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TLR2 as an essential molecule for protective immunity against *Toxoplasma gondii* infection

Hye-Seong Mun[1], **Fumie Aosai**[1], **Kazumi Norose**[1], **Mei Chen**[1], **Lian-Xun Piao**[1], **Osamu Takeuchi**[3], **Shizuo Akira**[3], **Hiroshi Ishikura**[2] and **Akihiko Yano**[1]

[1]Department of Infection and Host Defense, and [2]Department of Molecular Pathology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
[3]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

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Abstract

To investigate the role of the Toll-like receptor (TLR) family in host defense against *Toxoplasma gondii*, we infected TLR2-, TLR4- and MyD88-deficient mice with the avirulent cyst-forming Fukaya strain of *T. gondii*. All TLR2- and MyD88-deficient mice died within 8 days, whereas all TLR4-deficient and wild-type mice survived after i.p. infection with a high dose of *T. gondii*. Peritoneal macrophages from *T. gondii*-infected TLR2- and MyD88-deficient mice did not produce any detectable levels of NO. *T. gondii* loads in the brain tissues of TLR2- and MyD88-deficient mice were higher than in those of TLR4-deficient and wild-type mice. Furthermore, high levels of IFN-γ and IL-12 were produced in peritoneal exudate cells (PEC) of TLR4-deficient and wild-type mice after infection, but low levels of cytokines were produced in PEC of TLR2- and MyD88-deficient mice. On the other hand, high levels of IL-4 and IL-10 were produced in PEC of TLR2- and MyD88-deficient mice after infection, but low levels of cytokines were produced in PEC of TLR4-deficient and wild-type mice. The most remarkable histological changes with infiltration of inflammatory cells were observed in lungs of TLR2-deficient mice infected with *T. gondii*, where severe interstitial pneumonia occurred and abundant *T. gondii* were found.

Introduction

Protection against *Toxoplasma gondii*, an obligate intracellular parasitic protozoan, is believed to be due in part to the ability of IFN-γ together with a second signal of either tumor necrosis factor (TNF)-α or lipopolysaccharide (LPS) to activate macrophages (1–8). For *T. gondii*, this activation is associated with a microbicidal respiratory burst that includes the release of reactive oxygen radicals and the more recently described induction of reactive nitrogen intermediates (RNI), in particular NO (2–6). *T. gondii*-infected inducible NO synthase (iNOS) knockout (KO) mice or NOS inhibitor, L-N^G-monomethyl arginine (L-NMMA)-treated wild-type mice displayed increased parasite numbers in brain (2,3).

Toll-like receptors (TLR) play a critical role in innate immune responses in mammals (9–11). TLR2-deficient mice are highly susceptible to infection by *Staphylococcus aureus* and *Mycobacterium tuberculosis* (9,11). In mouse macrophages, bacterial lipoprotein activation of TLR2 leads to a NO-dependent killing of intracellular tubercle bacilli (11). C3H/HeJ mice, which possess a point mutation in TLR4, are susceptible to *M. tuberculosis* infection (10). Susceptibility to *M. tuberculosis* infection in these mice was associated with the delayed appearance of IFN-γ-producing CD4$^+$ T cells (10). It is also reported that LPS activation of TLR4 leads to NO release (12).

TLRs activate signal transduction cascades that lead to the expression of immune response genes following recognition of their respective ligands. MyD88, a cytoplasmic adapter protein, associates with all members of the IL-1R and TLR families (13). MyD88-deficient mice are highly susceptible to *T. gondii* infection (14,15). Chen *et al*. reported that high levels of anti-mouse heat shock protein (mHSP70) autoantibody and anti-*T.g.*HSP70 antibody production were detected in sera from MyD88-deficient mice (15). Scanga *et al*. reported that the induction of IL-12 by *T. gondii* depends on a unique

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Correspondence to: A. Yano; E-mail: yano@faculty.chiba-u.jp

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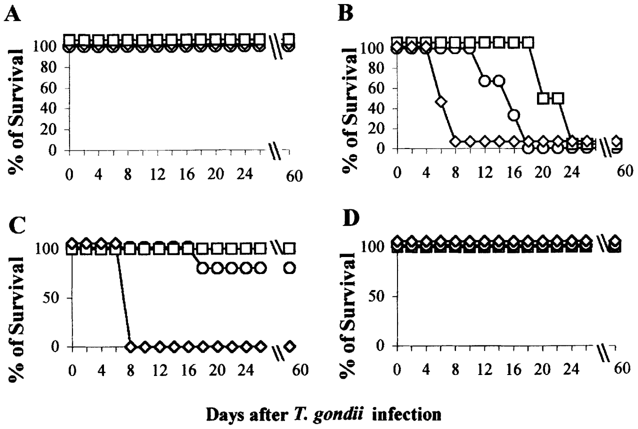

Fig. 1. TLR2-deficient mice fail to survive against *T. gondii* infection. Wild-type (A), MyD88- (B), TLR2- (C) and TLR4-deficient (D) mice were i.p. infected with 50 (squares), 100 (circles) and 300 (diamonds) cysts of *T. gondii*. Survival of the animals was then monitored daily and cumulative mortality was calculated. The experiment was performed 3 times and there were three to four mice in each group in each experiment.

mechanism involving both MyD88 and G protein-coupled signaling pathways, and that this reduced IL-12 production is not the result of impaired TLR2 or TLR4 signaling (14). Furthermore, NF-κB activation was abrogated in MyD88-deficient cells stimulated with ligands for TLR2, indicating that TLR family signaling requires MyD88 (16). However, NF-κB activation in response to LPS was retained in MyD88-deficient cells, although with delayed kinetics (17). Therefore, among IL-1R and TLR family members, TLR4 is unique in terms of its signaling, being able to lead to NF-κB activation in a MyD88-independent manner.

Here we report the involvement of TLR2 in NO-mediated protective immunity against infection by *T. gondii*.

Methods

Experimental animals

TLR2-, TLR4- and MyD88-deficient mice (9) with a C57BL/6 background, and wild type C57BL/6 mice (SLC, Hamamatsu, Japan) were used at 8–12 weeks of age.

Parasite, experimental infection and treatments

To analyze the effect of the release of NO on the protective immunity against *T. gondii* infection in TLR2-, TLR4- and MyD88-deficient, and wild-type mice, mice were i.p. injected with or without L-NMMA (175 mg/kg; Sigma, St Louis, MO) (18) 4 h before i.p. infection with 10, 50, 100 and 300 cysts of the avirulent cyst-forming Fukaya strain of *T. gondii* (19–21). Survival of the animals was then monitored daily and cumulative mortality was calculated.

Determination of NO

At 1 or 8 days after i.p. infection with 300 cysts of the avirulent cyst-forming Fukaya strain of *T. gondii*, peritoneal exudate cells (PEC) from TLR2-, TLR4- and MyD88-deficient, and wild-type mice were harvested, and peritoneal macrophages were obtained by adhering to plastic plates. The resulting peritoneal macrophage population was sorted to a purity of >97%. The culture supernatants were analyzed for the presence of NO_2^- by the use of the Griess reaction (6).

RT-PCR

The expression of mRNA from the PEC was investigated by RT-PCR as previously described (6). PCR was carried out with the following specific iNOS (22), indoleamine 2,3-dioxygenase (INDO) (23), IFN-γ, IL-12, IL-4 and IL-10 primers (24): iNOS, sense primer 5′-CCCTTCCGAAGTTTGGCAGCAGC-3′ and anti-sense primer 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′; INDO, sense primer 5′-CACTGAGCACGGACGGACTG-AGA-3′ and anti-sense primer 5′-TCCAATGCTTTCAGGTC-TTGACGC-3′; IFN-γ, sense primer 5′-TGAACGCTACACACT-GCATCTTGG-3′ and anti-sense primer 5′-CGACTCCTTTTC-CGCTTCCTGAG-3′; IL-12 sense primer 5′-GAGGACTTGA-AGATGTACCAG-3′ and anti-sense primer 5′-TCCTATCTG-TGTGAGGAGGGC-3′; IL-4, sense primer 5′-ATGGGTCTCA-ACCCCCAGCTAGT-3′ and anti-sense primer 5′-GCTCTTTA-GGCTTTCCAGGAAGTC-3′; IL-10, sense primer 5′-CGGGA-AGACAATAACTG-3′ and anti-sense primer 5′-CATTTCC-GATAAGGG-3′. GAPDH was used for internal control.

Quantitative competitive (QC)-PCR

T. gondii loads in brains of TLR2-, TLR4- and MyD88-deficient, and wild-type mice treated with or without L-NMMA were examined 8 days after i.p. infection with 300 cysts of *T. gondii*. The number of *T. gondii* in the brain of each mouse was measured using QC-PCR of the SAG1 gene as previously described (6,7,19–21,25,26). Gel electrophoresis of the PCR products was measured with an IPLab Gel Densitometer (Signal Analytical, Vienna, VA).

Cell-surface staining

PEC of mice before or 5 days after i.p. infection with 300 cysts of *T. gondii* were harvested, and incubated for 30 min at 4°C with various combinations of phycoerythrin (PE)-conjugated $CD8a^+$ and $CD11b^+$ (Caltag, Burlingame, CA), and U5A2-13 (NKT/NK, PharMingen, San Diego, CA) (27).

Detection of intracellular cytokines

PEC of mice before or 3 days after i.p. infection with 300 cysts of *T. gondii* were harvested and then subjected to cytoplasmic staining using Cytofix/Cytoperm kits (PharMingen, San Diego, CA). FITC-conjugated anti-mouse IFN-γ (XMG1.2, rat IgG1; PharMingen), IL-4 (BVD-24G2, rat IgG2b; Immunotech, Marseille, France) and IL-10 (JES5-16E3, rat IgG2b; PharMingen) mAb, and PE-conjugated anti-mouse IL-12 (p40/p70) (C15.6, rat IgG1; PharMingen) mAb were used. Specific mAb-labeled cells were analyzed by flow cytometry. FITC-conjugated rat IgG1 and rat IgG2b, and PE-conjugated rat IgG1 (PharMingen) were used as isotype controls.

Magnetic cell separation

PEC of mice before or 1 day after i.p. infection with 300 cysts of *T. gondii* were harvested. $CD11b^+$ and $CD8a^+$ cells from peritoneal cells of TLR2- and TLR4-deficient, and wild-type

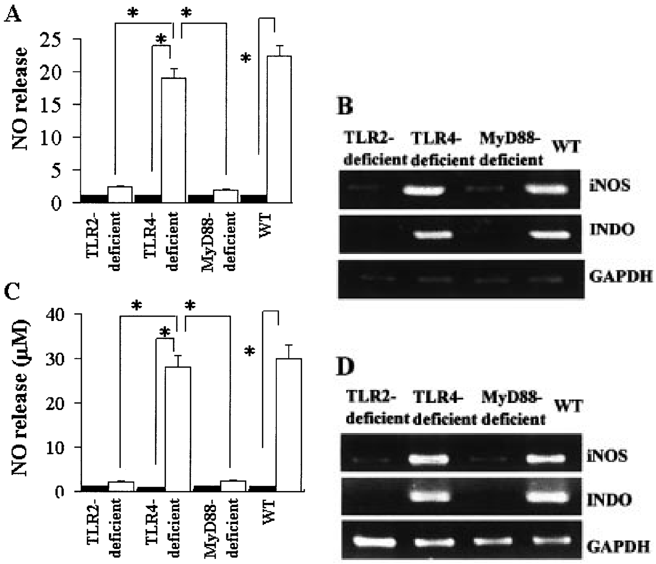

Fig. 2. TLR2 regulates *T. gondii*-induced NO release. (A and C) At 1 (A) or 8 (C) days after i.p. infection with (open bars) or without (closed bars) 300 cysts of *T. gondii*, peritoneal macrophages from TLR2-, MyD88- and TLR4-deficient, and wild-type mice were harvested. The culture supernatants were analyzed for the presence of NO_2^- by the use of the Griess reaction. (B and D) At 1 (B) or 8 (D) days after i.p. infection with *T. gondii*, peritoneal macrophages from TLR2-, MyD88- and TLR4-deficient, and wild-type mice were harvested. The iNOS and INDO mRNA expressions from PEC of mice were investigated by RT-PCR. $P < 0.05$ was taken as significant. The experiment was performed 3 times and there were three to four mice in each group in each experiment.

mice were obtained by magnetic cell separation (Miltenyi Biotec, Auburn, CA) as previously described (21). The purity of these cells was >98%.

ELISA

IFN-γ (PeproTech EC, London, UK), IL-12 (p70) (PeproTech EC) and IL-4 (Techne, Minneapolis, MN) levels were measured by commercially available ELISA kits.

Histopathology

On the day of, and at 8 days after, infection with 300 cysts of *T. gondii*, TLR2-deficient and wild-type mice were euthanized by asphyxiation with CO_2, and their lungs were fixed in a solution containing 10% formalin, 70% ethanol and 5% acetic acid. Sagittal sections of lungs were stained with hematoxylin & eosin.

Statistical analysis

Significance of differences between groups was determined by Student's *t*-test and the survival experiments were analyzed by the Kaplan–Meier method. $P < 0.05$ was taken as significant.

Results

TLR2-deficient mice fail to survive against T. gondii infection

All TLR4-deficient and wild-type mice infected i.p. with 300, 100 and 50 cysts of the avirulent cyst-forming Fukaya strain of *T. gondii* survived (Fig. 1A and D). All MyD88-deficient mice died within 8, 18 and 24 days after infection with 300, 100 and 50 cysts respectively (Fig. 1B). All TLR2-deficient mice died within 8 days after infection with 300 cysts, whereas 80 and 100% of TLR2-deficient mice survived after infection with 100 cysts and 50 cysts respectively (Fig. 1C). These results indicated that TLR2 is an essential molecule for protective immunity against high-dose *T. gondii* infection.

TLR2 regulates T. gondii-induced NO release

Peritoneal macrophages of *T. gondii*-infected TLR4-deficient and wild-type mice responded to *T. gondii* challenge with NO production, while TLR2- and MyD88-deficient mice failed to produce NO in response to the challenge. These results indicate that the *T. gondii* infection-induced release of NO (Fig. 2A and C) and expression of iNOS mRNA (Fig. 2B and D) are dependent on TLR2 and MyD88, but not TLR4. However, peritoneal macrophages of *T. gondii*-infected TLR4-deficient and wild-type, but not of TLR2- and MyD88-deficient mice, expressed INDO mRNA. This in turn starved tryptophan, an essential amino acid for *T. gondii*, resulting in the manifestation of anti-*T. gondii* activity. It is evident that the *T. gondii* infection-induced expression of INDO mRNA is dependent on TLR2 and MyD88, but not on TLR4 (Fig. 2B and D).

TLR2 regulates anti-T. gondii activity through NO

To test whether the survival system is NO dependent, mice were injected with L-NMMA, a NO synthase inhibitor, 4 h before infection. All of the L-NMMA-treated wild-type mice died within 8 days after infection with 300 cysts of *T. gondii* and 10 days after infection with 100 cysts of *T. gondii*, and 50% of the mice died with 14 days after infection with 50 cysts (Fig. 3A). L-NMMA-treated TLR2- and TLR4-deficient, and wild-type mice died within 8 days after infection with 300 cysts of *T. gondii* (Fig. 3B). Uninfected mice treated with L-NMMA survived >3 months (data not shown). Furthermore, *T. gondii* loads in the mice were shown to be NO dependent. *T. gondii* loads in the brain tissues of L-NMMA-treated TLR2- and MyD88-deficient mice were similar to those of L-NMMA-treated TLR4-deficient and wild-type mice, while the loads in the brain tissues of L-NMMA-untreated TLR2- and MyD88-deficient mice were greater than those of L-NMMA-untreated TLR4-deficient and wild-type mice ($P < 0.05$) (Fig. 3C).

Shift to T_h2 in T. gondii-infected TLR2-deficient mice

We next examined cytokine production in *T. gondii*-infected TLR2-, TLR4- and MyD88-deficient, and wild-type mice (Fig. 4). High levels of IFN-γ- and IL-12-producing cells were detected in PEC of TLR4-deficient and wild-type mice after infection, but low levels of IFN-γ- and IL-12-producing cells were detected in PEC of TLR2-deficient mice (Fig. 4A and B). IFN-γ and IL-12 production was not detected in PEC of MyD88-deficient mice (Fig. 4E). On the other hand, high levels of IL-4- and IL-10-producing cells were detected in PEC of TLR2- and MyD88-deficient mice after infection, but low levels of IL-4- and IL-10-producing cells were detected in PEC of wild-type and TLR4-deficient mice (Fig. 4C and E). No significant IFN-γ-, IL-12-, IL-4- and IL-10-producing cells were observed in these mice without *T. gondii* (data not shown).

To determine whether impaired resistance of TLR2-deficient mice stems from a defect in this pathway, we measured IL-4,

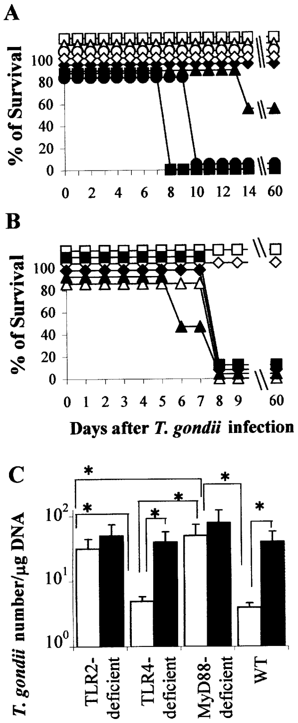

Fig. 3. TLR2 regulates anti-*T. gondii* activity through NO. (A) Wild-type mice were i.p. injected with (closed symbols) or without (open symbols) L-NMMA 4 h before *T. gondii* i.p. infection with 10 (diamonds), 50 (triangles), 100 (circles) and 300 (squares) cysts. (B) TLR2- (triangles) and TLR4-deficient (diamonds), and wild-type (squares) mice were i.p. injected with (closed symbols) or without (open symbols) L-NMMA 4 h before *T. gondii* i.p. infection with 300 cysts. (C) These mice injected with (solid squares) or without (open squares) L-NMMA before *T. gondii* infection with 300 cysts were killed at 8 days after infection and the number of *T. gondii* in the brain from each mouse was measured using QC-PCR targeting the SAG1 gene. $P < 0.05$ was taken as significant. The experiment was performed 3 times, and there were three to four mice in each group in each experiment.

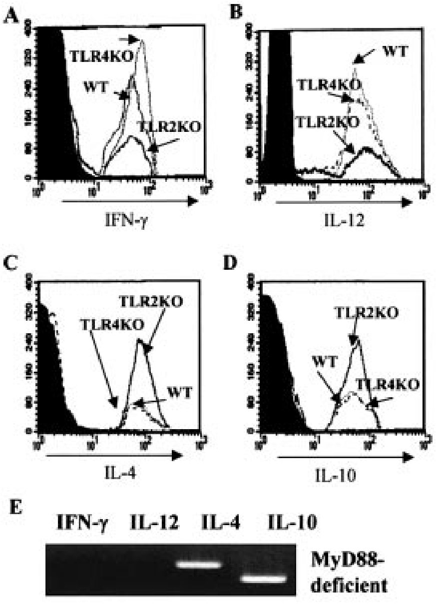

Fig. 4. The shifting to T_h2 in *T. gondii* infected TLR2-deficient mice. (A–D) Three days after i.p. infection with 300 cysts of *T. gondii*, intracellular IFN-γ (A), IL-12 (B), IL-4 (C) and IL-10 (D) production of PEC from TLR2- and TLR4-deficient, and wild-type was analyzed by FACScan as described in Methods. Negative control is shown by closed histograms. (E) Three days after i.p. infection with 300 cysts of *T. gondii*, IFN-γ, IL-12, IL-4 and IL-10 mRNA expression from PEC of MyD88-deficient mice was investigated by RT-PCR. The data are representative of three independent experiments.

IL-12 and IFN-γ in plasma from 5-day infected mice (Fig. 5A–C) by ELISA. Plasma IL-12 and IFN-γ levels in TLR2-deficient mice were much lower relative to wild-type and TLR4-deficient mice. On the other hand, plasma IL-4 levels in TLR2-deficient mice were much higher relative to wild-type and TLR4-deficient mice. Furthermore, plasma IL-12 and IFN-γ levels in MyD88-deficient mice were lower than in TLR2-deficient mice and plasma IL-4 levels in MyD88-deficient mice were higher than in TLR2-deficient mice (Fig. 5A–C). To confirm this defect at the cellular level, we examined IL-4, IL-12 and IFN-γ production by peritoneal macrophages from the same animals after infection with *T. gondii*. TLR2-deficient mice showed dramatically reduced IL-12 and IFN-γ production, but increased IL-4 production. No significant IFN-γ-, IL-12- and IL-4-producing cells were observed in these mice without *T. gondii* (data not shown). Then, the cell types of IFN-γ- and IL-4-producing cells in *T. gondii*-infected TLR2- and TLR4-deficient, and wild-type mice were analyzed by ELISA (Fig. 5D and E). IL-4-producing cells from PEC of TLR2-deficient mice were shown to be $CD11b^+$ cells (Fig. 5D), and IFN-γ-producing cells from PEC of TLR4-deficient and wild-type mice were shown to be $CD8a^+$ and $CD11b^+$ cells (Fig. 5E).

Histological changes in TLR2-deficient mice

Because of the remarkable difference in mortality between infected TLR2-deficient and wild-type mice after *T. gondii* infection, we performed histological studies of their organs at 8 days after infection with 300 cysts. The most remarkable histological changes were observed in the lung of TLR2-deficient mice, with severe interstitial pneumonia occurring and large numbers of *T. gondii* being found (Fig. 6B and C). *T. gondii* loads in lung tissues of MyD88-deficient mice were slightly higher than those of TLR2-deficient mice (Fig. 6E and F). Inflammation was observed in lungs of TLR2-deficient mice as well as of MyD88-deficient mice, but there were no inflammatory changes in lungs of infected wild-type mice.

Discussion

The mechanisms of IFN-γ-induced inhibition of *T. gondii* that have been demonstrated in macrophages include RNI, induction of NO production, tryptophan starvation and iron deprivation (2–6,28). *T. gondii*-infected iNOS KO mice or L-NMMA-treated wild-type mice displayed increased parasite numbers in brain (2,3), but the mice survived acute infection from a low-dose infection of *T. gondii*. However, the present study reveals the importance of NO against high-dose infection of *T. gondii*. The mice that fail to produce NO cannot survive acute infection with high-dose *T. gondii*. Khan *et al.* also reported that IFN regulatory factor (IRF)-1 KO mice

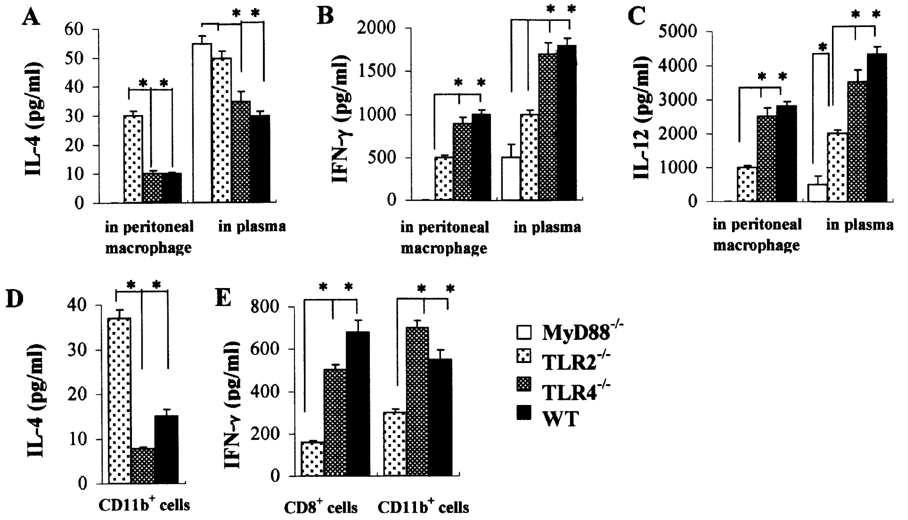

Fig. 5. PEC from TLR2-deficient mice elicited IL-4 production. (A–C) Five days after i.p. infection with 300 cysts of *T. gondii*, plasma IL-4 (A), IFN-γ (B) and IL-12 (C) production from MyD88-, TLR2-, TLR4-deficient, and wild-type was analyzed by ELISA as described in Methods. One day after i.p. infection with 300 cysts of *T. gondii*, peritoneal macrophages of mice were harvested and incubated for 72 h *in vitro*. Cellular IL-4 (A), IFN-γ (B) and IL-12 (C) production from TLR2- and TLR4-deficient, and wild-type was analyzed by ELISA. (D and E) One day after i.p. infection with 300 cysts of *T. gondii*, peritoneal macrophages of mice were harvested. CD11b$^+$ and CD8a$^+$ cells from peritoneal macrophages were obtained by magnetic cell separation (Miltenyi Biotec) as previously described. Cellular IL-4 (D) and IFN-γ (E) production was analyzed by ELISA. The experiment was performed 3 times and there were three to four mice in each group in each experiment.

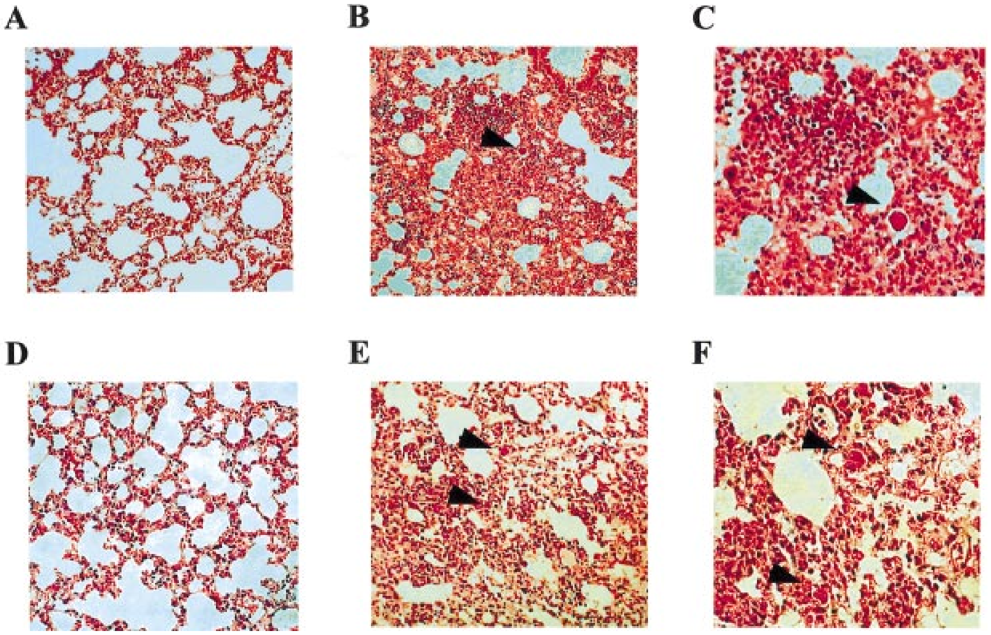

Fig. 6. Histological changes in lung of *T. gondii*-infected TLR2-deficient mice after *T. gondii* infection. TLR2- (A–C) and MyD88- (D–F) deficient mice were infected i.p. without (A and D) (×100) or with (B and E) (×100) and (C and F) (×200) 300 cysts of *T. gondii* and histological studies were performed 8 days after infection (hematoxylin & eosin). Each panel is representative of the histological changes observed in three TLR2- and MyD88-deficient mice or three control mice in three independent experiments. Arrowhead indicates a mass of *T. gondii*.

succumb to acute infection within 11 days after high-dose infection of *T. gondii*, but the mice survive acute infection after a low dose of infection (28). Furthermore, Bohne *et al.* reported that increasing L-NMMA concentrations resulted in continuously decreasing NO release accompanied by decreasing toxoplasmastatic activity of macrophages (2). These results indicate that the survival of mice depends on the doses of both L-NMMA and *T. gondii*.

All TLR2-deficient mice died within 8 days after infection with 300 cysts (Fig. 1C), whereas 80% survived after infection with 100 cysts (Fig. 1C) (15). The effect of TLR2 on survival of *T. gondii*-infected mice is dependent on the dose of *T. gondii*.

TLR2 cannot be the only MyD88-dependent receptor involved in resistance to *T. gondii* infection. TLR (except TLR3) or IL-1R require MyD88 as a cytoplasmic adapter protein for signal transduction (13). TLR2 is not an essential molecule for protective immunity to low-dose infections (50 and 100 cysts). On the other hand, TLR2 is an essential molecule for protective immunity to high-dose infections of *T. gondii* (300 cysts or more). The mice require several receptors that associate with MyD88 adaptor molecules for protective immunity against high-dose *T. gondii* infection.

Kawai *et al*. reported that several IFN-inducible genes, including a CXC chemokine, IFN-inducible protein 10 (IP-10), and IFN-regulated gene-1 (IRG-1), were induced in MyD88-deficient macrophages in response to LPS (29). IP-10 gene induction requires IRF-3 (30), and nuclear translocation of IRF-3 in response to LPS is detected in MyD88-deficient cells (31). Therefore, it is likely that IRF-3 activation contributes to the MyD88-independent pathway. At present, it is not known how IRF-3 is activated downstream of TLR4. Our results indicated that IFN-γ (Fig. 4), NO and INDO (Fig. 2B), which were induced by IRF-1, were not induced in TLR2- and MyD88-deficient macrophages in the challenge against *T. gondii* infection. Therefore, it is probable that IRF-1 activation contributes to the MyD88-dependent pathway. As for the activation of IRF-1 downstream of TLR2, the mechanism is as yet unknown.

There were several differences in experimental conditions between the study of Scanga *et al*. (14) and ours. (i) Different genetic background TLR2-deficient mice were used (Scanga *et al.* used 129/Ola × C57BL/6; we used C57BL/6). (ii) Different cell types of IL-12 production were examined (Scanga *et al*. used splenic DC; we used peritoneal adherent macrophages). (iii) Different stimulators were used for IL-12 production (Scanga *et al*. used soluble antigen from a cyst-non-forming virulent RH strain of *T. gondii*, while we used live bradyzoites from the cyst-forming Fukaya strain of *T. gondii*). *T. gondii* interconvert from bradyzoites to tachyzoites and from tachyzoites to bradyzoites in intermediate hosts like mice and humans. It is well known that pathogenicity of these two stages of *T. gondii* is different (6,32,33). Schade *et al*. reported that tachyzoites proved superior to bradyzoites prepared from the same *T. gondii* isolate in triggering macrophage production of IL-12 (32). These different experimental conditions might be responsible for the differences in conclusions.

Previous studies have indicated that the production of T_h2 cytokines such as IL-4 and IL-10 may be a contributory factor leading to death in the acute phase of *T. gondii* infection (34). NK cells (35,36), NKT cells (37,38) and T_h2 cells (39) are well known to be IL-4-producing CD11b$^+$ cells. Furthermore, IL-4 and IL-13 were implicated in the negative regulation of IFN-γ-induced anti-*Toxoplasma* activity such as NO release, INDO expression and tryptophan catabolism in human cells from fibroblast lineage (40).

According to pathological observations, TLR2 plays a role in the protective immunity against *T. gondii* infection in the lung, but its protective function in this organ remains to be clarified.

Taken together, the innate recognition of *T. gondii* by TLR family members plays an important role in the elimination of invading *T. gondii*. Mortality and *T. gondii* loads after *T. gondii* infection, and abrogation of NO release and iNOS expression in TLR2-deficient mice highlight the vital function of TLR2 in resistance against *T. gondii* infection.

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Abbreviations

INDO	indoleamine 2,3-dioxygenase
iNOS	inducible NO synthase
IP-10	IFN-inducible protein 10
IRF-3	IFN regulatory factor 3
KO	knockout
L-NMMA	L-N^G-monomethyl arginine
LPS	lipopolysaccharide
mHSP70	mouse heat shock protein
PEC	peritoneal exudate cells
QC	quantitative competitive
RNI	reactive nitrogen intermediates
TLR	toll-like receptor
TNF	tumor necrosis factor

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