Tyk2−/− mice were infected i.p. with 5 × 107 CFU of rLM-OVA 7, 14, 35, or 60 days previously. Spleen cells and PEC were isolated and cultured with 10 μg/ml brefeldin A in the presence of 5 μg/ml for OVA257−264 peptide for 5 h at 37°C. These cells were stained for expression of CD44, CD8, and IFN-γ. Data were analyzed by gated on CD8+ T cells. Each number indicates percentage of OVA257−264-positive CD8+ T cells ± SD of five mice. Data were obtained from three separate experiments. B, Kinetics of absolute number of OVA257−264-specific CD8+ Tc1 cells in Tyk2−/− mice infected with rLM-OVA. Absolute numbers were calculated by multiplying percentage of the CD8+ T cells positive for IFN-γ to total cells number in the spleen and PEC. Each point and vertical bar indicate mean ± SD of five mice of each group. Data of a representative are shown from three separate experiments. *, p < 0.05, **, p < 0.01, significantly different from the value for Tyk2+/− mice.

FIGURE 4. Memory CD8+ T cells generated in Tyk2−/− mice confer the protection against a challenge of rLM-OVA. CD8+ T cells (1 × 106) from Tyk2+/− or Tyk2−/− mice infected with 5 × 10^7 rLM-OVA 35 days previously were adoptively transferred to recipient mice via the tail vein. At 12 h after the transfer, mice were challenged with rLM-OVA (5 × 10^6 CFU), and 2 days later, the number of bacteria in the spleen and liver were counted. Each column and vertical bar indicate mean ± SD of five mice in each group. *, p < 0.05, **, p < 0.01, significantly different from the value for no transfer control mice.
impairs CD8 value for Tyk2 (p significantly higher in Tyk2 Tyk2 memory CD8 in Tyk2 and PEC (Fig. 3). To examine whether the memory CD8/H11001 to LM-OVA after the transfer, mice were challenged with rLM-OVA. The memory CD8/H11001/H11002 mice transferred with CD8 spleen, liver, and peritoneal cavity was significantly reduced in mice infected with 5 × 109 rLM-OVA 12 h later. On days 7 and 14 after infection, spleen cells and PEC were isolated and stained for expression of CD44, CD8, Ly5.2, and OVA257–264/Kb tetramer or IFN-γ. Data were analyzed after gated on CD8 T cells. Each number indicates mean percentage of OVA257–264/Kb tetramer-positive CD8 T cells. Expression of active form of caspase-3 in Ag-specific CD8 T cells or Tyk2+/− mice compared with no transfer control (p < 0.05). These results suggest that Ag-specific memory CD8 T cells in Tyk2−/− mice following rLM-OVA can serve to protect against a challenge with L. monocytogenes.

The memory CD8+ T cells generated in Tyk2−/− mice confer the protection against a challenge of rLM-OVA

To determine whether Tyk2 signaling is involved in apoptosis of effector CD8+ T cells at the contraction phase, we next examined active form of caspase-3 of the Ag-specific CD8+ T cells from Tyk2−/− and Tyk2+/− mice on days 7 and 10 after rLM-OVA infection. On day 7 after infection, the expression level of active caspase-3 in OVA257–264/H-2Kb+ CD8+ T cells were significantly lower in Tyk2−/− mice than in Tyk2+/− mice after 24 h of culture (p < 0.05, Fig. 5A), indicating that Tyk2 signaling play an important role in caspase 3 activation of the effector CD8+ T cells at contraction phase following Listeria infection.

We next examined intracellular expression levels of Bcl-2 in Bcl-2 in OVA257–264/H-2Kb tetramer-positive CD8+ T cells from Tyk2+/− and Tyk2−/− mice at expansion on day 7 or contraction phase on day 10 after infection. As shown in Fig 5B, the levels of the anti-apoptotic protein Bcl-2 in OVA257–264/H-2Kb tetramer-positive CD8+ T cells from Tyk2+/− mice were rapidly down-regulated on day 7 after Listeria infection, and then the level was gradually up-regulated during contraction phase on day 10 after infection. Bcl-2 expression level in the OVA257–264/H-2Kb tetramer-positive CD8+ T cells was almost the same in Tyk2−/− mice as in Tyk2+/− mice (Fig 5B). There was no significant difference in the levels of Bcl-xL expression in OVA257–264/H-2Kb tetramer-positive CD8+ T cells between Tyk2−/− and Tyk2+/− mice on day 7 after infection (data not shown).
Our results suggest that Tyk2 signaling is important for contraction of effector CD8⁺ T cells after Listeria infection. To determine whether Tyk2 signaling in CD8⁺ T cells or in other cells in the milieu of host environment affects contraction of the effector CD8⁺ T cells, we transferred the purified CD8⁺ T cells (1 × 10⁶) from Ly5.2⁺ Tyk2⁻/⁻ mice into Ly5.1⁺ Tyk2⁺/⁺ mice, which were subsequently infected with 5 × 10⁵ rLM-OVA. As shown in Fig. 6, there was no difference in relative numbers of OT-1 cells responding to a foreign Ag in vivo can be enhanced by IFN-γ produced in early inflammation after Listeria infection (17–19). These findings raise a possibility that impaired CD8⁺ T cell contraction may be due to reduced IFN-γ production in Tyk2⁻/⁻ mice.
mice during *Listeria* infection. To verify this, we examined effect of administration of exogenous IFN-γ on CD8+ T cell contraction in Tyk2−/− mice. Administration of rIFN-γ during the initial expansion phase (1–4 days) had no effect on contraction of the OVA257–264-specific CD8+ T cells positive for OVA257–264/H-2Kb tetramer or IFN-γ in Tyk2−/− mice on day 10 after infection (data not shown). In contrast, rIFN-γ administration during the initial contraction phase (6–9 days) significantly reduced the number of the OVA257–264-specific CD8+ T cells in Tyk2−/− mice (Fig. 8, p < 0.05). This effect was seen in cells isolated from a variety of lymphoid and nonlymphoid compartments, including spleen and PEC. Thus, these results suggest that impaired IFN-γ production during *Listeria* infection is at least partially responsible for down-regulation of CD8+ T cell contraction in Tyk2−/− mice after bacterial infection.

**Discussion**

Naive Ag-specific CD8+ T cells proliferate and differentiate into effector CD8+ T cells during expansion phase and most of the activated T cells die by apoptosis during contraction phase following infection (6–8). We showed in the present study that Ag-specific CD8+ T cells normally expanded in Tyk2−/− mice, as assessed by tetramer staining, whereas numbers of Ag-specific CD8+ T cells were significantly increased at contraction phase in Tyk2−/− mice after *Listeria* infection. These results demonstrate that Tyk2 signaling plays an important role in CD8+ T cell contraction following bacterial infection.

We and others found that Tyk2 plays an important role in signal transduction via receptors for IL-12 and contributes to IFN-γ production via the signals triggered by IL-12 in NK cells, CD4+ T cells and CD8+ T cells (2–5). It is notable that impaired CD8+ T cell contraction in Tyk2−/− mice is due at least partly to defect of Tyk2 signaling in milieu of the host environment during course of *Listeria* infection. A recent study has shown that death of the majority of activated T cells responding to a foreign Ag in vivo can be controlled by early inflammation, and IFN-γ in the early inflammation induced by *Listeria* infection plays a critical role in preventing CD8+ T cell death by ACAD during contraction phase (17–19). We have obtained direct evidence that exogenous IFN-γ restored CD8+ T cell contraction to a normal level in Tyk2−/− mice. Protection against *Listeria* infection depends mainly on NK cells, γδT cells and CD4+ Th1 cells (21–26), which produce IFN-γ within the few days following infection. Taken together, it is most likely that absence of Tyk2 results in impaired responses of these lymphocytes for IFN-γ production, which is critical for controlling infection and promoting CD8+ T cell contraction. We cannot completely exclude the possibility that IFN-γ produced by CD8+ T cells promotes apoptosis at contraction phase following *Listeria* infection. However, adoptive transfer experiments revealed that contraction of Tyk2−/− CD8+ T cells normally occurred in Tyk2−/− mice after *Listeria* infection, whereas contraction of CD8+ T cells carrying intact Tyk2 was impaired in Tyk2−/− mice following *Listeria* infection. These results suggested that Tyk2-mediated environment for IFN-γ production may play an important role in controlling CD8+ T cell contraction following *Listeria* infection.

Several studies have shown that Bcl-2 up-regulation in effector T cells plays a critical role in preventing activated T cell death during contraction phase (10, 11, 15). Bcl-2 expression was induced via signaling from the common cytokine receptor γ-chain (27, 28). We have recently reported that IL-15 plays a critical role in up-regulation of Bcl-2 and the survival of effector CD8+ T cells during the contraction phase after *Listeria* infection (16). In contrast, a variety of studies reported that IFN-γ enhances activated T cell death via and up-regulation of proapoptotic genes and down-regulation of survival signals (29–30). IFN-γ is known to induce apoptosis in effector T cells via altering expression of apoptotic molecules such as Bax and antiapoptotic molecules such as Bcl-2 (29–30). Thus, IFN-γ in milieu of the host environment may promote apoptosis of effector CD8+ T cells via up-regulation of proapoptotic genes and down-regulation of survival signals during the contraction phase after *Listeria* infection. However, we found no difference in Bcl-2 expression level in OVA257–264-specific CD8+ T cells in Tyk2−/− and Tyk2−/+ mice following rLM-OVA infection. Recently, Sercan et al. (31) have reported that CD11b+ macrophage-like cells are involved in the promotion of CD8+ T cell contraction in IFN-γ-dependent manner. Therefore, it is also possible that the macrophages in Tyk2−/− mice may have an impaired activity to promote CD8+ T cell contraction. In addition to IFN-γ production, IL-12 signaling is reported to be important for prolong survival of Ag-activated CD8+ T cells via IkappaB family member Bcl-3 up-regulation (32). Furthermore, type I IFNs are reported to provide a third signal to CD8+ T cells to stimulate clonal expansion and differentiation (33–35). In our study, we found that Ag-specific CD8+ T cells in Tyk2−/− mice were able to expand after *Listeria* infection, suggesting that Tyk2 signaling is dispensable for clonal expansion of CD8+ T cells following bacterial infection.

Another notable finding in the present study is that the size of memory CD8+ T cell pool is larger in Tyk2−/− mice following *Listeria* infection. We speculated that the increased size of memory CD8+ T cell pool in Tyk2−/− mice is due mainly to the increased amounts of surviving T cells from T cell contraction by apoptosis after *Listeria* infection. Gett et al. (36) have recently reported that naive CD8+ T cells receiving prolonged or strong stimulation of T cell receptors can differentiate into effector cells and survive as memory T cells by enhancing IL-15/IL-7 responsiveness. Therefore, it is possible that the larger number of bacteria at the early stage of *Listeria* infection in Tyk2−/− mice may affect the subsequent generation of memory CD8+ T cells. However, the fact that early inflammation induced by bacteria is reported to rather promote CD8+ T cell contraction (18) may exclude this possibility. Type I IFNs are reported to act on CD8+ T cells to allow memory formation (33–35). Although Tyk2 signaling is essential of responsiveness to a small amount of IFN-α, (2, 3), our results suggest that Tyk2 signaling is not involved in memory formation of CD8+ T cells following *Listeria* infection. We and others have previously reported that IL-15 play an important role in maintenance of memory CD8+ T cells in the absence of Ag via homeostatic proliferation (37–39). Zhang et al. have reported that selective reduction of aged memory CD8+ T cells is due to impaired responses to IL-15 and a direct anti-proliferative response mediated by increased level of type I IFNs (40, 41). Therefore, it is also possible that the memory CD8+ T cells in Tyk2−/− mice display a lack of responsiveness to a small amount of type I IFNs present in the milieu and exhibit an increased responsiveness to IL-15, resulting in increased number of memory CD8+ T cells in Tyk2−/− mice at the memory phase following bacterial infection. Further studies with adaptive transfer of OT-1 cells for a long period are needed to clarify this possibility.

In conclusion, Tyk2 signaling in milieu of the host environment plays a critical role in promoting contraction of effector CD8+ T cells following a microbial infection.

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Disclosures
The authors have no financial conflict of interest.

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