Toxoplasma gondii infection inhibits Th17-mediated spontaneous development of arthritis in IL-1 receptor antagonist-deficient mice

Takuya Washino1, Masataka Moroda1, Yoichiro Iwakura2, Fumie Aosai1*

1Department of Infection and Host Defense, Chiba University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan
2Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108-8670, Japan

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*Corresponding Author. Telephone: +81-43-226-2073; Fax: +81-43-226-2076. E-mail address: aosai@faculty.chiba-u.jp.
Abstract

IL-1 receptor antagonist (IL-1Ra)-deficient BALB/c mice develop spontaneous arthritis resembling human rheumatoid arthritis. We herein report that infection with *Toxoplasma gondii*, an intracellular protozoan, is capable of ameliorating the spontaneous development of arthritis in IL-1Ra-deficient mice. The onset of arthritis development was delayed and the severity score of arthritis was significantly suppressed in *T. gondii*-infected mice. Expression of IL-12p40 mRNA from CD11c+ cells of mesenteric lymph nodes (mLN) and spleen markedly increased at 1 week after peroral infection. While CD11c+ cells also produced IL-10, IL-1β and IL-6, CD4+ T cells from *T. gondii*-infected mice expressed significantly high levels of T-bet and IFN-γ mRNA at both mLN and spleen. Levels of GATA-3/IL-4 mRNA or RORγt/IL-17 mRNA decreased in the infected mice, indicating Th1 polarization and the reduction of Th2 and Th17 polarization. The severity of arthritis was related to Th1 polarization accompanied with Th17 reduction, demonstrating the protective role of *T. gondii*-derived Th1 response against Th17-mediated arthritis in IL-1Ra-deficient mice.
**Introduction**

Toxoplasma gondii is an obligate intracellular protozoan parasite. In intermediate hosts such as humans and mice, perorally (p.o.) infected encysted *T. gondii* bradyzoites undergo stage conversion to rapidly dividing tachyzoites that are responsible for acute toxoplasmosis in immunocompromised individuals and intrauterine infected-fetuses (43).

Furthermore, chronic infection with *T. gondii* is one of the most common worldwide parasitic infections in humans since *T. gondii* survives in hosts during all their lives by forming tissue cysts in organs. Yet, the associations between chronic infection with *T. gondii* and human disease (and human behavior) have been under explored.

We have previously demonstrated that *T. gondii* infected mice produce anti-murine heat shock protein (HSP) 70 antibody which cross-reacts to *T. gondii* HSP 70, a virulent molecule specific for *T. gondii* tachyzoites (10, 11, 33), from VH1-JH1 B-1 cells of the infected BALB/c (a resistant strain) and C57BL/6 (B6, a susceptible strain) mice (5, 7). Furthermore, we have revealed that *T. gondii* infection was capable of preventing the development of lupus nephritis in (New Zealand Black x New Zealand White) F1 mice (NZBW F1 mice), a mouse model of systemic lupus erythematosus (SLE), i.e., an autoimmune disease characterized by B cell hyperactivity (6).

On the other hand, IFN-γ production by CD4⁺ T, CD8⁺ T and NK cells has been proven to be essential in inducing and maintaining host protective immunity against *T. gondii* infection (13, 21, 38, 43), and antigen presenting cells (APC) such as dendritic cells (DC) play a crucial role in initiating IL-12 synthesis and determining a highly polarized Th1 type response triggered by *T. gondii*- infection (1, 23, 31, 42). Therefore, in this study, we have attempted to examine the effects of *T. gondii* infection on T cell-mediated autoimmunity by using IL-1 receptor antagonist (IL-1Ra)-deficient BALB/c mice, i.e., a
rheumatoid arthritis mouse model (19). IL-1 is a key mediator of a series of host responses to infection and inflammation known as acute-phase response, and affects a wide range of cells and organs (9). IL-1Ra competes with IL-1α and IL-1β in binding IL-1 receptors and has been considered to be a negative regulator of IL-1 signals (9, 22). Excess IL-1 signal due to a deficiency in IL-1Ra causes autoimmunity (19, 22). The evidence that the arthritis in IL-1Ra-deficient BALB/c mice starts at 4-5 weeks of age and almost all mice are affected by 12 weeks (19) has prompted us to analyze the roles of T. gondii-infection on the development of arthritis in IL-1Ra-deficient BALB/c mice.

Since spontaneous development of arthritis in IL-1Ra-deficient mice has been proven to be IL-17-dependent (17, 26, 36), effects of T. gondii infection on DC activation and successive Th polarization in IL-1Ra-deficient BALB/c mice have been evaluated. The application of T. gondii HSP70 gene vaccine that induces DC activation and Th1 polarization (31) will be discussed.

**Materials and Methods**

**Mice and T. gondii strain.** A generation of IL-1Ra-deficient mice with BALB/c background has previously been described (19). Wild type (WT) BALB/c mice were purchased from SLC (Hamamatsu, Japan). Cysts of an avirulent Fukaya strain of T. gondii were prepared as described (30). Ten T. gondii cysts of Fukaya strain were suspended in 200 μl of phosphate buffered saline (PBS), and 2.5-week-old WT or IL-1Ra-deficient BALB/c mice were p.o. infected with 10 T. gondii cysts with a specially prepared small size needle with a round head.
Clinical evaluation and histological examination. Development of arthritis was examined weekly with a clinical macroscopic evaluation and the severity score of arthritis was determined as described (19). For a histological examination, ankle joints were fixed in 10% phosphate-buffered formalin, decalcified in 10% EDTA-4Na, and embedded in paraffin. Sections (4 mm) were stained with hematoxylin/eosin as described (19).

Reverse transcription PCR. WT and IL-1Ra-deficient BALB/c mice with or without *T. gondii*-infection were euthanized at 1 week P.I., and CD11c+ or CD4+ cells were fractionated from either mesenteric lymphnodes (mLN) or spleens of the mice by Vario MACS separator system (Miltenyi Biotec, Auburn, CA, USA) with microbead-conjugated anti-CD11c or anti-CD4 monoclonal antibody (mAb) (Miltenyi Biotec) according to the company’s instructions. CD4+ cells were similarly fractionated from LN (popliteal, inguinal, and axillary LN) or spleen of uninfected or infected IL-1Ra-deficient BALB/c mice at 8 weeks P.I. Total RNA from CD11c+ or CD4+ cells of the LN or spleen was isolated and transcribed to cDNA by reverse transcription (RT), and cDNA was used for real-time quantitative PCR using Taqman PCR system (Applied Biosystems, Tokyo, Japan). Primers and 6-carboxyfluorescein (FAM)-labeled probes (Applied Biosystems) specific for IL-12 p40, IL-10, IL-6 and IL-1β were used for CD11c+ cells, and those specific for IFN-γ, IL-4, IL-17, T-bet, GATA-3 and RORγt were used for CD4+ cells. GAPDH was used for internal control. The primer/probe sets were purchased from Applied Biosystems. We normalized each set of samples using the difference in threshold cycles (ΔC_T) between the sample gene and the internal control gene (GAPDH): ΔC_T = C_T sample − C_T GAPDH. The calibrator sample (ΔC_T calibrator) was assigned as the sample with the highest ΔC_T in each set. The relative scale of mRNA measurement was represented on the Y-axes by the expression of
log-transformed $2^{\Delta \Delta CT}$, where $\Delta \Delta CT = \Delta CT_{\text{sample}}(n) - \Delta CT_{\text{calibrator}}(n)$. Each reaction was done in triplicate.

Intracellular cytokine staining. LN or spleen cells from uninfected or infected IL-1Ra-deficient BALB/c mice were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Tokyo, Japan) plus 1 μg/ml ionomycin (Sigma-Aldrich) for 6 hours in the presence of Golgi-Stop (BD Biosciences) that was added for the last 4 hours, and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 mAb (clone GK1.5) and fixed & permeabilized using Cytofix/Cytoperm (BD biosciences), followed by the staining with phycoerythrin (PE)-conjugated anti-IFN-γ (XMG1.2), anti-IL-4 (11B11) or anti-IL-17 (TC11-18H10) mAb. PE-conjugated isotype control IgG (rat IgG1, κ) was used for staining. All mAbs used for the staining were purchased from BD Biosciences. Intracellular cytokine expression was analyzed by FACScalibur flow cytometer (Becton Dickinson).

Statistics. Significance of differences between groups was determined by 2x2 contingency $\chi^2$ test or Student’s t-test. $P$ values less than 0.05 were considered statistically significant.

Results

Effects of *T. gondii* infection on spontaneous development of arthritis in IL-1Ra-deficient mice

Almost all IL-1Ra-deficient mice with BALB/c background, but not with C57BL/6 background, develop spontaneous arthritis closely resembling rheumatoid
arthritis by 12 weeks of age without gender difference (19). As BALB/c mice are a resistant strain against *T. gondii*-infection and not lethal by infection, we took the advantage of using IL-1Ra-deficient BALB/c mice for the study. Since arthritis in the mice starts at 4-5 weeks of age (19), we p.o. infected the mice at 2.5 weeks with 10 *T. gondii* cysts. Compared with the uninfected mice, the incidence of spontaneous arthritis development was delayed for 2-3 weeks in the *T. gondii*-infected mice until 10-12 weeks of age (Fig. 1A). Also the severity score of arthritis was significantly suppressed in *T. gondii*-infected mice of all ages (*p*<0.05 at 8-14 weeks and *p*<0.01 at 15-20 weeks of age between infected and uninfected mice) (Fig. 1B).

Consistent with a low degree of clinical severity, histological findings of ankle joints in uninfected IL-1Ra-deficient mice which demonstrated a remarkable infiltration of inflammatory cells, hyperplasia of synovial membrane, and erosive destruction of the bone (Fig. 1C a-c) were markedly reduced in the *T. gondii*-infected mice (Fig. 1C d-f).

Thus, *T. gondii* infection is capable of ameliorating the spontaneous development of arthritis in IL-1Ra-deficient mice.

**Effect of *T. gondii* infection on DC activation in IL-1Ra-deficient mice**

Since DC plays a central role in determining Th polarization that regulates the acquired immunity, effects of *T. gondii* infection on DC activation in IL-1Ra-deficient mice were comparatively analyzed with that of WT mice at 1 week P.I. By *T. gondii* infection, production of IL-12p40 mRNA from CD11c<sup>+</sup> cells of IL-1Ra-deficient mice markedly increased both in the mLN and spleen (Fig. 2A) as well as that of WT mice (Fig. S1). Also levels of surface expression of CD86, an accessory molecule for T cell activation, and MHC class II molecule on CD11c<sup>+</sup> cells in the mLN of *T. gondii*-infected
IL-1Ra-deficient mice were up-regulated at 1 week P.I. compared to that of uninfected mice (data not shown). Thus, in vivo maturation of DC was induced in the draining LN (dLN) after p.o. *T. gondii* infection at 1 week P.I.

On the other hand, mRNA expression of IL-10 from CD11c+ cells of *T. gondii*-infected IL-1Ra-deficient mice also increased in the spleen more than in the mLN (Fig. 2B). Expressions of pro-inflammatory cytokines such as IL-1β and IL-6 from CD11c+ cells also increased in *T. gondii*-infected IL-1Ra-deficient mice (Fig. 2C and D).

Effects of *T. gondii* infection on IL-10, IL-1β and IL-6 mRNA expression of CD11c+ cells in IL-1Ra-deficient mice were similar to that observed in WT mice (Fig. S1). Thus, similar to WT mice, anti- or pro-inflammatory cytokines were also produced from DC of regional LN and spleen of IL-1Ra-deficient mice at an early stage of *T. gondii* infection.

**Effect of *T. gondii* infection on Th polarization in IL-1Ra-deficient mice**

Since development of arthritis in IL-1Ra-deficient mice was ameliorated by *T. gondii* infection and the spontaneous development of arthritis in IL-1Ra-deficient mice was proven to be IL-17-dependent (17, 26, 36), the effect of *T. gondii* infection on Th polarization in IL-1Ra-deficient mice was comparatively examined with that of WT mice. CD4+ T cells were isolated from the mLN or spleen of WT and IL-1Ra-deficient mice with or without *T. gondii*-infection at 1 week P.I., and Th polarization was evaluated by measuring IFN-γ, IL-4 or IL-17 mRNA expression of CD4+ cells as representative Th1, Th2, and Th17 cytokines. CD4+ T cells from *T. gondii*-infected IL-1Ra-deficient mice expressed significantly higher levels of IFN-γ mRNA at both mLN and spleen compared to that of uninfected mice (Fig. 3A). Conversely, levels of IL-4 or IL-17 mRNA decreased in infected IL-1Ra-deficient mice (Fig. 3B and C). The effect of *T. gondii* infection on
IFN-γ, IL-4 or IL-17 mRNA expression of CD4+ cells in IL-1Ra-deficient mice was similar to that observed in WT mice (Fig. S2). Thus, *T. gondii*-infection induced marked Th1 polarization not only at dLN but also at spleen in IL-1Ra-deficient mice as well as in WT mice at an early phase of infection, and Th1 polarization was accompanied with reduction of Th2 and Th17 polarization.

Regulation of transcription factors for Th polarization by *T. gondii* infection

DC of *T. gondii*-infected WT and IL-1Ra-deficient mice produced not only IL-12 but also IL-10, IL-1β and IL-6. Since IL-10 is known as a negative regulator of IFN-γ, it suppresses differentiation to Th1. Also IL-1β and IL-6 play a role that helps differentiation to Th17. Therefore, Th polarization was further evaluated by measuring mRNA expression of T-bet, GATA-3 and RORγt, the specific transcription factors of Th1, Th2 and Th17, in CD4+ T cells isolated from the mLN or spleen of WT and IL-1Ra-deficient mice with or without *T. gondii*-infection at 1 week P.I. CD4+ cells from *T. gondii*-infected IL-1Ra-deficient mice expressed significantly higher levels of T-bet mRNA at both mLN and spleen compared to that of uninfected mice (Fig. 4A). In contrast, levels of GATA-3 or RORγt mRNA expression of CD4+ cells decreased in infected mice compared to that of uninfected mice (Fig. 4B and C). The effect of *T. gondii* infection on T-bet, GATA-3 or RORγt mRNA expression of CD4+ cells in IL-1Ra-deficient mice was similar to that observed in WT mice (Fig. S3). Thus, although levels of IL-10 mRNA expression of DC increased in IL-1Ra-deficient mice at 1 week P.I., neither Th2 nor Th17 but Th1 polarization of CD4+ T cells was confirmed at the transcription factor level of infected mice (Fig. 4A-C). Likewise, although levels of IL-1β and IL-6 mRNA expression of DC increased in IL-1Ra-deficient mice at 1 week P.I., mRNA expression of RORγt did not increase (Fig.
Relation of Th polarization and the severity of arthritis.

In order to clarify the relation of Th polarization and severity of arthritis, Th polarization of CD4+ T cells from IL-1Ra-deficient mice with or without *T. gondii*-infection was analyzed by intracellular cytokine staining. In *T. gondii*-infected mice that did not develop arthritis till 8 weeks P.I., numbers and mean fluorescent intensities (MFI) of IFN-γ positive splenic CD4+ cells markedly increased compared to that in the same aged-uninfected mice with arthritis (Fig. 5A). On the other hand, IL-4 and IL-17 positive CD4+ cells from spleen of infected mice decreased compared to that of uninfected mice (Fig. 5B and C). Differences were not obvious between uninfected and infected mice that developed arthritis (data not shown). The effect of *T. gondii* infection on CD4+ cells at LN (data not shown) was similar to that observed at spleen (Fig. 5).

To confirm the relation of Th polarization and arthritis, mRNA expressions of cytokines and transcription factors specific for Th1, Th2 and Th17 were measured in CD4+ cells that were isolated from LN or spleen of either “uninfected mice with arthritis” or “infected mice without arthritis at 8 week P.I.” CD4+ T cells from infected IL-1Ra-deficient mice expressed significantly higher levels of IFN-γ mRNA at spleen compared to that of uninfected mice (Fig. 6A). Conversely, levels of IL-4 or IL-17 mRNA decreased in infected IL-1Ra-deficient mice (Fig. 6B and C). Also CD4+ cells from infected mice expressed higher levels of T-bet mRNA compared to that of uninfected mice (Fig. 7A). In contrast, levels of GATA-3 or RORγt mRNA expression decreased in infected mice compared to that of uninfected mice (Fig. 7B and C). Thus, infection-induced Th1 polarization with reduction...
of Th2 and Th17 was shown in the asymptomatic mice at 8 week P.I.

Th1 dominancy shown in T. gondii-infected IL-1Ra-deficient IL-1Ra-deficient mice at 8 weeks P.I. persisted for more than 20 weeks P.I. in mice as long as clinical arthritis did not appear (data not shown). Thus, it has been confirmed that the amelioration of spontaneous development of arthritis in IL-1Ra-deficient mice by T. gondii-infection corresponds to the induction of Th1 polarization that accompanies reduction of Th17 polarization.

Discussion

In the present study, we have demonstrated that T. gondii infection ameliorated remarkably the spontaneous development of arthritis in IL-1Ra-deficient BALB/c mice. The early signs of arthritis could be detected at the ankle joints of the hind limbs as early as 4-5 weeks of age and the incidence of arthritis gradually increased to more than 80% at 8 weeks and almost all mice were affected by 13 weeks of age in IL-1Ra-deficient BALB/c mice, whereas the incidence of arthritis was around 30% even at 48 weeks of age in IL-1Ra-deficient C57BL/6 mice (19). Since BALB/c mice are a resistant strain against T. gondii-infection and not lethal by infection, we took advantage of using IL-1Ra-deficient mice with BALB/c background, and have revealed that T. gondii infection is capable of ameliorating the spontaneous development of arthritis in IL-1Ra-deficient mice.

Among autoimmune diseases, rheumatoid arthritis has been known to be a T cell-driven autoimmune disease (4, 18, 28) and T. gondii-infection affects on Th polarization of hosts. Therefore, we examined the effects of T. gondii-infection on the activation of DC since DC is a professional APC crucial for initiating T cell-mediated adaptive immunity (3).
DC functions as a director of Th polarization in the dLN for the subsequent adaptive response, and IL-12 selectively promotes the differentiation of Th0 to Th1 cells. The IL-12 production from CD11c^+ cells of both WT and IL-1Ra-deficient mice markedly increased in the mLN at 1 week P.I. after p.o. infection, indicating the \textit{in vivo} activation of DC which triggers Th1 polarization in the dLN at an early stage of \textit{T. gondii} infection. As we have measured IL-12p40 mRNA expression of CD11c^+ cells, one may argue that IL-12 and IL-23 share common p40 subunit (20). However, increased IL-12p40 mRNA expression measured in CD11c^+ cells of the mLN from \textit{T. gondii}-infected WT and IL-1Ra-deficient mice was confirmed to be IL-12 production but not IL-23 production because of the subsequent INF-\gamma production and the lack of IL-23-promoted IL-17 production from CD4^+ T cells. Also, levels of surface expression of CD86 and MHC class II molecules on CD11c^+ cells in the mLN of \textit{T. gondii}-infected IL-1Ra-deficient mice were up-regulated, confirming DC maturation in the dLN of infected mice.

Additionally, production of pro- and anti-inflammatory cytokines such as IL-1\beta, IL-6 and IL-10 from DC rose in \textit{T. gondii}-infected WT and IL-1Ra-deficient mice. Especially, the production of IL-10 mRNA from DC increased significantly in mLN and spleen, indicating that IL-10 production from DC of \textit{T. gondii}-infected mice would down-regulate the spontaneous development of arthritis that is known to be IL-17-dependent. IL-10, first described as a cytokine that inhibits IFN-\gamma expression in Th1 cells, plays a role in preventing exaggerated inflammatory and immune responses and protects the host from immune-mediated damage as an anti-inflammatory cytokine (39, 40).

In fact, consistent with our report, Gu Y et al reported that IL-10 negatively regulates the expression of IL-17 (14) and Heo YJ et al further revealed that IL-10 suppresses Th17 cells and promotes regulatory T cells in the CD4^+ T cell population of rheumatoid arthritis.
patients (16). On the other hand, augmentation of pro-inflammatory cytokine expression such as IL-1β, IL-6 and TNF-α was reported in arthritic joints of IL-1Ra-deficient mice compared with that of WT mice (19). Although pro-inflammatory cytokine production from DC increased in IL-1Ra-deficient mice at 1 week P.I. in this study, it is considered that the increase of IL-1β and IL-6 reflects acute inflammation caused by *T. gondii* infection but not regional inflammation in the joints. In addition, though IL-1β might induce the expression and secretion of IL-23 (8), the level of IL-1β mRNA from DC was not statistically different between infected IL-1Ra-deficient and WT mice.

By measuring mRNA expression of representative cytokines (IFN-γ, IL-4 or IL-17) and transcription factors (T-bet, GATA-3 and RORγt) specific for Th1, Th2 and Th17, Th polarization was comparatively evaluated in WT and IL-1Ra-deficient mice at 1 week P.I. (i.e., 3.5-week-old). *T. gondii* infection was shown to induce marked Th1 polarization not only at the dLN but also at the spleen in IL-1Ra-deficient mice as well as in WT mice. Th2 or Th17 polarization was reduced. Since existence of IL-6 plus TGF-β together with IL-1β, TNFα and IL-23 is known to induce Th differentiation to Th17 (4, 28), the increase of IL-1β and IL-6 mRNA production from DC in both WT and IL-1Ra-deficient mice after *T. gondii* infection might trigger Th17 differentiation. However, mRNA expressions of not only IL-17 but also RORγt decreased in this study, indicating the down-regulation to Th17 differentiation at the transcription level. The increase of pro-inflammatory cytokines after infection is clearly an acute phase response to infection and has no effect on Th polarization of the host, i.e. the protective immune responses of infected mice.

In this study, we noticed that IL-17 production was reduced in CD4⁺ T cells from the mLN and spleen of WT and IL-1Ra-deficient BALB/c mice after *T. gondii*-infection, whereas Guiton et al reported that IL-17 expression was increased at the ileum of WT.
C57BL/6 mice and that the IL-17 contributed to the ileitis and other inflammatory responses after *T. gondii*-infection (15). It is possible that the IL-17 response against *T. gondii*-infection is different between C57BL/6 and BALB/c mice because massive necrosis was observed in the ileum of C57BL/6 mice, but not in that of BALB/c mice. This had been already reported in acute toxoplasmosis (29). Since it was reported that IL-10 is required for the prevention of necrosis in the small intestine (39), increased IL-10 production and decreased IL-17 expression may explain why *T. gondii*-infection ameliorates inflammation in IL-1Ra-deficient BALB/c mice. Since spontaneous development of arthritis in IL-1Ra-deficient mice is IL-17-dependent (17, 26, 36), we conclude that Th1 polarization with reduced Th17 polarization by *T. gondii* infection down-regulates the severity of arthritis in IL-1Ra-deficient mice. *T. gondii* infection contributes in delaying onset of the symptoms indicating that the infection affects the early stage of arthritis only and would not be effective on established arthritis. In regards to this, it was reported that IL-17 is involved in the recruitment of neutrophils to the site of inflammation (27), activation of T cells (35), stimulation of antibody production (34, 41), and enhancement of osteoclastogenesis (37).

We have developed a DNA vaccine encoding a virulent HSP70 molecule specific for tachyzoites (i.e., pathogenic stage of *T. gondii* at acute phase or at acute exacerbation of the chronic phase of infection) targeting epidermal and dermal DC against *T. gondii* infection (25, 31, 32). *T. gondii* HSP70 gene vaccine is proven to induce *in vivo* DC activation and successive Th1 polarization in the dLN, and the vaccine effects persist to the chronic phase of toxoplasmosis (31). The DNA vaccine by gene gun activates antigen-specific CD4+ T cell response with a coding antigen gene (12). Thus, *T. gondii* HSP70 gene vaccine with a gene gun will be applied on arthritis in IL-1Ra-deficient mice.
We herein report a positive effect of *T. gondii*-infection on Th17-mediated spontaneous development of arthritis in IL-1Ra-deficient mice, which may give insight into the development of protozoan-derived prophylactic medicine for T cell-mediated autoimmune diseases. Th1 dominancy with reduced Th17 responses still persisted at chronic phase of infection in asymptomatic *T. gondii*-infected mice. This study warrants further association studies between chronically infected human individuals and autoimmune diseases like rheumatoid arthritis.

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Fig. 1. Effects of *T. gondii* infection on spontaneous development of arthritis in IL-1Ra-deficient mice. (A) IL-1Ra-deficient BALB/c mice were p.o. infected with 10 *T. gondii* cysts at 2.5 weeks and development of arthritis in uninfected (open circle) and infected (closed circle) mice was examined weekly as described in Materials and Methods. Incidence (%) was calculated from 11 uninfected and 29 *T. gondii*-infected mice. (B) The severity score of arthritis in uninfected (open circle) and infected (closed circle) mice was determined weekly as described in Materials and Methods. The average scores ± SD were obtained from 11 uninfected and 29 *T. gondii*-infected mice. Statistical differences between uninfected and *T. gondii*-infected groups were shown as # *p* < 0.05 by 2x2 contingency chi-squared test, and * *p* < 0.05 and ** *p* < 0.01 by Student’s *t*-test. (C) Histopathology of the ankle joints of uninfected (a-c) and *T. gondii*-infected (d-f) IL-1Ra-deficient BALB/c mice; (a) Swelling and redness of the ankle joint of uninfected IL-1Ra-deficient BALB/c mice. (b)-(c) Parafin-embedded tissue sections of ankle joints from uninfected IL-1Ra-deficient mice were stained with hematoxylin/eosin, and the microscopic observation magnified at 40x and 100x was shown. Marked infiltration of inflammatory cells (arrow), hyperplasia of synovial membrane (star), and erosive destruction of the bone (arrowhead) were observed. (d) Ankle joint of *T. gondii*-infected IL-1Ra-deficient mice. Swelling and redness of the ankle joint were not observed. (e)-(f) Microscopic observation of the ankle joint of *T. gondii*-infected IL-1Ra-deficient mice without infiltration of inflammatory cells.

Fig. 2. Effects of *T. gondii* infection on DC activation in IL-1Ra-deficient mice. CD11c⁺ cells were isolated from the mLN or spleen of IL-1Ra-deficient mice with or
without T. gondii infection at 1 week P.I., and (A) mRNA expression of IL-12p40 of
CD11c+ cells from uninfected (white column) or T. gondii-infected (black column) of
IL-1Ra-deficient mice was evaluated by reverse transcription-PCR as described in
Materials and Methods. (B) IL-10 mRNA, (C) IL-6 mRNA and (D) IL-1β mRNA
expression of CD11c+ cells were similarly measured. The results are normalized with
GAPDH in the same sample. Results represent three independent experiments with three
to five mice in each experimental group. Statistical differences by Student’s t-test between
groups are shown as *p<0.05 and ***p<0.005.

Fig. 3. Effect of T. gondii infection on Th polarization in IL-1Ra-deficient mice. CD4+
cells were isolated from either the mLN or spleen of IL-1Ra-deficient mice with or
without T. gondii infection at 1 week P.I., and mRNA expressions of IFN-γ (A), IL-4 (B)
and IL-17 (C) were comparatively measured as described in Materials and methods. The
results from mice uninfected (white column) or T. gondii-infected (black column) are
normalized with GAPDH in the same sample. Results represent three independent
experiments with three to five mice per group. Statistical differences between groups are
shown as *p<0.05, **p<0.01 and ***p<0.005.

Fig. 4. Regulation of transcription factors for Th polarization by T. gondii infection.
CD4+ cells were isolated from either the mLN or spleen of IL-1Ra-deficient mice with or
without T. gondii infection at 1 week P.I., and mRNA expressions of T-bet (A), GATA-3
(B) and RORγt (C) were comparatively measured as described in Materials and Methods.
The results from mice uninfected (white column) or T. gondii-infected (black column) are
normalized with GAPDH mRNA in the same sample. Results represent three
independent experiments with three to five mice per group. Statistical differences between

groups are shown as *p<0.05, **p<0.01 and ***p<0.005.

Fig. 5. Flow cytometric analyses at chronic phase of infection. Spleen cells of T. gondii-infected IL-1Ra-deficient mice without development of arthritis at 8 weeks P.I. were stained with those of age-matched uninfected mice for intracellular IFN-γ (A), IL-4 (B) or -IL-17 (C) as described in Materials and Methods. Isotype control (D) was used for staining. Numbers shown in each square are the percentages of cells contained in gated CD4+ cells. The mean fluorescent intensity (MFI) is given in parentheses. Experiments were repeated three times with similar results.

Fig. 6. Th polarization in the mice without arthritis at 8 weeks P.I. CD4+ cells were isolated from either LN or spleen of IL-1Ra-deficient mice without development of arthritis at 8 week P.I., and mRNA expressions of IFN-γ(A), IL-4 (B) and IL-17 (C) were comparatively measured with that of uninfected mice as described in Materials and methods. The results from uninfected (white column) or T. gondii-infected (black column) mice are normalized with GAPDH mRNA in the same sample. Results represent three independent experiments with three mice per group. Statistical differences between groups are shown as *p<0.05 and ***p<0.005.

Fig. 7. Transcription factors for Th polarization in the mice without arthritis at 8 weeks P.I. CD4+ cells were isolated from either LN or spleen of IL-1Ra-deficient mice without development of arthritis at 8 week P.I., and mRNA expressions of T-bet (A), GATA-3 (B) and RORγt (C) were comparatively measured with that of uninfected mice as described in
Materials and Methods. The results from mice uninfected (white column) or *T. gondii*-infected (black column) are normalized with GAPDH mRNA in the same sample. Results represent three independent experiments with three mice per group. Statistical differences between groups are shown as *p < 0.05 and **p < 0.01.