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Tyk2-Dependent Bystander Activation of Conventional and Nonconventional Th1 Cell Subsets Contributes to Innate Host Defense against *Listeria monocytogenes* Infection

Tomomitsu Hashiguchi,*,† Akiko Oyamada,† Koji Sakuraba,*,† Kazuya Shimoda,‡ Keiichi I. Nakayama,† Yukihide Iwamoto,§ Yasunobu Yoshikai,* and Hisakata Yamada*

IL-12, which is produced in response to intracellular bacteria, such as *Listeria monocytogenes*, promotes the development of pathogen-specific Th1 cells that play an important role in host defense. However, it has also been known that CD44^{high} memory-phenotype CD4 T cells with Th1 functions naturally occur in naive mice, and that lymphopenia-induced proliferation of naive CD4 T cells generates memory-phenotype CD4 T cells with Th1 functions, although their differentiation mechanism and contribution to host defense are unclear. In this study, we analyzed the development and the functions of the different subsets of Th1 cells by using mice lacking tyrosine kinase 2 (Tyk2), a member of the Janus kinase family critically involved in IL-12 signaling. In contrast with the case of conventional Ag-specific Th1 cells, the development of naturally occurring Th1 cells was not impaired in Tyk2-deficient mice. In addition, Th1 cells were normally generated from Tyk2-deficient naive CD4 T cells via lymphopenia-induced proliferation. Nevertheless, all these Th1 subsets, including conventional Ag-induced Th1 cells, produced IFN-γ in response to IL-12 in a Tyk2-dependent manner. Importantly, such Tyk2-dependent bystander IFN-γ production of any Th1 subsets conferred early protection against *L. monocytogenes* infection. Thus, Tyk2-mediated IL-12 signaling is differentially required for the development of different Th1 cell subsets but similarly induces their bystander IFN-γ production, which contributes to innate host defense against infection with intracellular bacteria. *The Journal of Immunology*, 2014, 192: 4739–4747.

T he induction of pathogen-specific CD4 and CD8 T cells that produce IFN-γ is critical for the elimination of intracellular bacteria, such as *Listeria monocytogenes* (1). However, because clonal expansion and functional differentiation of Ag-specific T cells, that is, adaptive immune response, require several days, Ag-nonspecific innate IFN-γ production plays a role at an early phase of *L. monocytogenes* infection. A variety of IFN-γ–producing lymphocytes, including NK cells, NK-T cells, and γδ T cells, are involved in innate host defense against *L. monocytogenes* infection (2–4). It is also known that CD8 T cells that express memory markers confer innate protection against *L. monocytogenes* infection (5).

Such memory phenotype (MP) CD8 T cells can be found in naive mice, even in Tec kinase–deficient mice, which lack naive conventional CD8 T cells (6–8). Therefore, the naturally occurring MP CD8 T cells are thought to belong to a lineage distinct from

conventional CD8 T cells and are also called innate-like CD8 T cells (9). In fact, MP CD8 T cells in naive mice produced IFN-γ in response to IL-12, which was enhanced by an addition of IL-18 (10, 11). They were also activated during *L. monocytogenes* infection and conferred significant protection (5, 8, 12, 13). In addition to the naturally occurring MP CD8 T cells, MP CD8 T cells in naive mice might also include CD8 T cells that have acquired MP and effector functions after lymphopenia-induced proliferation (LIP) (14) and bona-fide memory CD8 T cells specific for self- or environmental Ags. Interestingly, it was demonstrated that effector CD8 T cells specific for an unrelated Ag, for example, OVA, produced IFN-γ during listerial infection and contributed to host defense (5). Thus, even Ag-induced CD8 T cells can be involved in both innate and adaptive immune responses.

MP CD4 T cells, a portion of which are functionally Th1 cells, are also found in naive mice (15–17), but their functions are less known compared with MP CD8 T cells. Similar to MP CD8 T cells, such naturally occurring Th1 cells developed in the absence of Tec kinases, and were activated and produced IFN-γ in response to IL-12 in vitro or during *L. monocytogenes* infection in vivo (15). However, in vivo relevance of the bystander activation of Th1 cells in innate host defense remains unclear. It is also unknown whether LIP-induced MP CD4 T cells, a portion of which is functionally Th1 type (18), as well as conventional Ag-induced Th1 cells exert bystander IFN-γ production.

Tyrosine kinase 2 (Tyk2), a member of the Janus kinase family, is involved in various cytokine signalings and is particularly important for transducing signals from IL-12Rβ1 chain, the common subunit for IL-12Rs and IL-23Rs (19). Hence IL-12–induced IFN-γ production by NK cells was diminished in the absence of Tyk2 (20). Tyk2-deficient mice failed to mount Ag-specific Th1 responses after immunization or infection (21, 22), supporting the importance of IL-12 signaling in the differentiation of Th1 cells. However, interestingly, we have found that differentiation of

*Division of Host Defense, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan; †Department of Orthopedic Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan; ‡Department of Gastroenterology and Hematology, Faculty of Medicine, University of Miyazaki, Miyazaki 889-1692, Japan; and §Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan

Address correspondence and reprint requests to Dr. Hisakata Yamada, Division of Host Defense, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan. E-mail address: hisakata@bioreg.kyushu-u.ac.jp

The abbreviations used in this article: KO, knockout; LIP, lymphopenia-induced proliferation; LN, lymph node; MP, memory phenotype; Tyk2, tyrosine kinase 2; WT, wild-type.

IFN-γ–producing effector CD8 T cells did not require Tyk2 (23). In line with this observation, comparable or even larger numbers of IFN-γ–producing CD8 T cells were detected in IL-12–deficient mice (24, 25). Thus, the requirement of Tyk2-mediated signaling differs among different IFN-γ–producing T cell populations. In this regard, it is of interest to examine whether the Th1 cell subsets that arise in noninfectious conditions, namely, naturally occurring Th1 cells and LIP-generated Th1 cells, are dependent on Tyk2 signaling. In addition, Tyk2-deficient mice would be useful for evaluating the importance of IL-12–induced bystander activation of Th1 cells in vivo.

In this study, we examined the differentiation mechanism and the functions of different Th1 subsets by using Tyk2-deficient mice. We found that nonconventional naturally occurring and LIP-generated Th1 cells did not require Tyk2-mediated signaling for their development, whereas Tyk2-dependent bystander activation of these Th1 cell subsets contributes to the innate protection against *L. monocytogenes* infection.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Breeding Laboratories (Yokohama, Japan). Tyk2 knockout (KO) mice were generated as previously described (21). OT-II (transgenic mice expressing TCR specific for $OVA_{323-339}$ peptide on I-A^b), RAG1 KO, IFN-γ KO, and CD45.1-congenic C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in specific pathogen-free conditions in our institute. Sex- and age-matched mice were used between 6 and 8 wk of age, except for the experiments analyzing neonatal thymus in which 2-d-old mice were used. This study design was approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine (Kyushu University). Experiments were carried out under the control of the Guidelines for Animal Experimentation.

Abs and flow cytometric analysis

Fluorochrome-conjugated mAbs and reagents used for flow cytometric analysis were as follows: FITC-conjugated anti-CD44 (IM-7), anti-CD45.2 (104), anti-TCRδ (GL3), Alexa Fluor 488–conjugated anti–IFN-γ (XMF1.2), PE-conjugated anti–IFN-γ (XMF1.2), anti-CD62L (MEL-14), anti-CD4 (GK1.5), anti-Vα2 (B20.1), allophycocyanin-conjugated anti-CD3 (17A2), anti-CD8 (53.6.7), anti-CD45.1 (A20), anti-CD45.2 (104), PerCP-Cy5.5–conjugated anti-CD4 (CT-CD4), and anti-NK1.1 (PK136) mAbs were purchased from BD Biosciences (San Jose, CA). To detect cytokine production, we cultured cells for 5 h with PMA (25 ng/ml) and ionomycin (1 μg/ml; Sigma-Aldrich, St. Louis, MO) or for 18 h with IL-12 (5 ng/ml; PeproTech, Rocky Hill, NJ) or IL-18 (10 ng/ml; PeproTech), or combination of both. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added to the cultures for the last 4 h. The cell culture medium used in this study was RPMI 1640 (Pure Chemicals) supplemented with 10% FBS (Cell Culture Technologies, Gravesano, Switzerland), 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.5 mM 2-ME. Intracellular staining was performed using BD Cytofix/Cytoperm Kit (BD Biosciences). Stained cells were run on a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using BD CellQuest software (BD Biosciences).

Cell purification

Naive CD4 T cells were purified from lymph node (LN) cells in OT-II or wild-type (WT) C57BL/6 mice. Cells were first stained with anti-CD8 (2.43) and anti–I-A/E (M5/114.112.5) mAbs, followed by an incubation with Dynabead sheep anti-rat IgG (Invitrogen, Carlsbad, CA) to negatively select CD4 T cells. Then naive CD4 T cells were purified by using anti-CD62L microbeads and an autoMACS cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD44^{low} CD62L^{high}CD4$^+$ T cells was >95%. For purifying CD44^{high} and CD44^{low} CD4 T cells, negatively selected CD4 T cells from the spleens and the LNs were stained with FITC-conjugated anti-CD44, PE-conjugated anti-CD4, and allophycocyanin-conjugated anti-CD3 mAbs. CD44^{high} or CD44^{low} CD3$^+$ CD4$^+$ T cells were sorted by FACSAriaII (BD Biosciences). The purity of CD44^{high} or CD44^{low} CD4 T cells was >97%. For the experiments transferring bulk CD4 T cell population, CD4 T cells were directly purified from splenocytes and LN cells by using anti-CD4 microbeads and an autoMACS cell separator (Miltenyi Biotec). The purity of CD4$^+$ T cells was >90%.

In vivo priming of OT-II cells

Naive CD4 T cells purified from WT (CD45.1/1) and Tyk2 KO (CD45.2/2) OT-II mice were mixed (5×10^5 cells/each) and injected i.v. into WT C57BL/6 mice (CD45.1/2). On the next day, the recipient mice were injected s.c. into the base of the tail with an emulsion containing 100 μg OVA and CFA (DIFCO, Detroit, MI). Mice were killed after 1 wk, and splenocytes were used for the analysis.

LIP of CD4 T cells

Naive CD4 T cells purified from WT (CD45.1/1) or Tyk2 KO (CD45.2/2) mice were mixed (1×10^6 cells/each) and injected i.v. into RAG1 KO mice or sublethally irradiated WT (CD45.1/2) mice. After 2 wk, when most of the transferred CD4 T cells had converted to CD44^{high}, splenocytes were harvested from the RAG1 KO recipients and used for the in vitro analysis, whereas the irradiated recipients were subjected to the infection experiments.

L. monocytogenes infection

Mice were inoculated i.p. with 3×10^5 CFU *L. monocytogenes* strain EGD, which correspond to one tenth of the LD_{50} for WT C57BL/6 mice, in 0.2 ml PBS. Peritoneal cavity was lavaged with 3 ml PBS, whereas the spleens were removed and dispersed in 3 ml PBS to measure bacterial number. Various dilutions of the samples were spread on trypto-soya agar plates (Nissui Pharmaceutical, Tokyo, Japan), and colonies were counted after incubation for 24 h at 37°C. Heat-killed listeria was prepared by incubating viable *L. monocytogenes* at 72°C for 60 min.

Induction of Th1 cells in vitro

LN cells from WT OT-II mice were cultured with 10 ng/ml $OVA_{323-339}$ peptide, 1 ng/ml recombinant mouse IL-12 (PeproTech), and 10 μg/ml anti–IL-4 mAb (11B11) at 5–7 × 10^5 cells/well in 48-well plates. At day 4, cells were harvested, washed with PBS, and used for the transfer experiments. Differentiation to Th1 was confirmed by intracellular staining of IFN-γ after stimulation with PMA and ionomycin.

Statistics

Statistical significance was calculated by the two-tailed Mann–Whitney *U* test using Prism software (GraphPad Software). A *p* value <0.05 was considered statistically significant.

Results

Tyk2-dependent differentiation of Ag-induced Th1 cells from naive CD4 T cells

It has been shown that the induction of Ag-specific Th1 responses was impaired in Tyk2-deficient mice (21, 22, 26). However, because IL-12 could also induce T cell proliferation (21), it is possible that the reduced Th1 responses in Tyk2-deficient mice resulted from an insufficient clonal expansion of Ag-specific CD4 T cells, but not from an impaired differentiation into Th1 cells. To test this possibility, we compared in vivo Th1 differentiation of OVA-specific TCR transgenic CD4 T (OT-II) cells of Tyk2-deficient (KO) and WT mice. Naive WT and KO OT-II cells were cotransferred into CD45-congenic recipient mice, which were subsequently immunized with OVA (Fig. 1A). One week after immunization, Th1 differentiation of donor cells in the spleen was examined by detecting IFN-γ production after stimulation with PMA and ionomycin. We found that, although total number of OT-II cells did not differ, the frequency and the number of IFN-γ–producing cells was much lower in KO than in WT cells (Fig. 1B, 1C). Thus, Tyk2-mediated IL-12 signaling is critical for the differentiation of Ag-induced Th1 cells from naive CD4 T cells.

We then examined whether the Ag-specific Th1 cells generated from WT mice produce IFN-γ in response to IL-12 without antigenic stimulation. The spleen cells harvested in the earlier experiment were stimulated with IL-12 or IL-12 and IL-18 in the presence of brefeldin A. We detected IFN-γ production of WT
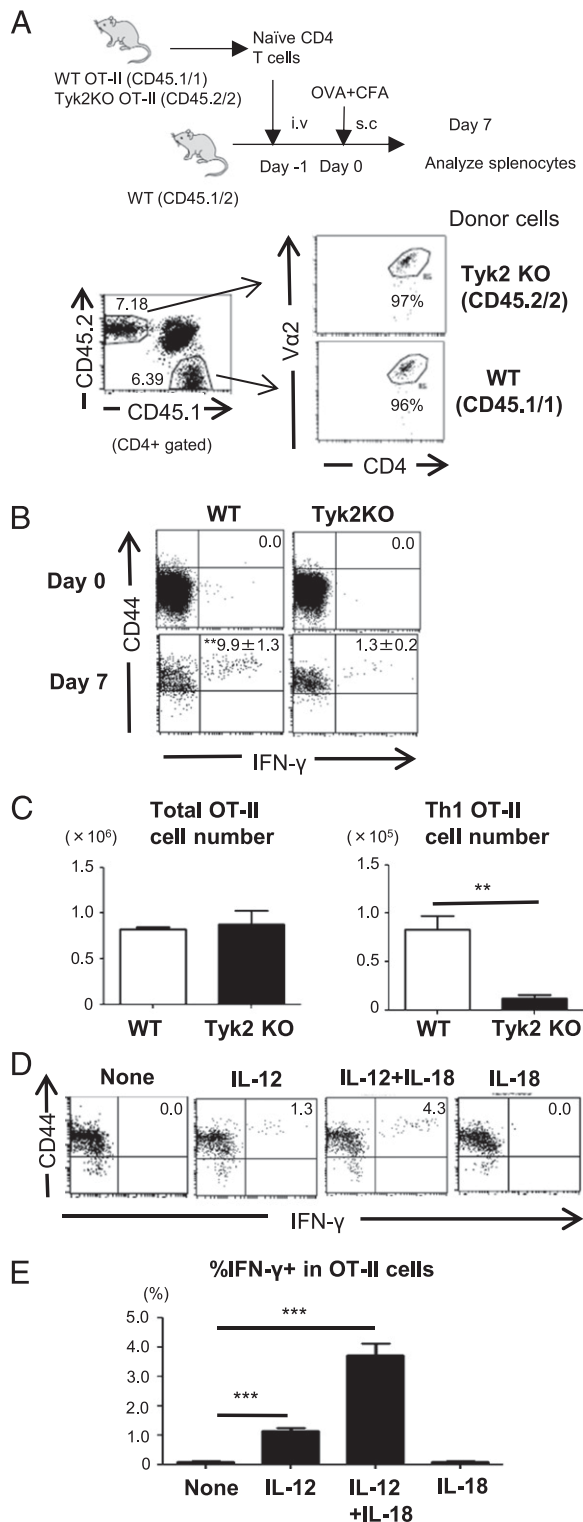
FIGURE 1. Tyk2-dependent differentiation of Ag-induced Th1 cells. (**A**) Naive CD4 T cells from WT (CD45.1/1) and Tyk2 KO (CD45.2/2) OT-II mice were cotransferred i.v. into WT (CD45.1/2) mice (*n* = 3), which were immunized with OVA on the next day. The spleen cells were harvested 1 wk after immunization and were subjected to flow cytometric analysis or were restimulated for functional assays. Lower panels show the expression of OT-II TCR (Vα2) and CD4 in each donor cell population. (**B**) Intracellular staining of IFN-γ after stimulation with PMA and ionomycin on days 0 (before transfer) and 7. Representative dot plots are shown after gating on CD45.2⁻ (WT) or CD45.1⁻ (Tyk2 KO) CD4⁺ T cells. The number in each panel indicates the mean ± SEM percentage of IFN-γ⁺ cells in donor OT-II cells. (**C**) The number of total (*left panel*)

OT-II cells stimulated with IL-12, which was augmented by the addition of IL-18 (Fig. 1D, 1E). Stimulation with IL-18 alone did not induce IFN-γ production. These results indicate that IL-12 signaling induces not only development but also bystander IFN-γ production of Ag-induced Th1 cells.

Tyk2-independent development of naturally occurring Th1 cells in naive mice

We next examined the requirement of Tyk2-mediated signaling for the development of Th1 cells that naturally occur in naive mice. Splenocytes and thymocytes of naive WT or Tyk2 KO C57BL/6 mice were stimulated with PMA and ionomycin to detect Th1 cells. We found comparable numbers of CD44^high CD4 T cells in the spleen or the thymus of WT and Tyk2 KO mice (Fig. 2A). There was no difference in the percentage of IFN-γ–producing cells among CD44^high CD4 T cells in WT and KO mice (Fig. 2B, 2C). Virtually no CD44^low cells produced IFN-γ. Such Th1-type MP CD4 T cells were found even in the neonatal thymus, suggesting their naturally occurring origin (data not shown). Although MP CD4 T cells in both the spleen and the thymus included NK1.1⁺ cells, there was no difference in the percentage of IFN-γ⁺ cells in either NK1.1⁺ or NK1.1⁻ CD4 T cell population between WT and KO mice (Fig. 2D, 2E). Thus, NKT cells and naturally occurring Th1 cells in naive mice develop independently of Tyk2.

These data suggest differential requirements of IL-12 signaling for the development of naturally occurring Th1 cells and conventional Ag-induced Th1 cells, but it is also possible that the requirement of Tyk2 for IL-12 signaling differs between these two Th1 cell populations. So, we examined the responsiveness of naturally occurring Th1 cells to IL-12 in vitro. As reported previously (15), stimulation with IL-12 induced IFN-γ production from CD44^high CD4 T cells in naive WT mice, which was augmented by the presence of IL-18 (Fig. 2F, 2G). However, Tyk2-deficient CD44^high CD4 T cells produced much less IFN-γ in response to IL-12 signaling. Therefore, IL-12 signaling in naturally occurring Th1 cells was actually Tyk2 dependent. This, in turn, indicates that their development is IL-12 independent.

Tyk2-independent differentiation of naive CD4 T cells into Th1 cells by LIP

MP T cells found in the periphery of naive mice might include those that have been generated via LIP, in addition to those that naturally occur in the thymus. In this study, we investigated the involvement of Tyk2 in the differentiation of Th1 cells generated via LIP. Naive Tyk2 KO or WT CD4 T cells were transferred into RAG1 KO mice, and spleen cells were harvested 2 wk later (Fig. 3A). Most of the transferred CD4 T cells from both Tyk2 KO and WT mice converted to express high levels of CD44 in the lymphopenic recipients (Fig. 3B). There was also no difference in the number between Tyk2 KO and WT donor cells (Fig. 3C). By examining cytokine production after stimulation with PMA and ionomycin, we found no difference in the percentage and the number of donor cells producing IFN-γ (Fig. 3B, 3C). Similar results were obtained by using naive OT-II cells as the donor (data not shown). Thus, LIP-induced Th1 cells resemble the naturally occurring Th1 cells

and IFN-γ–producing (*right panel*) donor OT-II cells in the spleen is shown. Data are mean ± SEM. ****p* < 0.01. (**D**) The splenocytes were stimulated with IL-12, IL-18, or IL-12 and IL-18 for 18 h and were tested for intracellular staining of IFN-γ. Data are shown after gating on CD45.2⁻ WT OT-II cells. (**E**) Mean percentages of IFN-γ⁺ cells in OT-II cells are shown (*n* = 3). Error bars represent SEM. ***p < 0.001. Data are representative of three independent experiments.
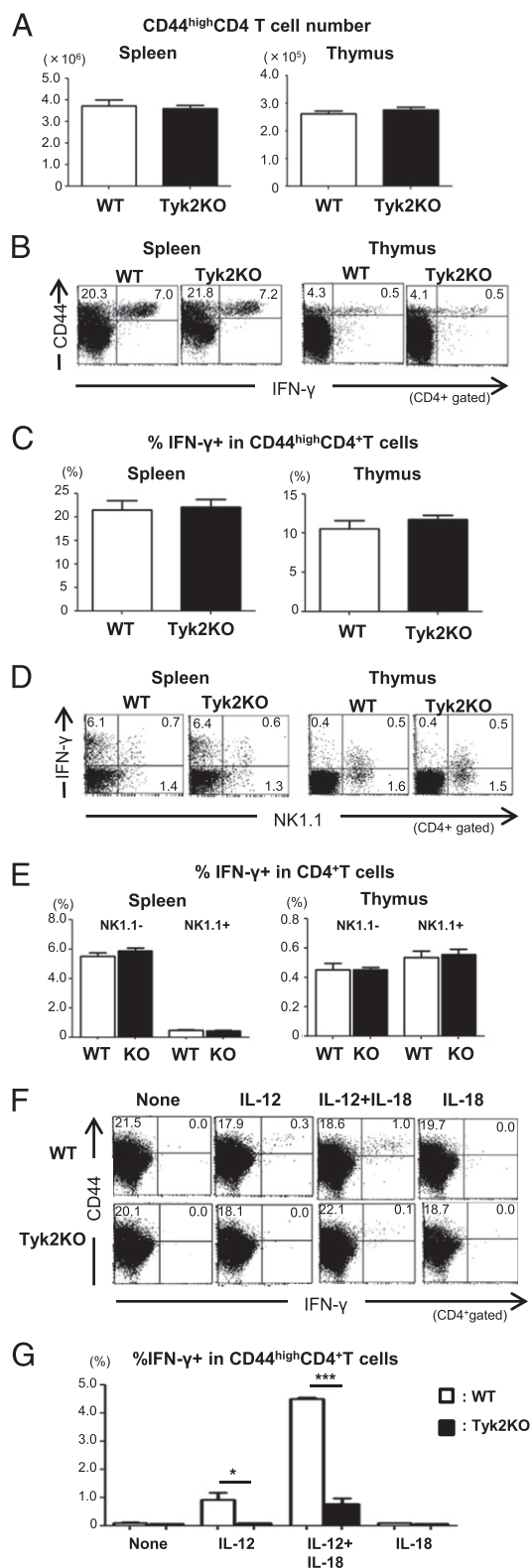
in naive mice in that they develop independently of Tyk2. We tested the in vitro response to IL-12 of the LIP-generated Th1 cells and found that only WT donor cells produced IFN-γ in response to IL-12 (Fig. 3D, 3E). Thus, LIP-generated Th1 cells can also produce IFN-γ in a bystander manner and their IL-12 signaling is Tyk2 dependent.

Tyk2-dependent bystander IFN-γ production from naturally occurring Th1 cells confers protection against bacterial infection

Tyk2-deficient mice showed an impaired early protection against *L. monocytogenes* infection (27). Although Tyk2-dependent IFN-γ production by NK cells, NK-T cells, MP CD8 T cells, as well as TCR γδ T cells might be involved in the early protection against listerial infection, earlier results also suggest a role of Th1 cells. We reported that in situ IFN-γ production of CD8 T cells during *L. monocytogenes* infection was detected by staining intracellular IFN-γ after brief culture with brefeldin A (28). By using this technique, we detected IFN-γ production from CD44^{high} CD4 T cells in WT, but not in KO, mice as early as at day 2 after listerial infection (Fig. 4A), suggesting bystander IFN-γ production of naturally occurring Th1 cells. We also found that listerial Ag-specific IFN-γ production, which was detected by adding heat-killed bacteria to the culture, was impaired in Tyk2 KO CD4 T cells at day 7 postinfection (Fig. 4A), indicating that the development of bacterial Ag-specific Th1 cells was also Tyk2 dependent. Bacterial number in the spleen and the peritoneal cavity of Tyk2 KO mice was significantly increased compared with WT mice (Fig. 4B), indicating that the impaired IFN-γ production of KO CD4 T cells was not due to a decreased bacterial burden. The difference in bacterial number between WT and Tyk2 KO mice was smaller compared with our previous study (27), possibly because of the altered bacterial virulence and different number of inoculated bacteria. By examining IFN-γ production of various cell subsets at day 2 postinfection, it was revealed that the majority of IFN-γ–producing cells were NK cells, which were reduced in Tyk2 KO mice (Fig. 4C). Notably, the proportion of CD4 T cells in IFN-γ–producing cells exceeded that of CD8 T cells, NKT cells, or γδ T cells. Most IFN-γ–producing CD4 T cells were NK1.1[−] (data not shown).

To verify the relevance of Tyk2-dependent bystander IFN-γ production of naturally occurring Th1 cells in host defense, we transferred CD4 T cell subsets from naive WT mice into Tyk2 KO mice before *L. monocytogenes* infection (Fig. 5A). We found that transferring CD44^{high} CD4 T cells, but not CD44^{low} CD4 T cells, conferred significant protection against listerial infection in Tyk2 KO mice (Fig. 5B). Depleting NK1.1^{+} cells from the donor CD4 T cells did not affect the degree of protection (data not shown). To confirm that the protection provided by CD44^{high} CD4 T cells was mediated by IFN-γ, we transferred WT or IFN-γ KO CD4 T cells and found that IFN-γ KO CD4 T cells could not reduce bacterial burden in Tyk2 KO mice (Fig. 5C, 5D). There was no difference in the proportion of CD44^{high} cells between WT and IFN-γ KO CD4

FIGURE 2. Tyk2-independent development of naturally occurring Th1 cells in naive mice. (**A**) The absolute number of CD44^{high} CD4 T cells in the spleen and thymus of naive WT and Tyk2 KO mice was calculated after flow cytometric analysis. Data are mean ± SEM (*n* = 3). (**B**) Splenocytes and thymocytes from naive WT and Tyk2 KO mice were tested for intracellular staining of IFN-γ after stimulation with PMA and ionomycin. Representative dot plots are shown after gating on CD4^{+} cells. (**C**) The percentages of IFN-γ^{+} cells in CD44^{high}CD4^{+} splenocytes (*left*) or CD44^{high}CD4^{+}CD8^{−} thymocytes (*right*) are shown. Data are mean ± SEM. (**D**) Representative dot plots depicting IFN-γ production from

NK1.1^{+} and NK1.1^{−} CD4^{+}splenocytes or CD4^{+}CD8^{−} thymocytes (*right*) after PMA and ionomycin stimulation. Data are shown after gating on CD4^{+} cells. (**E**) Mean percentages of IFN-γ^{+} cells NK1.1^{+} and NK1.1^{−} CD4^{+} splenocytes (*left*) or CD4^{+}CD8^{−} thymocytes (*right*) are shown. (**F**) Splenocytes from naive WT or Tyk2 KO mice were stimulated with IL-12, IL-18, or IL-12 and IL-18 for 18 h and were tested for intracellular staining of IFN-γ. Representative dot plots are shown after gating on CD4^{+} cells. (**G**) Mean percentages of IFN-γ^{+} cells in CD44^{high} CD4^{+} T cells are shown. *$p < 0.05$, ***$p < 0.001$. Data are representative of three independent experiments.
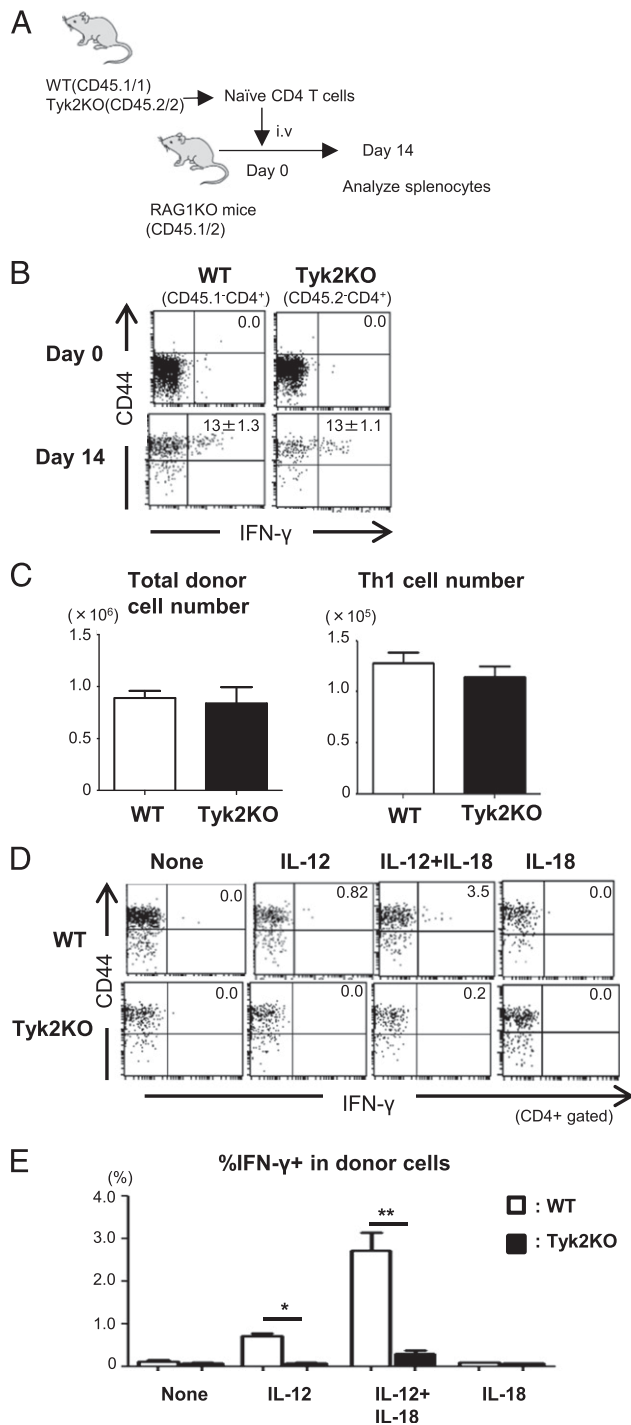
FIGURE 3. Tyk2-independent differentiation of naive CD4 T cells into Th1 cells by LIP. (**A**) Naive CD4 T cells from WT (CD45.1/1) and Tyk2 KO (CD45.2/2) mice were transferred i.v. into RAG1 KO (CD45.1/2) mice ($n = 3$). Two weeks after transfer, splenocytes were harvested and restimulated before flow cytometric analysis. (**B**) The splenocytes were stained for intracellular IFN-γ after stimulation with PMA and ionomycin. Representative dot plots are shown after gating on CD4+CD45.2− (WT) or CD4+CD45.1− (Tyk2 KO) cells. The upper panels show donor T cells before transfer. The numbers in the panels are the mean percentages of IFN-γ–producing cells in CD4+ cells. (**C**) Absolute number of total (*left*) and Th1 (*right*) donor CD4 T cells in the spleen is shown. Data are mean ± SEM. (**D**) The splenocytes were stimulated with IL-12, IL-18, and IL-12 + IL-18 for 18 h and were tested for intracellular staining of IFN-γ. Representative dot plots are shown after gating on donor CD4+ cells. (**E**) Mean percentages of IFN-γ–producing cells in donor CD4 T cells are shown. *$p < 0.05$, **$p < 0.01$. Data are representative of three independent experiments.

T cells (data not shown). We also found that CD4 T cells from naive WT, but not Tyk2 KO mice, decreased bacterial burden in IFN-γ KO mice (data not shown). These results suggest that Tyk2-dependent bystander IFN-γ production by naturally occurring Th1 cells is involved in the host defense against listerial infection.

Tyk2-dependent host defense by LIP-induced Th1 cells

To test whether LIP-induced Th1 cells can also confer innate protection against listerial infection, we transferred naive CD4 T cells from WT or Tyk2 KO mice into irradiated IFN-γ KO mice that were infected with *L. monocytogenes* 2 wk later (Fig. 6A). We confirmed that both WT and Tyk2 KO CD4 T cells differentiated into Th1 cells after LIP in the irradiated IFN-γ KO recipients (Fig. 6B). However, only WT CD4 T cells spontaneously produced IFN-γ in response to listerial infection. Furthermore, bacterial number was decreased in mice transferred with WT, but not Tyk2 KO CD4 T cells (Fig. 6C). Thus, LIP-induced Th1 cells can also be involved in host defense against *L. monocytogenes* in a Tyk2-dependent manner.

Bystander IFN-γ production from Ag-induced Th1 cells can be involved in host defense against bacterial infection

Although IL-12–induced bystander IFN-γ production was also detected in conventional Ag-specific Th1 cells (Fig. 1D, 1E), its in vivo relevance remains unclear. To verify this, we transferred WT or Tyk2 KO naive OT-II cells into IFN-γ KO mice that were subsequently immunized with OVA. Then the mice were challenged with *L. monocytogenes* infection, and bacterial number in the peritoneal cavity and the spleen was examined at day 2 (Fig. 7A). We detected IFN-γ production by the transferred WT OT-II cells (data not shown) and also found that transferring WT, but not Tyk2 KO OT-II cells significantly decreased bacterial burden in IFN-γ KO mice (Fig. 7B). By transferring the same number of in vitro–induced Th1 or naive OT-II cells from WT mice into Tyk2 or IFN-γ KO mice before *L. monocytogenes* infection (Fig. 7C), we found that Th1, but not naive WT OT-II cells conferred protection against *L. monocytogenes* infection in both Tyk2 KO mice and IFN-γ KO mice (Fig. 7D, 7E). These results indicate that even conventional Ag-induced Th1 cells can contribute to innate host defense against listerial infection via bystander IFN-γ production.

Discussion

Differentiation of naive CD4 T cells to various Th cell subsets is strongly influenced by the cytokine milieu. It is established that naive CD4 T cells primed in the presence of IL-12 preferentially differentiate into Th1 cells (29). We confirmed by using OT-II cells that the differentiation of Th1 cells was impaired in the absence of Tyk2, an essential molecule for IL-12 signaling. Induction of the bacterial Ag-specific Th1 cells during listerial infection was also reduced in Tyk2 KO mice. These results seem reasonable because immunization with CFA or listerial infection induces IL-12 production from APCs, and thereby provides an environment suitable for Th1 differentiation. However, there are subsets of Th1 cells that develop under noninfectious conditions. These include naturally occurring MP CD4 T cells in naive mice and MP CD4 T cells generated via LIP (18). We demonstrated in this study by using Tyk2-deficient mice that these two Th1 cell subsets differentiated independently of Tyk2-mediated cytokine signaling, including IL-12. They might use alternative cytokines for differentiation, but it is also possible that they do not require any cytokine signals. Interestingly, IL-12– or Tyk2-independent differentiation of naive CD8 T cells to IFN-γ–producing Tc1 cells was also reported (23–25). In addition, we have found that naturally occurring IL-17–
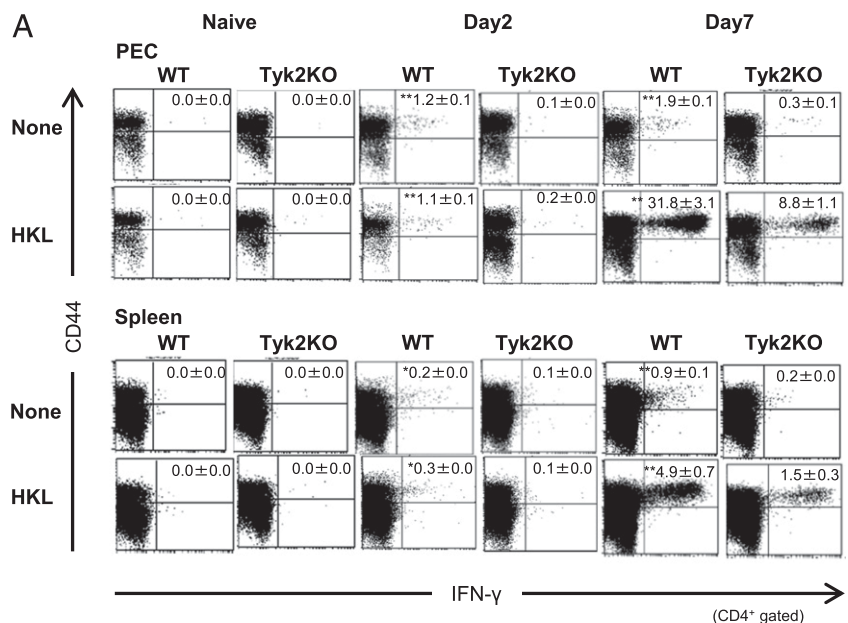
FIGURE 4. Tyk2-dependent bystander IFN-γ production from naturally occurring Th1 cells during bacterial infection. (**A**) Peritoneal exudate cells (PECs) and spleen cells in WT (*n* = 3) and Tyk2 KO (*n* = 3) mice were harvested on days 2, 3, and 7 after i.p inoculation with *L. monocytogenes*. Cells were cultured for 5 h with or without stimulation with heat-killed listeria in the presence of brefeldin A and were subjected to intracellular staining of IFN-γ. Representative dot plots are shown after gating on CD4⁺ cells. The numbers in the panels are the mean percentages of IFN-γ–producing cells in CD4⁺ cells. (**B**) Bacterial numbers in the peritoneal cavity and the spleen are shown. (**C**) Absolute number of IFN-γ–producing cell population in PECs and spleen of WT (white bars, *n* = 3) and Tyk2 KO (black bars, *n* = 3) mice 2 d after *L monocytogenes* infection. Intracellular IFN-γ was detected after 5-h culture with brefeldin A. Data are mean ± SEM. *$p <$ 0.05, **$p <$ 0.01, ***$p <$ 0.001. Data are representative of three separate experiments.
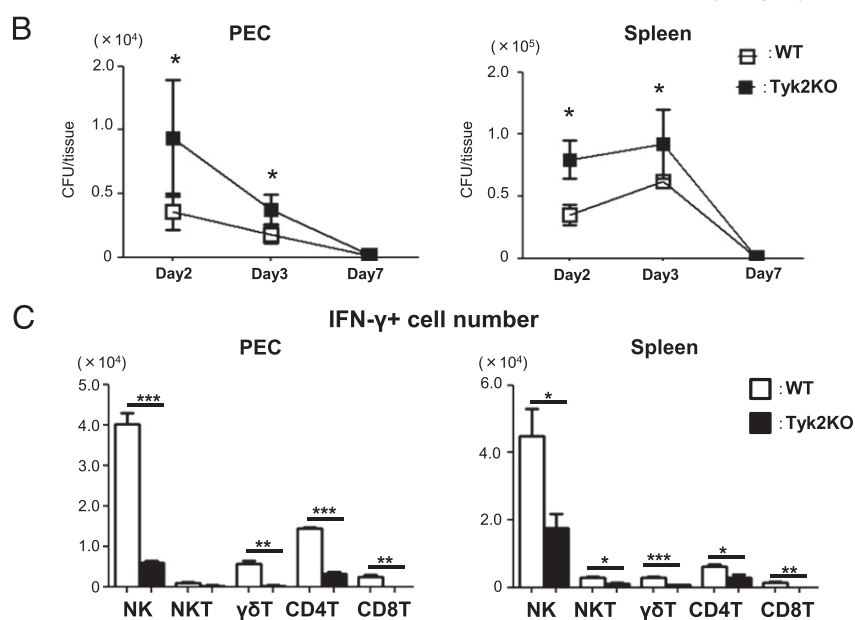
producing TCR γδ T cells do not require STAT3, which mediates IL-6 signaling and is critical for Th17 differentiation (30). Thus, there are differences in the requirement for cytokine signaling in the differentiation of effector T cell populations.

The origin of MP CD4 T cells in naive mice, a portion of which are functionally Th1 cells, is unclear. They might include non-conventional naturally occurring Th1 cells differentiated in the thymus (15), Th1 cells generated via LIP (18), as well as conventional memory Th1 cells induced by environmental Ags/pathogens. In this regard, our results provide a clue to understand their origin. Thus, most of these T cells might not be conventional Ag-induced Th1 cells but nonconventional naturally occurring Th1 cells or LIP-induced Th1 cells, because they can develop in the absence of Tyk2. Younes et al. (17) also reported that MP CD4 T cells in naive mice are distinct from Ag-induced memory CD4 T cells with regard to proliferation rate and TCR repertoires. However, it is difficult to discriminate the first two nonconventional Th1 subsets, because there have been no specific markers or master

regulators of their differentiation. Expression of PLZF, a transcription factor involved in NKT cells (31), was demonstrated in MP CD4 T cells in MHC class II transgenic mice, but they are not expressed in those in WT mice (32). Interestingly, it was recently reported that innate MP CD8 T cells and LIP-induced MP CD8 T cells showed a distinct pattern of gene expression (33). A similar approach would be applied for CD4 T cells in the future experiments.

Bystander activation of MP T cells is well recognized for CD8 T cells. We have also reported that naturally occurring MP CD8 T cells produce IFN-γ in response to IL-12 without antigenic stimulation (34). It was also shown that OVA-specific Tc1 cells were activated by IL-12 in the absence of the Ag (5). In contrast, there have been a few studies showing bystander activation of CD4 T cells, but one study showed IFN-γ production of naturally occurring MP CD4 T cells by stimulation with IL-12 in vitro or during *L. monocytogenes* infection in vivo (15). In this study, we extend these findings by demonstrating IL-12–induced IFN-γ
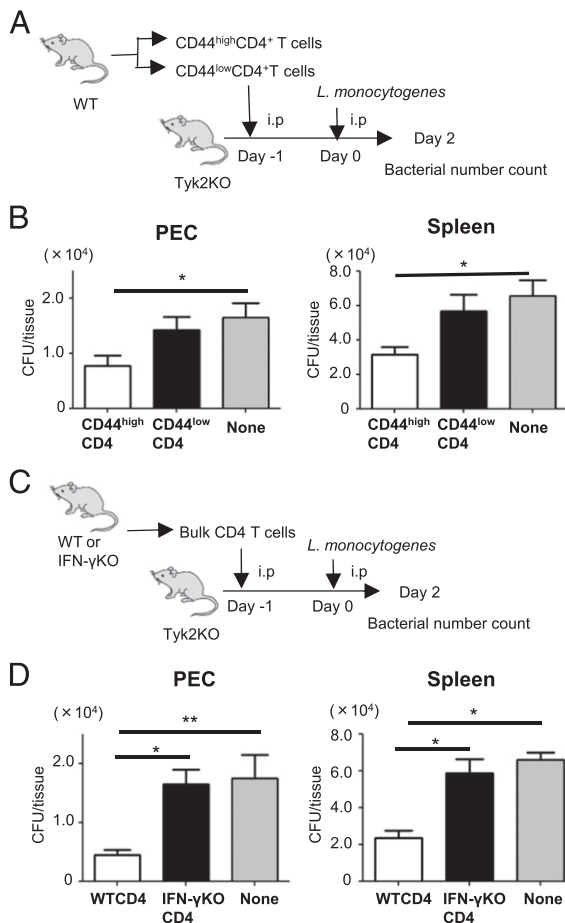
FIGURE 5. WT MP CD4 T cells confer protection against LM infection in Tyk2-deficient mice. (**A**) CD44^{high} or CD44^{low} CD4^{+} T cells from WT mice were injected i.p. into Tyk2 KO (*n* = 5, in each group) mice 1 d before *L. monocytogenes* infection. Bacterial number in the peritoneal cavity (peritoneal exudate cells) and the spleen was measured 2 d after *L. monocytogenes* infection (**B**). (**C**) WT or IFN-γ KO CD4 T cells were injected i.p. into Tyk2 KO mice (*n* = 5, in each group) 1 d before *L. monocytogenes* infection. Bacterial number in the peritoneal cavity and the spleen was measured 2 d after *L. monocytogenes* infection (**D**). Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$. Data are representative of three independent experiments.

FIGURE 6. Tyk2-dependent innate host defense by LIP-induced Th1 cells. (**A**) Naive CD4 T cells from WT (CD45.1/1) or Tyk2 KO (CD45.1/1) mice were transferred i.v. into sublethally irradiated IFN-γ KO (Ly5.2/5.2) mice (*n* = 4 in each group). Two weeks after the transfer, the recipients were infected with *L. monocytogenes*, and the spleen and peritoneal exudate cells (PECs) were harvested from the recipient mice 2 d postinfection. (**B**) The PECs were cultured for 18 h without stimulation (*upper panels*) or stimulated with PMA and ionomycin for 5 h (*lower panels*) in the presence of brefeldin A; then the cells were tested for intracellular staining of IFN-γ. Representative dot plots are shown after gating on the donor CD4^{+} cells. The numbers in the panels are the mean ± SEM percentages of IFN-γ^{+} cells in CD4^{+} cells. (**C**) Bacterial counts in the peritoneal cavity and the spleen of the recipient IFN-γ KO mice are shown. Data are mean ± SEM. *$p < 0.05$. Data are representative of two separate experiments.

production of Th1 cells generated by LIP and that of conventional Ag-induced Th1 cells. Thus, bystander activation is not a unique function of naturally occurring MP T cells, which might be called "innate-like" T cells, but rather a general feature of T cells expressing memory/activation markers.

It has been assumed that CD4 T cells participate in host defense against *L. monocytogenes* infection as a part of adaptive immune system specific for listerial Ags. However, it has also been known that listerial Ag-specific CD4 T cell responses become detectable when bacteria are almost eradicated (35). In addition, depleting CD4 T cells significantly increased bacterial burden from early stages after listerial infection (36, 37). Although an involvement of a small number of listerial Ag-specific CD4 T cells cannot be excluded, our data suggest an importance of IL-12–induced bystander IFN-γ production in CD4 T cell–mediated early host defense against *L. monocytogenes*. The degree of protection conferred by innate CD4 T cell activation was modest in the cell transfer experiments. This would have been improved by increasing the number of transferred CD4 T cells. However, it might overestimate their role, because CD4 T cells were not the main source of early IFN-γ production as shown in Fig. 4C. Nevertheless, it would be of interest to examine the relative importance of listerial
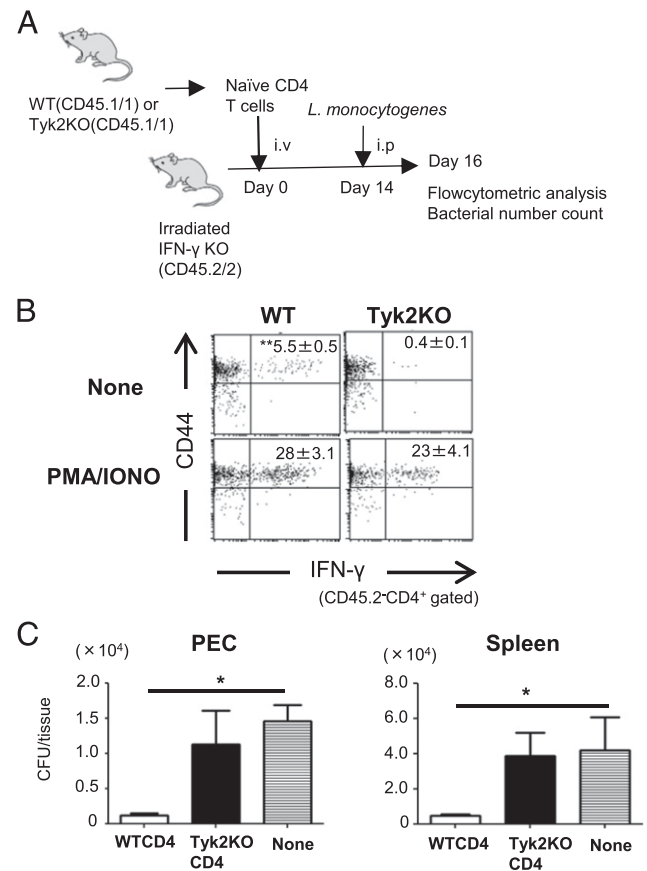
Ag-specific CD4 T cells and bystander MP CD4 T cells during primary *L. monocytogenes* infection. The adaptive CD4 T cell responses might play a role only in secondary infection as memory CD4 T cells.

Berg et al. (5) demonstrated that OVA-specific CD8 T cells conferred protection against *L. monocytogenes* infection. In this study, we demonstrated in a similar system that OVA-specific CD4 T cells can confer protection against listerial infection through bystander IFN-γ production. This not only formally demonstrates that the CD4 T cell can be activated in an Ag-independent manner, but also indicates that cells in adaptive immune system can be involved in innate host defense. Thus, irrespective of whether CD4 or CD8 T cells, there is no difference in the innate functions not only in vitro but also in vivo between conventional Ag-induced effector/memory T cells and nonconventional T cells.

Our results indicate that Tyk2-mediated signaling in CD4 T cells is involved in both adaptive and innate host defense against intracellular pathogens. This is noteworthy because Tyk2 deficiency causes severe immune deficiency in humans (38). Although we examined only an acute infection model in this study, it is also
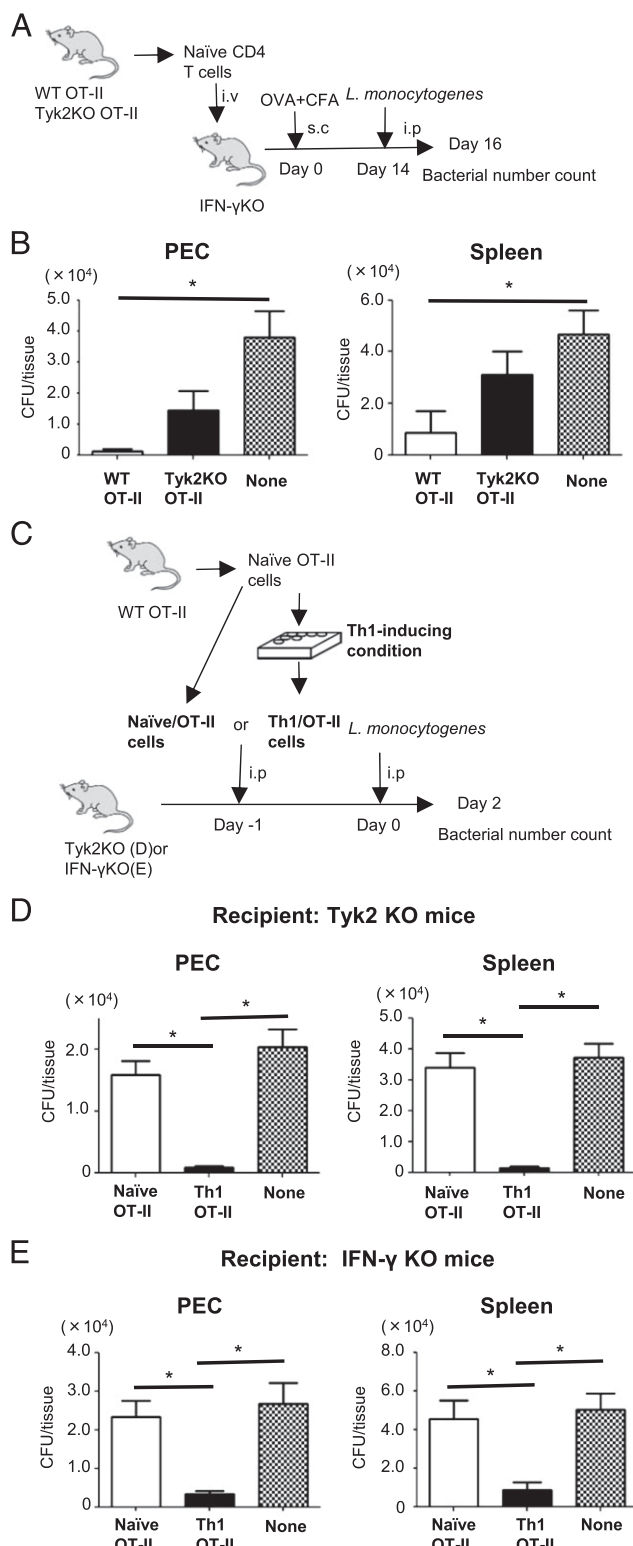
FIGURE 7. Bystander IFN-γ production of conventional Ag-induced Th1 cells can also confer protection against bacterial infection. (**A**) Naive CD4 T cells purified from WT or Tyk2 KO OT-II mice were transferred i.v. into IFN-γ KO mice (*n* = 3 in each group), which were immunized with OVA on the next day. Two weeks after immunization, the recipient mice were infected i.p. with *L. monocytogenes*, and the spleen and peritoneal exudate cells (PECs) were harvested on day 2 postinfection. (**B**) Bacterial number in the peritoneal cavity and the spleen are shown. Data are mean ± SEM. **p* < 0.05. (**C**) LNs cells from WT OT-II mice were stimulated with OVA peptide under a Th1-inducing condition for 4 d. Cells were collected and injected i.p. into Tyk2 KO or IFN-γ KO mice 1 d before *L. monocytogenes* infection. Control groups of

possible that Tyk2-mediated bystander Th1 cell activation takes place during chronic infection and also in chronic inflammatory conditions such as autoimmunity. Thus, our data also provide important information for analyzing the Ag specificity of Th1 cells in the pathogenesis of these inflammatory diseases.

Disclosures

The authors have no financial conflicts of interest.

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mice were either transferred with the same number of naive OT-II cells or not receiving cell transfer. Bacterial number in the peritoneal cavity and the spleen of Tyk2 KO (**D**) or IFN-γ KO mice (**E**) was measured on day 2 postinfection. Data are mean ± SEM (*n* = 3 in each group). **p* < 0.05. Data are representative of three independent experiments.

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