Roles of the small intestine for induction of toll-like receptor 4-mediated innate resistance in naturally acquired murine toxoplasmosis

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Abstract

Peroral infection of *Toxoplasma gondii* is thought to reflect the typical infection route of naturally acquired toxoplasmosis in humans. We have investigated possible differential roles of toll-like receptor 2 (TLR2) and TLR4 in host defense against naturally acquired murine toxoplasmosis. After peroral inoculation of *T. gondii* ME49 cysts, TLR4-deficient C3H/HeJ mice were more susceptible to infection than wild-type (WT) C3H/HeN mice, as shown by increased cyst number and low production of cytokines, which are the key factors in protective immunity. When mice were inoculated by intra-peritoneal inoculation of *T. gondii*, there were no significant differences in the number of brain cysts and cytokine productions between C3H/HeJ and C3H/HeN mice. Histopathologic examination revealed severe inflammation in the small intestine of C3H/HeJ (TLR4-deficient) mice, while an increased number of TLR4-positive mononuclear cells was found in C3H/HeN (WT) mice. To confirm these phenomena, TLR2$^{-/-}$ or TLR4$^{-/-}$ mice were infected perorally with *T. gondii* cysts. TLR4$^{-/-}$ mice were more susceptible to infection compared with TLR2$^{-/-}$ and C57BL/6 mice. Nuclear factor-kappa B activation through TLR4 agonistic activity of *T. gondii* ME49 was demonstrated by luciferase assay using stably expressing mouse (m) TLR2 or mTLR4/mMD-2 transfectants. We demonstrate here for the first time that innate immune recognition by TLR4 is involved in protective mechanisms against peroral infection with *T. gondii* ME49. These results suggest that the small intestine plays an important role in the induction of innate immunity in naturally acquired toxoplasmosis.

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite occurring worldwide in both human and animal hosts. Under normal conditions, infection is largely asymptomatic. However, in immunocompromised hosts, such as patients with AIDS, the parasite can become widely disseminated and cause severe toxoplasmosis or encephalitis (1). Natural infection with *T. gondii* is acquired orally by ingestion of undercooked or raw meat-containing parasite cysts or oocysts. Following ingestion, bradyzoites or sporozoites are released from the cysts and rapidly invade the intestinal mucosa and convert into tachyzoites. After multiplication and

disruption of infected cells, tachyzoites invade neighboring cells and disseminate via the blood and lymphatic system. For reasons mentioned above, the peroral route of *T. gondii* infection is thought to reflect the typical infection that occurs in naturally acquired toxoplasmosis in humans, and it is important to investigate the mechanism of host resistance against the parasite using animals infected via peroral route.

Recently, it has been reported that toll-like receptor 2 (TLR2) and the TLR4/MD-2 complex are highly expressed in the gastrointestinal mucosa of mice, and play important roles in innate immunity against pathogens (2, 3). The TLR family

has been identified as key host molecules in the induction of innate immune responses to microbial ligands (4, 5). TLR2 and TLR4 react to bacterial cell-wall compounds (6, 7). TLR2 is activated by a variety of ligands, such as bacterial lipopeptides, as well as fungal and mycobacterial components (8), while TLR4 is activated not only by bacterial LPS but also apparently by other ligands, such as viral proteins (9–11). So far, little information is available regarding roles of the small intestine in the induction of innate immunity mediated by TLRs in naturally acquired toxoplasmosis.

In the present study, we therefore decided to investigate the possible differential role of TLR2 and TLR4 in mediating host defense against *T. gondii* infection, using animal models of peroral infection with *T. gondii*. Our results indicate that TLR4 mediates innate immunity against toxoplasmosis induced by peroral, but not intra-peritoneal infection of *T. gondii* ME49 cysts, and that the small intestine plays an important role in this process.

Methods

Experimental animals

TLR2$^{-/-}$ and TLR4$^{-/-}$ mice with C57BL/6 background (12, 13) were bred and maintained under SPF conditions at the Animal Center, Institute of Medical Science, University of Tokyo, Japan. C57BL/6, TLR4-deficient C3H/HeJ and the wild-type (WT) C3H/HeN mice were purchased from Japan SLC (Hamamatsu, Japan). Female mice between 5 and 6 weeks old were used for experiments. The animal experiments were approved by the Committee for Animal Experiment of the Institute of Medical Science, University of Tokyo.

Parasites and experimental infection

Brain tissue containing *T. gondii* ME49 cysts was prepared by homogenization in saline, and the homogenate was used as an inoculum for infection after the number of cysts in homogenates had been counted. Mice were infected with various doses of cysts of the avirulent *T. gondii* ME49 strain, by peroral or intra-peritoneal inoculation (p.o.i. or i.p.i.). The number of cysts in infected brains was determined by microscopic examination of brain homogenates made with saline. Brains from uninfected control mice were also removed in parallel, and an equal volume of infected brains was administered by p.o.i. or i.p.i. as a control. *Toxoplasma gondii* ME49 tachyzoites were obtained by tissue culture using Vero cells as previously described, and they were used as a soluble *Toxoplasma gondii* ME49 tachyzoite antigen (SA) after solubilization by sonication (14). To examine the possibility that normal Vero cell lysate induces cytokine production or nuclear factor-kappa B (NF-κB) luciferase activity, cytokine production or NF-κB luciferase activity was also performed using normal Vero cell lysate. Since their activities were comparable with those of controls (medium alone), it was concluded that contamination of normal Vero cells in SA did not significantly affect cytokine production or NF-κB luciferase activity.

Measurement of cytokine production in spleen cells

For measurement of *in vitro* cytokine production, single-cell suspensions were prepared from spleens of *T. gondii* ME49-infected mice at 5 days after p.o.i. or i.p.i. Spleen cells were cultured at 1×10^6 cells per well in 1 ml RPMI1640 containing 10% FCS and antibiotics with or without SA (20 μg ml^{-1}), and then supernatants were collected 24 h later. The levels of IFN-γ, IL-4, IL-6, IL-10 and IL-12p40 in the culture supernatants were measured by sandwich ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Samples were assayed in triplicate, and the data were expressed as the mean ± SD.

Histopathology and immunohistochemistry

The major organs and small intestines of mice were fixed in 10% neutral buffered formalin and then embedded in paraffin. Sections were made and stained with H&E for evaluation of pathologic changes. For immunohistochemistry, deparaffinized sections were stained with anti-mouse TLR2- or TLR4/MD-2-specific antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were then washed in PBS and incubated with anti-rat antibody labeled with biotin, streptavidin labeled with peroxidase (Histofine, Nichirei Corp., Tokyo, Japan) and then incubated with diaminobenzidine substrate. Normal rabbit IgG instead of primary antibody was used as a negative control. Finally, sections were mounted in Mount-Quick (Daido Sangyo, Co., Ltd, Tokyo, Japan) and then observed. Quantification of mononuclear cell or neutrophil numbers in the lesions of small intestine was determined by counting these cells in 20 high-magnification fields of sections, and the results were presented as fold increase as compared with normal mice.

Luciferase assay

The Ba-F3 cell line stably expressed mouse (m) TLR2 or mTLR4/mMD-2 and p55Ig κLuc with an NF-κB-dependent luciferase reporter construct (15). For the luciferase assay, the transfectants were washed and cultured in RPMI1640 containing 10% FCS and IL-3. Individual wells containing 1×10^5 cells were left untreated or stimulated with SA. In some experiments, SA was pre-treated with 10 mg of polymyxin B (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 37°C for 2 h to deactivate LPS activity. LPS (100 ng ml^{-1}, Sigma, St Louis, MO, USA) or Pam$_3$CSK$_4$ (100 ng ml^{-1}, ECM PG Microcollections) was used as a ligand for TLR4 or TLR2. After 4 h stimulation with ligand or SA, cells were harvested, washed and then lysed in 100 μl lysis buffer. Luciferase activity was measured using 10 μl lysate and 50 μl luciferase substrate (Nippon Gene, Toyama, Japan), quantified as relative light units on a luminometer (Berthold Japan, Tokyo, Japan). The results were presented as fold increase as compared with control wells.

Antibiotics

Penicillin G potassium salt (1500 U ml^{-1}, Wako Pure Chemical Industries, Ltd) and streptomycin sulfate (2 mg ml^{-1}, Wako Pure Chemical Industries, Ltd) were administered via sterilized drinking water to remove the intestinal microflora of mice until fecal bacteria disappeared. During the treatment with antibiotics, cages, bottles for drinking water and antibiotic solutions given to the mice were renewed daily. Feces from each cage of mice were collected individually and placed

into sterile tubes. The fecal bacteria were checked daily by Giemsa stain to examine the effect of antibiotic treatment. Fecal bacteria had disappeared at 3 days after the treatment, and then *Toxoplasma* infection was performed.

Statistical analyses

Statistical significance was established using an unpaired, two-tailed Student's *t* test. Statistically significant data are indicated in the figures by an asterisk, and the corresponding *P* values are listed in the figure legends.

Results

Number of brain cysts in C3H/HeJ and C3H/HeN mice infected with T. gondii ME49

Since C3H/HeJ mice are functionally TLR4-deficient for LPS signaling, we sought to determine whether these mice were susceptible to *Toxoplasma* infection compared with WT C3H/HeN mice. C3H/HeJ and C3H/HeN mice were infected with *T. gondii* ME49, either by p.o.i. as a natural route of infection or by i.p.i. as an unnatural route of infection. The number of brain cysts was examined 30 days after infection. The number of brain cysts in C3H/HeJ mice infected by p.o.i was significantly higher than C3H/HeN mice, suggesting that TLR4 might be required for innate resistance to murine toxoplasmosis. In striking contrast, in both strains following i.p.i. of *T. gondii* cysts, the number of cysts in the brains was low and not significantly different between the two mouse strains (Fig. 1). This indicates that TLR4-mediated innate resistance was not involved in the response to i.p.i. of *T. gondii*. Since innate immunity mediated by TLR4 might contribute to host resistance to *T. gondii* infection when parasites are inoculated via the peroral route but not the intra-peritoneal route, it appears that induction of innate resistance to murine toxoplasmosis is dependent on the route of parasite inoculation.

Production of pro-inflammatory cytokines in C3H/HeJ and C3H/HeN mice infected with T. gondii ME49

To compare the level of pro-inflammatory cytokines, IFN-γ, IL-12 and IL-6 produced in response to *T. gondii* infection,
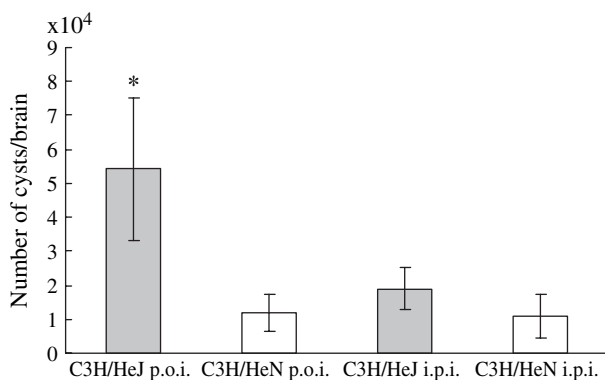

Fig. 1. Growth of *T. gondii* ME49 cysts in the brains of TLR4-deficient C3H/HeJ and WT C3H/HeN mice. C3H/HeJ and C3H/HeN mice were infected with 10 cysts of *T. gondii* ME49 by p.o.i. or i.p.i., and then the number of cysts in the brain was examined at 30 days after the infection (mean ± SD, $n = 6$, *$P < 0.01$). This experiment was repeated three times with similar results.

spleen cell cultures were prepared at 5 days after infection and cultured with SA for 24 h. Supernatants were collected from each well, and then cytokine production was measured by sandwich ELISA. When C3H/HeJ and C3H/HeN mice were inoculated perorally with *T. gondii* ME49 cysts, the production of IFN-γ and IL-12 in C3H/HeJ (TLR4-deficient) mice was significantly lower than that in C3H/HeN mice. However, when these mice were inoculated intra-peritoneally with *T. gondii* ME49 cysts, IFN-γ and IL-12 were produced at the same level in both strains, and their levels were almost comparable with those of C3H/HeN mice with p.o.i. of *T. gondii* ME49, which showed resistance to *T. gondii* infection (Fig. 2). These results suggest that increased susceptibility to *T. gondii* in C3H/HeJ mice infected perorally is associated with reduced levels of IFN-γ and IL-12, which are the key factors for resistance to murine toxoplasmosis. Regarding IL-6 production, the level of IL-6 was significantly lower in C3H/HeJ (TLR4-deficient) mice than that in C3H/HeN mice after p.o.i. of cysts; however, IL-6 production was low in both C3H/HeJ and C3H/HeN mice following i.p.i. of *T. gondii*.

Histopathology and immunohistochemistry

In histopathologic examinations following peroral infection with *T. gondii* in C3H/HeJ and C3H/HeN mice, both necrosis and remarkable cellular inflammation were observed in the small intestine of C3H/HeJ (TLR4-deficient) mice at 5 days post-infection. Although the infiltration of neutrophils in the small intestines of C3H/HeJ and C3H/HeN mice was comparable, the infiltration of monocytes in C3H/HeJ mice was less prominent than that in C3H/HeN mice (Fig. 3A and B). However, such inflammatory changes were usually not marked in either C3H/HeJ or C3H/HeN mice following i.p.i. of *T. gondii* ME49. After p.o.i. of cysts, TLR4 was expressed in mononuclear cells in the lamina propria of the intestines. The number of TLR4-positive cells was increased in resistant C3H/HeN mice (Fig. 3C, arrows) compared with susceptible C3H/HeJ mice (Fig. 3D). It was hypothesized that the appearance of TLR4-positive cells might have been induced by the infection in C3H/HeN mice and reflects innate immunity to *T. gondii*. However, when C3H/HeJ or C3H/HeN mice were infected by i.p.i. of parasites, TLR4-positive cells were not usually observed in the lamina propria (Fig. 3E and F). TLR2-positive cells were not observed in C3H/HeJ and C3H/HeN mice after p.o.i. of cysts (Fig. 3G and H). No TLR2 or TLR4 immunoreactivity was found in the small intestine of normal C3H/HeJ and C3H/HeN mice (data not shown). The small intestines of *T. gondii*-infected C3H/HeJ and C3H/HeN mice were also not stained when control rabbit IgG was used as the primary antibody (data not shown). To quantify inflammatory cell recruitment, the number of mononuclear cells or neutrophils in the small intestinal lesions was examined by counting these cells in minimum of 20 high-magnification fields of sections, and the results were expressed as fold increase compared with those of normal C3H/HeJ and C3H/HeN mice. Following p.o.i. of *T. gondii* ME49, the number of mononuclear cells infiltrated in C3H/HeJ (TLR4-deficient) mice was significantly lower than that in C3H/HeN mice, although the infiltration of neutrophils in the lesion was similar in C3H/HeJ and C3H/HeN mice (Fig. 4).
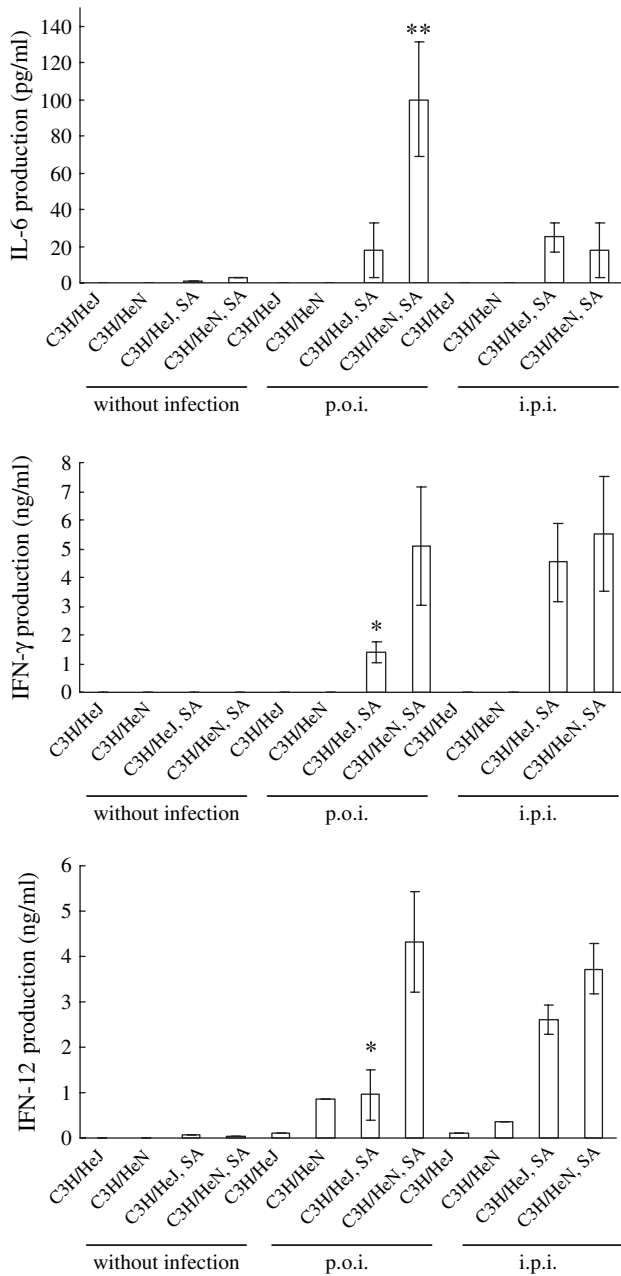
Fig. 2. Production of IFN-γ, IL-12 and IL-6 in spleen cells from *T. gondii* ME49-infected C3H/HeJ and C3H/HeN mice. C3H/HeJ and C3H/HeN mice were infected with 10 cysts of *T. gondii* ME49 by p.o.i. or i.p.i., and then single-cell suspensions were prepared from spleens of the mice at 5 days after the infection. Spleen cells were cultured at 1×10^6 cells per well in 1 ml RPMI1640 containing 10% FCS in the presence or absence of SA (20 μg ml^{-1}), and then supernatants were collected 24 h later. The concentration of IFN-γ, IL-12 and IL-6 in spleen cells was measured by sandwich ELISA (mean ± SD, $n = 5$, *$P < 0.05$, **$P < 0.01$). This experiment was repeated three times with similar results.

Differential roles of TLR2 and TLR4 in mediating the induction of host defense to T. gondii infection

To confirm further the possible differential role of TLR2 and TLR4 in mediating the induction of innate immunity against *T. gondii* infection, TLR2$^{-/-}$ or TLR4$^{-/-}$ mice were inoculated
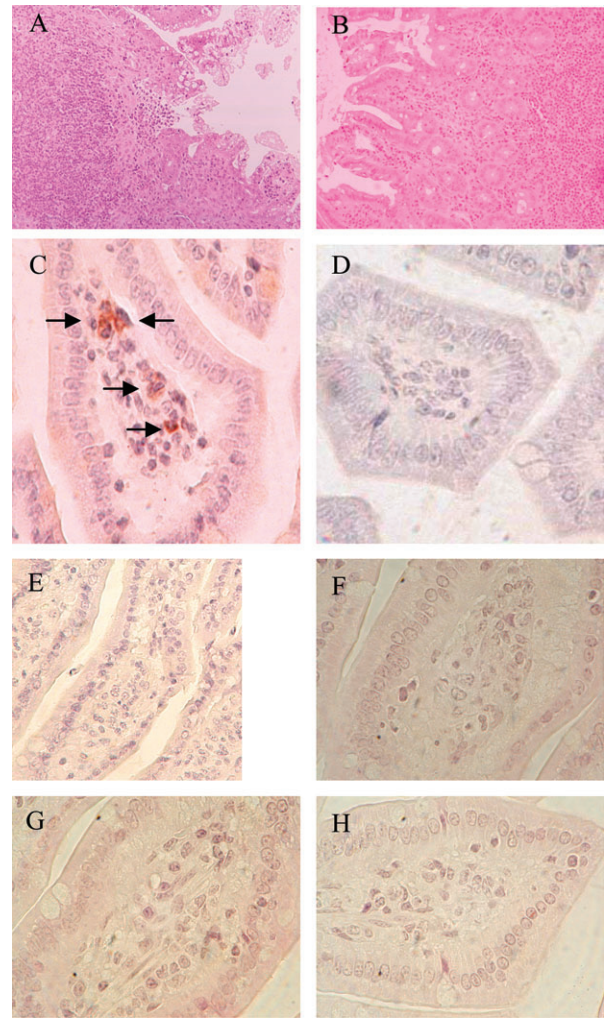
Fig. 3. Histopathology of the small intestines in C3H/HeJ and C3H/HeN mice infected with *T. gondii* ME49. In histopathologic examinations following peroral infection with *T. gondii* in C3H/HeJ and C3H/HeN mice, necrosis and remarkable cellular inflammation were observed in the small intestine of C3H/HeJ (TLR4-deficient) mice at 5 days post-infection (A and B, original magnification ×200). Although the infiltration of neutrophils in the small intestines of C3H/HeJ and C3H/HeN mice was comparable, the infiltration of monocytes in C3H/HeJ mice was less prominent than that in C3H/HeN mice. However, such inflammatory changes were usually not marked in either C3H/HeJ or C3H/HeN mice following i.p.i. of *T. gondii* ME49. TLR4-positive cells stained by anti-TLR4 antibody were observed in the lamina propria of C3H/HeN mice (C, arrows, original magnification ×400), but these cells were few or absent in the lamina propria of C3H/HeJ mice (D, original magnification ×400). No positive cells stained by anti-TLR4 antibody were observed in the lamina propria of C3H/HeJ and C3H/HeN mice infected by i.p.i. with *T. gondii* ME49 cysts (E and F, original magnification ×400). No positive cells stained by anti-TLR2 were observed in the small intestines of C3H/HeJ and C3H/HeN mice with p.o.i. of cysts (G and H, original magnification ×400). No TLR2 or TLR4 immunoreactivity was found in the small intestine of normal C3H/HeJ and C3H/HeN mice (data not shown). The small intestines of *T. gondii*-infected C3H/HeJ and C3H/HeN mice were also not stained when control rabbit IgG was used as the primary antibody (data not shown).

by p.o.i. or i.p.i. with 20 cysts of *T. gondii* ME49 to examine the growth of brain cysts in animals. When cysts were inoculated by p.o.i., a significant increase of parasites was found in the
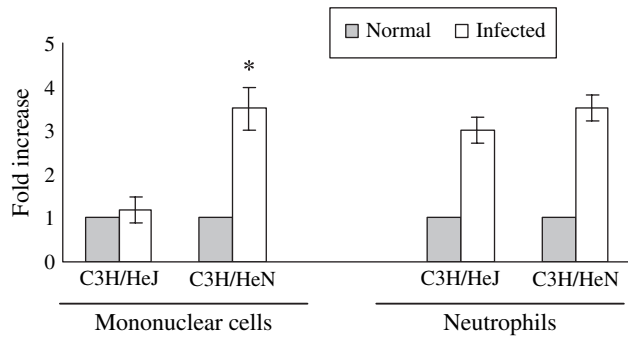
Fig. 4. Number of mononuclear cells and neutrophils in the lamina propria of the small intestine. Number of mononuclear cells and neutrophils in the small intestine of C3H/HeJ and C3H/HeN mice was counted by microscopy in 20 high-magnification fields of sections stained with H&E at 5 days after peroral *T. gondii* ME49 infection. Data were presented as fold increase as compared with the infected C3H/HeJ mice (mean ± SD, $n = 5$, *$P < 0.01$).

TLR4$^{-/-}$ mice compared with TLR2$^{-/-}$ or WT C57BL/6 mice at 30 days after the infection (Fig. 5A). However, when cysts were inoculated by i.p.i., the number of brain cysts in TLR4$^{-/-}$ mice was comparable to those of TLR2$^{-/-}$ and WT mice. To further evaluate the role of TLR2 and TLR4 in host resistance to *T. gondii*, TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were infected with different doses of *T. gondii* ME49 cysts, by p.o.i. or i.p.i. as indicated in Fig. 5(B and C). When 20 cysts were inoculated by p.o.i., TLR4$^{-/-}$ mice were more susceptible than TLR2$^{-/-}$ or WT mice. All TLR4$^{-/-}$ mice died between 34 and 38 days after parasite inoculation, whereas TLR2$^{-/-}$ or WT mice survived >45 days (Fig. 5B). However, these differences in susceptibility between TLR2$^{-/-}$ and TLR4$^{-/-}$ mice disappeared when mice were inoculated with a higher dose of cysts (200 cysts); all mice died within 15 days after infection (Fig. 5B). In contrast to p.o.i., no remarkable differences were observed between TLR2$^{-/-}$ and TLR4$^{-/-}$ mice receiving low- or high-dose i.p.i. (40 or 200 cysts, Fig. 5C). When TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were inoculated with low dose of cysts by i.p.i., all mice survived >45 days, while those mice inoculated with high dose of cysts by i.p.i., all died within 15 days. These data suggest that TLR4 serves as a protective function in the host response to *Toxoplasma* infection, and that TLR4 signaling is required to control the growth of *T. gondii* infection when parasites are inoculated by p.o.i.

Production of pro-inflammatory cytokines in TLR4$^{-/-}$ mice infected by p.o.i. of T. gondii ME49

To compare the level of cytokines (IFN-γ, IL-4, IL-6, IL-10 and IL-12) produced in response to *T. gondii* infection, spleen cells were prepared at 5 days after the infection of *T. gondii* ME49 cysts by p.o.i., and cultured with SA for 24 h. Supernatants were collected, and then cytokine production was measured by sandwich ELISA. When TLR4$^{-/-}$ mice were inoculated by p.o.i. with *T. gondii* ME49 cysts, the production of IFN-γ, IL-12 and IL-6 in TLR4$^{-/-}$ mice was significantly lower than that in TLR2 or WT mice, which are resistant to murine toxoplasmosis, although production of IL-4 and IL-10 in TLR4$^{-/-}$ was comparable with those in TLR2$^{-/-}$ and WT mice. These results suggest that the increased production of
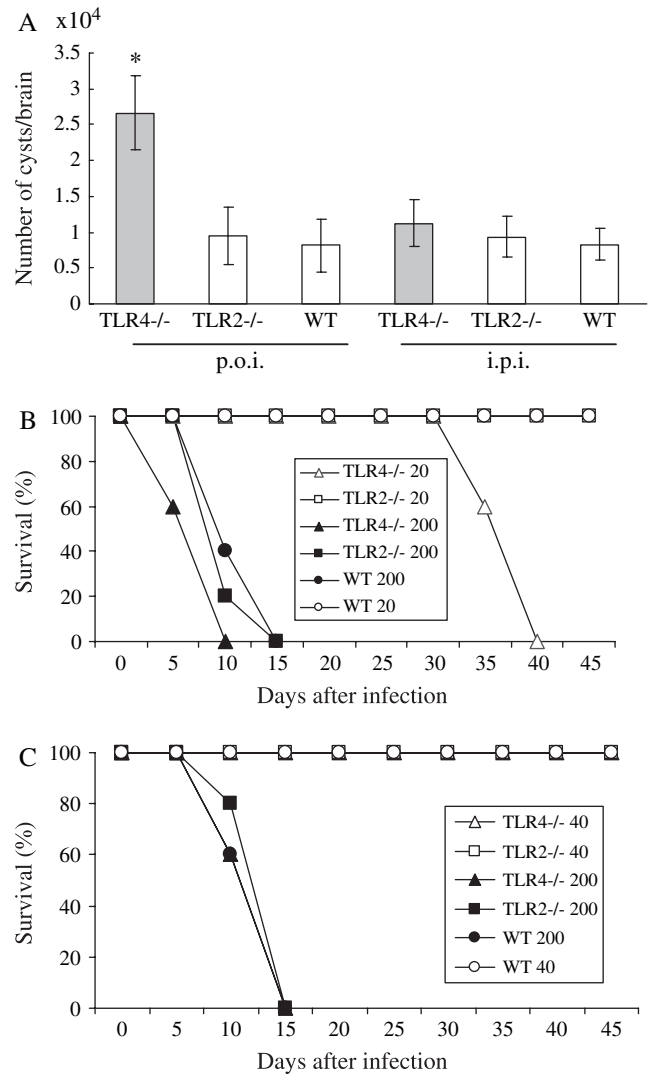

Fig. 5. Growth of *T. gondii* ME49 cysts in the brains of TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice infected by p.o.i. or i.p.i. (A) TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were infected with 20 cysts of *T. gondii* ME49 by p.o.i. or i.p.i., and then number of cysts in the brain was examined at 30 days after the infection (mean ± SD, $n = 5$, *$P < 0.05$). This experiment was repeated three times with similar results. Survivals of TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice after *T. gondii* ME49 infection. TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were infected perorally (B) or intra-peritoneally (C) with 20 cysts of *T. gondii* ME49 as indicated in each figure, and then the survival was monitored. Data are representative of three times experiments, each with five female mice per group.

IFN-γ, IL-12 and IL-6 mediated by TLR4 is associated with resistance to murine toxoplasmosis (Fig. 6) and is consistent with the data showing that production of cytokines in C3H/HeJ (TLR4-deficient) mice infected perorally with *T. gondii* is lower than that in C3H/HeN mice (Fig. 2).

Induction of TLR4-dependent signaling pathway by T. gondii ME49 infection

To further characterize the activation of TLR4 by *T. gondii* ME49, a stable transfectant-expressing mTLR2 or mTLR4/mMD-2 was stimulated with SA to examine whether *T. gondii*

ME49 influences signaling via mTLR4/mMD-2, and then NF-κB activation was examined by luciferase assay. SA induced NF-κB activation in a dose-dependent manner in the transfectant-expressing mTLR4/mMD-2 but not in the

transfectant-expressing mTLR2 (Fig. 7). These activities of SA were not abolished by treatment with polymyxin B to deactivate LPS. These results suggest that *T. gondii* ME49 has a TLR4 agonistic activity.
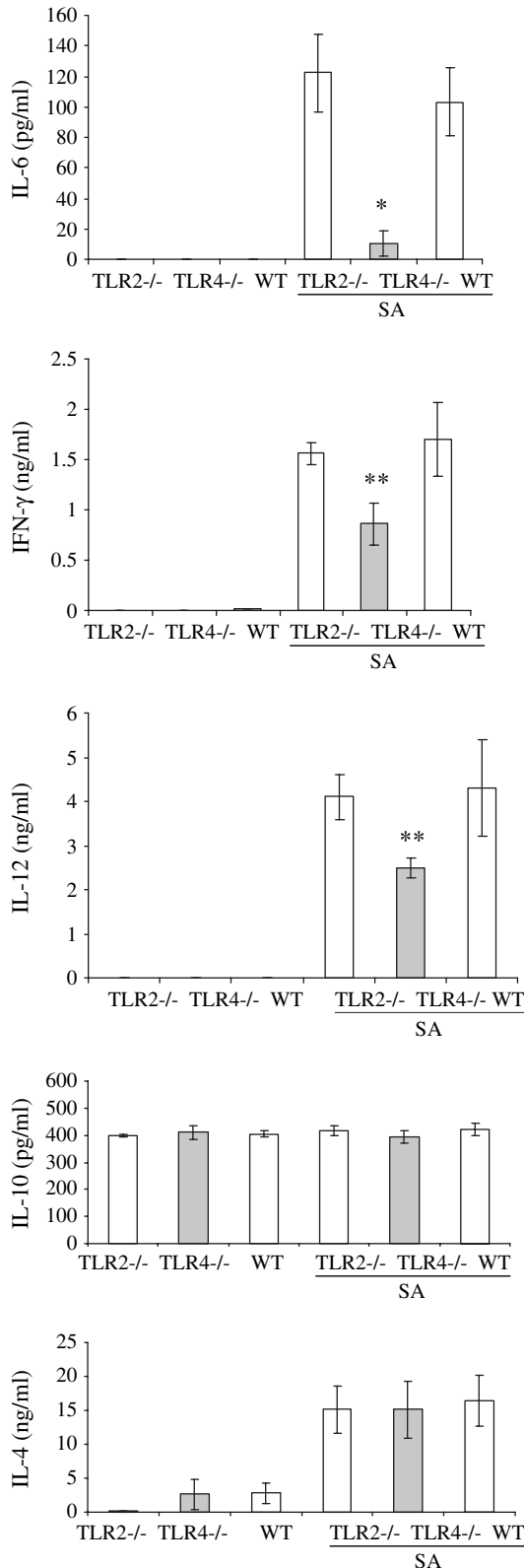
Influence of normal intestinal microflora on TLR4-mediated innate resistance against T. gondii ME49 infection

To consider the possibility that the normal intestinal microflora, which is mostly gram-negative bacteria, affects TLR4-mediated innate resistance during peroral infection with *T. gondii*, penicillin G and streptomycin sulfate were administered via sterilized drinking water to remove intestinal microflora of mice. After TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice had been treated with antibiotics, *T. gondii* cysts were inoculated orally into these mice, and then the number of brain cysts was examined. Since a high susceptibility to *T. gondii* infection was still observed in TLR4$^{-/-}$ mice treated with antibiotics compared with TLR2$^{-/-}$ or WT mice (Fig. 8), we conclude that TLR4-mediated host
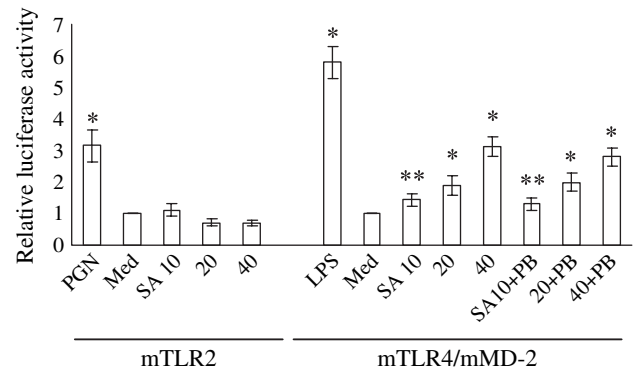

Fig. 7. Induction of TLR4-dependent signaling pathway by *T. gondii* ME49. Ba-F3-transfected cells were cultured in RPMI1640 containing 10% FCS and IL-3. The Ba-F3 cell line was stably expressing mouse (m) TLR2 or mTLR4/mMD-2 and p55Ig κLuc with NF-κB-dependent luciferase reporter construct. Individual wells containing 1×10^5 cells were left untreated or stimulated with SA (20 μg ml^{-1}) as indicated. Luciferase assays were performed using the Luciferase Assay System according to the manufacturer's instructions. LPS (100 ng ml^{-1}) or Pam$_3$CSK$_4$ (PCSK, 100 ng ml^{-1}) was added as positive control for mTLR4 or mTLR2. In some experiments, SA was pretreated with 10 mg of polymyxin B (PB) at 37°C for 2 h to deactivate LPS activity (mean ± SD, *$P < 0.01$ and **$P < 0.05$ for mTLR2 or mTLR4/mMD-2 without SA). The results were presented as fold increase as compared with control wells. The experiment was performed three times with similar results.

Fig. 6. Production of IFN-γ, IL-4, IL-6, IL-10 and IL-12 in spleen cells from TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice after p.o.i. of *T. gondii* ME49. TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were infected orally with 20 cysts of *T. gondii* ME49, and then single-cell suspensions were prepared from spleens of the mice at 5 days after the infection. Spleen cells were cultured at 1×10^6 cells per well with or without SA (20 μg ml^{-1}), and then supernatants were collected 24 h later. Concentration of IFN-γ, IL-4, IL-6, IL-10 and IL-12 in spleen cells from the infected mice was measured by sandwich ELISA (mean ± SD, $n = 5$, *$P < 0.01$, **$P < 0.05$). The cytokine production in spleen cells from normal TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice was also examined by sandwich ELISA as mentioned before, but the production of cytokines was not detected in these normal mice (data not shown). This experiment was repeated three times with similar results.
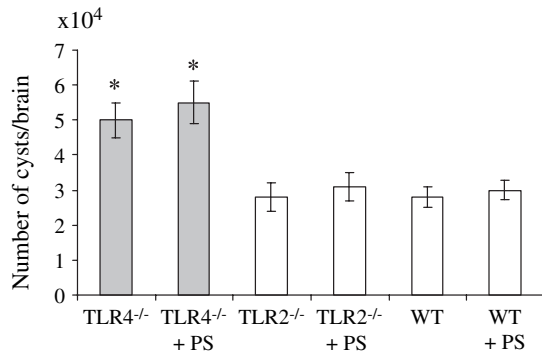
Fig. 8. Influence of normal intestinal microflora on TLR4-mediated innate resistance against *T. gondii* ME49 infection. After TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were treated with penicillin G (1500 U ml^{-1}) and streptomycin sulfate (2 mg ml^{-1}) (PS) via sterilized drinking water, 20 cysts of *T. gondii* were inoculated orally into these mice, and then the number of brain cysts was examined at 30 days after the infection (mean ± SD, $n = 5$, *$P < 0.01$ for TLR2$^{-/-}$ or WT mice with or without antibiotics). Data are representative of three times experiments.

resistance is not influenced by the presence of normal intestinal microflora.

Discussion

We demonstrate here that innate immune recognition by TLR4 is involved in protective mechanisms against naturally acquired *T. gondii* ME49 infection via the oral route. In addition, the small intestine plays important roles in this process. Since the peroral route of *T. gondii* infection has been considered to reflect the typical infection route that occurs in naturally acquired toxoplasmosis in humans, it is important to study the mechanism of resistance against the parasite using animals infected via peroral route. We therefore decided to characterize the potential role of innate immunity against toxoplasmosis in mice infected by p.o.i. of *T. gondii* ME49.

To investigate the role of the TLR in host defense against *T. gondii*, we infected both TLR-deficient and WT mice with *T. gondii* ME49. Upon i.p.i., TLR4-deficient C3H/HeJ and control C3H/HeN mice had almost identical number of cysts on the brain and produced similarly high amounts of IFN-γ and IL-12, which are the key factors for resistance to *T. gondii* infection. This suggests that innate immunity mediated by TLR4 was not critical in these mice. In striking contrast, upon p.o.i. with *T. gondii* ME49, C3H/HeJ mice were significantly more susceptible to *T. gondii* infection than C3H/HeN mice. This was associated with a significant decrease of pro-inflammatory response in C3H/HeJ mice as evidenced by reduced production of IFN-γ and IL-12 at 5 days after infection. These results might suggest that IFN-γ and IL-12 production in mice after peroral infection is mediated by TLR4 and confers resistance to the infection. In the case of i.p.i. of parasites, it is likely that immunity is assessed in central lymphoid organs (16). Since intra-peritoneal infection with *T. gondii* leads to rapid induction of a strong systemic immune response (such as cell-mediated immunity), it is possible that the local immune response (such as innate immunity in the small intestine) is limited following intra-peritoneal infection of mice (17, 18). Regarding mucosal immune responses of the small

intestine, we have so far examined IFN-γ production in lamina propria cells as well as spleen cells after p.o.i. of *T. gondii* ME49. Although increased IFN-γ production by lamina propria cells in C3H/HeN mice was consistently observed as compared with C3H/HeJ mice (data not shown), these differences were not significant. The reasons why significant differences were not observed between C3H/HeJ and C3H/HeN mice were not clear. Despite our careful attention, we cannot rule out the possibility that, during the preparation of lamina propria cells from the small intestines, there may have been contamination with small amounts of bacteria or bacterial components such as LPS or other toxins that then influenced cytokine production.

To confirm further the importance of TLR4 for resistance to *T. gondii* infection, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were inoculated perorally or intra-peritoneally with *T. gondii* cysts. TLR4$^{-/-}$ but not TLR2$^{-/-}$ mice proved to be susceptible to *T. gondii* infection when parasites were inoculated by peroral route (Fig. 5). These data clearly implied that TLR4 is involved in host resistance against murine toxoplasmosis via the peroral route of infection.

Recently, other researchers have reported the importance of innate immunity against *Toxoplasma* parasites using murine models. Scanga *et al.* (19) reported that the induction of IL-12 by *T. gondii* depends on a unique mechanism involving both MyD88 and G protein-coupled signaling pathways, without participation of TLR2 or TLR4 signaling. On the other hand, Mun *et al.* (20) reported that the effect of TLR2 on survival of *T. gondii* Fukaya-infected mice is dependent on the dose of *T. gondii*: TLR2 is not an essential molecule for protective immunity to low-dose infections (50 and 100 cysts), but is essential for protective immunity to high-dose infections of *T. gondii* (≥300 cysts). In the present study, we inoculated 20 cysts of *T. gondii* ME49 into mice via the intra-peritoneal route, and found that the number of brain cysts in TLR2$^{-/-}$ mice was comparable with TLR4$^{-/-}$ and WT mice (Fig. 5A). For a low-dose infection, this suggests that protective immunity to *T. gondii* ME49 is not dependent on TLR2, which may be consistent with the data reported by Mun *et al.* (20). However, there were several differences in the experimental conditions between the studies of Mun *et al.* and our own. They inoculated intra-peritoneally with cysts of *T. gondii* Fukaya strain, and the number of brain cysts in mice was estimated using PCR amplification of the SAG1 gene at 8 days after the infection. Cytokine production *in vitro* was measured in peritoneal macrophages stimulated with live bradyzoites of *T. gondii* Fukaya at 1 day after the infection. On the other hand, we used cysts of *T. gondii* ME49 strain, and the number of cysts in brain homogenates was counted directly by microscopy at 30 days after infection. We measured cytokine productions in the spleen cells with stimulation of sonicated SA at 5 days after the infection. The differences of experimental conditions might correlate with the discrepancies of data reported.

In addition to these innate resistances to *T. gondii* infection, recent data reported by Yarovinsky *et al.* (21) indicate that *T. gondii* profilin activates dendritic cells through TLR11 and that TLR11 is required *in vivo* for parasites-induced IL-12 production and optimal resistance to infection. Furthermore, Debierre-Grockiego *et al.* (22) reported that glycosylphosphatidylinositol molecules in *T. gondii* stimulate cytokine

production in macrophages, and they can serve as TLR2 as well as TLR4 agonists (23).

In the present study, remarkable histopathological changes in the small intestine were found in mice infected with *T. gondii* ME49 by p.o.i. but not i.p.i. These findings are consistent with data previously reported, showing that local immune responses in the gut during acute phase infection are limited following intra-peritoneal infection (18, 24). *Toxoplasma gondii*-infected TLR4-deficient C3H/HeJ mice showed an increasing infiltration of neutrophils with necrosis and low number of mononuclear cells (including TLR4-positive cells) in the small intestine, together with increased numbers of brain cysts (Fig. 4). This finding might be correlated with data suggesting macrophage defects and dysfunction of inflammatory cell recruitment in C3H/HeJ mice susceptible to *Salmonella* infection (25). Severe neutrophil infiltration in the small intestine of C3H/HeJ mice was found after p.o.i., suggesting TLR4-independent neutrophil migration. Haziot *et al.* (26) demonstrated that dramatically enhanced neutrophil recruitment in response to either LPS or *Escherichia coli* is TLR4 independent. It is not clear why the number of TLR4-expressing mononuclear cells was increased during the infection. However, Ortega-Cava *et al.* (2) reported that the TLR4/MD-2 complex is mainly found in mononuclear cells that infiltrated two different areas of the mucosa, crypt epithelial cells and lamina propria, and that TLRs and protein levels, as well as CD14, are up-regulated during dextran sodium sulfate-induced inflammation. After oral infection with cysts, bradyzoites are released from cysts and rapidly invade the intestinal epithelial and lamina propria cells (27). In the present study, TLR4-positive cells increased in the lamina propria after p.o.i. and were associated with resistance to infection as shown by decreased numbers of cysts and high cytokine production in C3H/HeN mice. We postulate that the increase of TLR4-expressing cells in the small intestine might reflect resistance to *T. gondii* infection generated by the mucosal immune system.

Acknowledgements

Abbreviations

i.p.i.	intra-peritoneal inoculation
NF-κB	nuclear factor-kappa B
p.o.i.	peroral inoculation
SA	soluble *Toxoplasma gondii* ME49 tachyzoite antigen
TLR	toll-like receptor
WT	wild-type

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