

# Impaired Innate Immunity in Mice Deficient in Interleukin-1 Receptor-Associated Kinase 4 Leads to Defective Type 1 T Cell Responses, B Cell Expansion, and Enhanced Susceptibility to Infection with *Toxoplasma gondii*

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Interleukin-1 receptor (IL1R)-associated kinase 4 (IRAK4) is a member of the IRAK family and has an important role in inducing the production of inflammatory mediators. This kinase is downstream of MyD88, an adaptor protein essential for Toll-like receptor (TLR) function. We investigated the role of this kinase in IRAK4-deficient mice orally infected with the cystogenic ME49 strain of *Toxoplasma gondii*. IRAK4<sup>-/-</sup> mice displayed higher morbidity, tissue parasitism, and accelerated mortality than the control mice. The lymphoid follicles and germinal centers from infected IRAK4<sup>-/-</sup> mice were significantly smaller. We consistently found that IRAK4<sup>-/-</sup> mice showed a defect in splenic B cell activation and expansion as well as diminished production of gamma interferon (IFN- $\gamma$ ) by T lymphocytes. The myeloid compartment was also affected. Both the frequency and ability of dendritic cells (DCs) and monocytes/macrophages to produce IL-12 were significantly decreased, and resistance to infection with *Toxoplasma* was rescued by treating IRAK4<sup>-/-</sup> mice with recombinant IL-12 (rIL-12). Additionally, we report the association of IRAK4 haplotype-tagging single nucleotide polymorphisms (tag-SNPs) with congenital toxoplasmosis in infected individuals (rs1461567 and rs4251513,  $P < 0.023$  and  $P < 0.045$ , respectively). Thus, signaling via IRAK4 is essential for the activation of innate immune cells, development of parasite-specific acquired immunity, and host resistance to infection with *T. gondii*.

*Toxoplasma gondii* is a ubiquitous intracellular protozoan parasite that infects a broad range of vertebrate hosts, including humans and domestic animals (18). Clinically, toxoplasmosis may be asymptomatic or present signs and symptoms that vary depending on the host's genetic background and immunological status (22, 49) and the type of infective parasite (44). *Toxoplasma* infection in an immunocompetent host is controlled by an effective immune response and causes little or no overt signs of disease. However, in immunocompromised patients or during pregnancy, *T. gondii* may emerge as a serious infection which, if not treated, often leads to mortality (52).

A hallmark of *T. gondii* infection is the induction of a strong Th1-mediated immunity, especially with production of gamma interferon (IFN- $\gamma$ ), which keeps in check the tachyzoites, the rapidly multiplying form of the parasite. The control of tachyzoite replication allows the development of the chronic and asymptomatic stage of disease, which is maintained by the bradyzoite stage of the parasite (10). While the CD4<sup>+</sup> and CD8<sup>+</sup> T cells are critical for maintaining chronic toxoplasmosis, innate immunity seems to play an important role in host resistance at the early stages of infection. Activation of the cellular compartment of the innate immune system triggers effector mechanisms that are directly responsible for the control of replication of the intracellular parasites. In addition, cytokines produced by innate immune cells will direct the development of acquired immunity, which is responsible for immune surveillance at later stages of infection (10).

The activation of innate immune cells is achieved through en-

gaging an array of germ line-encoded pattern recognition receptors to detect invariant pathogen motifs (51). During infection with *T. gondii*, the production of interleukin-12 (IL-12) by myeloid cells is induced by the activation of Toll-like receptors (TLRs), which triggers signaling through the adaptor protein MyD88 (9, 31, 43, 55). Following MyD88 association with TLRs, the former recruits IL-1 receptor-associated kinase 1 (IRAK1) (4), IRAK2 (4), IRAK-M (53), and IRAK4 (23, 46) to TLRs through interaction of the death domains (50) of both molecules. Several recent studies using transgenic animals indicated that each IRAK protein has a distinct function (47). Unlike deficiencies in IRAK1 or -M, a deficiency in IRAK4 leads to a serious flaw in the activation of transcription factors (NF- $\kappa$ B, AP1, cJUN) and the production of several inflammatory cytokines induced by signaling through TLRs (46). In humans, IRAK4 has 460 amino acids, sharing 84% similarity with the murine IRAK4 protein. The role of IRAK4 in the activation of the immune system was

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demonstrated in a murine model of viral infection (47) and in humans who suffer from recurrent infections with pyogenic bacteria (30, 35, 39).

In the present study, we observed that both activation of innate immunity and development of acquired immunity were highly compromised in IRAK4-deficient mice. In particular, defects in IL-12 production by myeloid cells and IFN- $\gamma$  production by T cells were shown to be the primary mechanisms responsible for the enhanced susceptibility of IRAK4<sup>-/-</sup> mice to infection with *T. gondii*. Finally, the relevance of our findings for human disease is highlighted by our findings that two haplotype-tagging single nucleotide polymorphisms (tag-SNPs) in the *IRAK4* gene were significantly associated with congenital toxoplasmosis in humans.

## MATERIALS AND METHODS

**Mice.** IRAK4<sup>+/-</sup> mice (46) in the C57BL/6 and 129 genetic backgrounds were kept as heterozygote breeders to generate both the homozygote and the heterozygote mice used in our studies. IRAK4<sup>-/-</sup>, IRAK4<sup>+/-</sup>, and 129/Ola mice were bred and maintained in microisolators at the Federal University of Minas Gerais and the René Rachou Research Institute. Male mice between 8 and 12 weeks of age were used in all experiments. All procedures were conducted according to institutional guidelines for animal ethics.

**Antibodies.** I-A/I-E-biotin (2G9), I-A/I-E-allophycocyanin (APC) (M5/114.15.2), CD86-PECy7 (IT2.2), NK1.1-fluorescein isothiocyanate (FITC) (PK136), CD3-PerCP5.5 (145-2C11), CD19-FITC (1D3), CD69-phycoerythrin (PE) (H1.2F3), CD8-PECy7 (53-6.7), CD62L-APC (MEL-14), FC Block, IL-12-PE (C17.8), TNF- $\alpha$ -PECy7 (TN3-19), IFN- $\gamma$ -PE (XMG1.2), and Ki67-PerCP5.5 (20Raj1) were from BD Pharmingen, CD19-APC-eFluor780 (1D3), CD86-APC (B7-2), CD11c-Alexa 700 (N418), CD40-PECy5 (1C10), GRI/Ly-6G and Ly-6C-PECy5 (RB6-8C5), CD4-PO (RM4-5), CD44-PB (IM7), CD11b-FITC (MI/70), F4/80-APC (BM8), and recombinant IL-12 (rIL-12) were from eBioscience, and streptavidin-QD605 (Q10101MP) was from Invitrogen.

**Parasites, soluble *Toxoplasma* antigen, and experimental infections.** Infective cysts of the ME49 strain were obtained from the brain tissue of Swiss mice at 30 to 40 days postinfection. Soluble *T. gondii* antigen (STAg) was obtained from tachyzoites of the RH strain of *T. gondii*, which is maintained in Swiss mice by passage at regular periods of 48 to 72 h. Briefly, a parasite suspension obtained from the peritoneal cavity was sonicated and centrifuged, the supernatant was collected, and the protein content was determined by the Bradford method (15). For our experiments, mice were infected orally with 0.1 ml of brain homogenates adjusted to contain 20 cysts and either monitored for mortality/morbidity for 30 days or euthanized at days 7, 9, or 20 to 30 (for cyst counts) after infection, when their blood, brains, lungs, livers, and spleens were collected. Morbidity was assessed based on the clinical parameters that we scored as follows: bright and active (0), ruffled coat (1), 10% weight loss (2), hunched, tottering gait, staring, stiff coat (3), reluctance to move and 20% weight loss (4), and death (5).

**In vivo treatment with rIL-12.** The IRAK4<sup>-/-</sup> mice were challenged orally with the ME49 strain and treated daily with intraperitoneal injections of rIL-12 (150 ng/mouse) (eBioscience) for 7 days beginning on day 1 postinfection. As controls, IRAK4<sup>-/-</sup> mice were infected and received phosphate-buffered saline (PBS) on an identical schedule (40).

**Quantitative real-time PCR.** Spleens and livers were harvested from the mice at 7 or 9 days after infection and frozen in liquid nitrogen. Total DNA was extracted using the phenol-chloroform protocol and quantified by UV spectrophotometry (NanoDrop spectrophotometer; Thermo Scientific). Quantitative PCRs were setup in a final volume of 20  $\mu$ l containing 25 ng of total tissue DNA, 10 ng of each primer, and 1  $\times$  iQ SYBR green supermix (Bio-Rad) and performed on a 7000 sequence detection system (Applied Biosystems). The results are expressed in pg of parasite DNA per 25 ng of tissue DNA. The primers used for amplification of the *T. gondii*

B1 gene were 5'-CTGGCAAATACAGGTGAAATG-3' (forward) and 5'-GTGTACTGCGAAAATGAATCC-3' (reverse) (31).

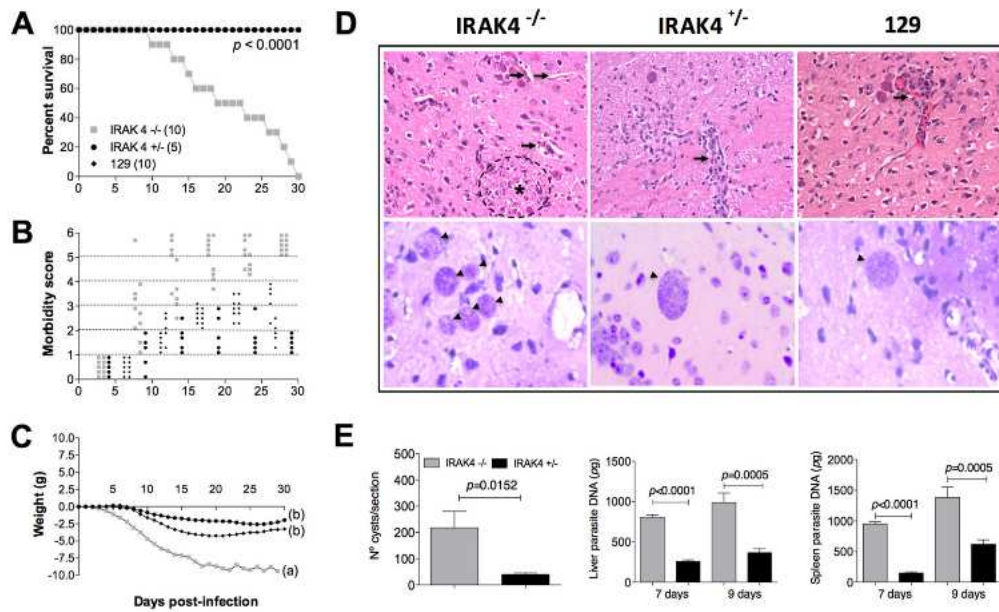
**Histopathology.** Brains, lungs, livers, and spleens were fixed with 10% buffered formaldehyde and processed for paraffin embedding. Tissue sections were cut with a microtome and stained with hematoxylin and eosin or Giemsa stain. The histological aspects of parenchyma tissue were evaluated for inflammation (endothelial reactivity, perivascular cell infiltration and necrosis) and parasitism (tissue cysts) by light microscopy. Morphometric aspects of the spleen, such as the diameter of the follicle and the germinal center, were evaluated using the program KS 300 (Zeiss, Germany).

**Isolation of splenic leukocytes.** Spleens from control and infected mice were incubated with 0.4 mg/ml Liberase CI solution (Roche Biochemicals) for 30 min at 37°C. Single-cell suspensions were then passed through a 100- $\mu$ m nylon cell strainer. Erythrocytes were then lysed and splenocytes were resuspended in RPMI 1640 medium supplemented with 40 mg gentamicin sulfate and 10% fetal bovine serum (Gibco). Cells were then cultured for 48 h in 96-well plates in the presence or the absence of exogenous stimuli for cytokine measurement with a flow cytometer.

**Cytokine measurements.** Cytokine levels were assessed in the supernatants of splenocyte cultures and serum samples harvested from IRAK4<sup>-/-</sup> and IRAK4<sup>+/-</sup> mice at 7 days postinfection with the ME49 strain of *T. gondii*. IL-12p70 levels were measured with an enzyme-linked immunosorbent assay kit (eBioscience), and the IFN- $\gamma$ , tumor necrosis factor alpha (TNF- $\alpha$ ), IL-10, IL-6, and monocyte chemoattractant protein 1 (MCP-1) levels were determined using the BD cytometric bead array mouse inflammation kit according to the manufacturer's instructions.

**Flow cytometry.** To analyze the phenotype of splenocyte subpopulations, cell suspensions were stained with monoclonal antibodies specific for cell surface markers or cytokines. Two million freshly isolated splenocytes were analyzed for surface markers expressed by dendritic cells (DCs), monocytes/macrophages, and CD4, CD8, B, and NK cells. To assess cytokine production by DCs and macrophage/monocytes, splenic cells were cultured in the presence of brefeldin A (GolgiPlug protein transport inhibitor; BD Biosciences, San Jose, CA) for 6 h at 37°C in 5% CO<sub>2</sub> with lipopolysaccharide (LPS) (100 ng/ml), STAg (5  $\mu$ g/ml), or medium alone. To assess T lymphocytes and NK cells, 1  $\times$  10<sup>6</sup> splenic cells were cultured for 20 h in the presence of STAg (5  $\mu$ g/ml), anti-CD3 (1  $\mu$ g/ml), or medium alone. Brefeldin A was added to the cultures in the last 12 h before staining. To assess B cells, 1  $\times$  10<sup>6</sup> splenic cells were analyzed without stimulus. Cell suspensions were then washed and stained with different combinations of the antibodies described above. Cell suspensions were then washed, fixed, and permeabilized for the intracellular cytokine or proliferation marker staining (anti-IL-12p40, anti-TNF- $\alpha$ , anti-IFN- $\gamma$ , or Ki67) according to the manufacturer's instructions. Finally, the cells were suspended in 200  $\mu$ l of 2% paraformaldehyde, and the data were collected in an LSR II with Diva (BD Biosciences) and analyzed with FlowJo (Tree Star Inc., Ashland, OR) v 8.8.6 software.

**Families studied and their evaluations.** Since 1981, persons with congenital toxoplasmosis and their families have participated in a longitudinal study of congenital toxoplasmosis, which takes place across their lifetimes (27–29, 32, 36, 42). Enrollment begins when children are first diagnosed, which can be as early as at the time of birth (or in some cases *in utero*). They are then reexamined at 1, 3.5, 5, 7.5, 10, 15, and  $\geq$ 20 years of age (27–29, 32, 36, 42). Serologic testing is performed in the Remington U.S. Reference Laboratory at the Palo Alto Medical Foundation-Stanford University or at the Institut de Puericulture in Paris, France (for U.S. expatriate families who returned from France to the United States). A general pediatric evaluation, ophthalmologic, neurologic, and hearing testing, developmental pediatric standardized testing according to a pre-specified protocol, and a hematologic examination were performed for every child at birth and at each follow-up visit. Full fundus retinal photographs, optical coherence tomography (OCT), and visual fields were obtained for children who were at the age or cognitive capacity appropriate



**FIG 1** IRAK4 mediates control of parasite replication and influences disease outcome during primary infection with *Toxoplasma gondii*. Groups of IRAK4<sup>-/-</sup>, IRAK4<sup>+/-</sup>, and 129/Ola mice were orally infected with 20 cysts of the ME49 strain. Mortality (A), morbidity (B), and weight loss (C) (different letters in parentheses indicate  $P$  of  $< 0.0001$ ) were assessed for 30 days after infection. Numbers within the parentheses indicate the number of animals used in the experiment shown in panels A and C. In panel B, each dot at one time point corresponds to one animal. Morbidity was scored as follows: b bright and active, 0; ruffled coat, 1, 10% weight loss, 2, hunched, tottering gait, staring, stiff coat, 3; reluctance to move and 20% weight loss, 4, and death, 5. Data are representative of two independent experiments that yielded similar results. (D) Tissue cysts in IRAK4<sup>-/-</sup> (left panels), IRAK4<sup>+/-</sup> (middle panels), and 129 (right panels) mice are indicated with black arrowheads. Black arrows indicate the absence or presence of perivascular inflammatory infiltrates in IRAK4<sup>-/-</sup>, IRAK4<sup>+/-</sup>, and 129 mice. The asterisk indicates an area of moderate necrosis in the brain tissue of IRAK4<sup>-/-</sup> mice. (E) Cyst counts in histological sections of the brain from IRAK4<sup>-/-</sup> and IRAK4<sup>+/-</sup> mice infected with *T. gondii* (left) and quantification of parasite DNA in the livers and spleens of IRAK4<sup>-/-</sup> (middle) and IRAK4<sup>+/-</sup> (right) mice at 7 and 9 days after infection. The presented results are an average of data from eight mice per group, and the  $P$  values were calculated for a 95% confidence interval. The experiments were repeated three times with similar results.

for cooperation with such testing. A computed tomographic brain scan was obtained for every infant at birth or at the time of diagnosis for other congenitally infected persons (27–29, 32, 36, 42). Of the 179 infected children who were genotyped for this study, 124 (83%) had clinically confirmed brain calcifications with or without hydrocephalus and/or retinal lesions at birth or the time of diagnosis. This study was performed by the National Collaborative Chicago-Based Congenital Toxoplasmosis Study (NCCCTS) Group (27–29, 32, 36, 42). Physician and scientists who conducted these evaluations in the NCCCTS are listed in Acknowledgments. All procedures were performed in person, in accordance with institutional and NIH guidelines, and with informed consent from parents or legal guardians and adult congenitally infected persons.

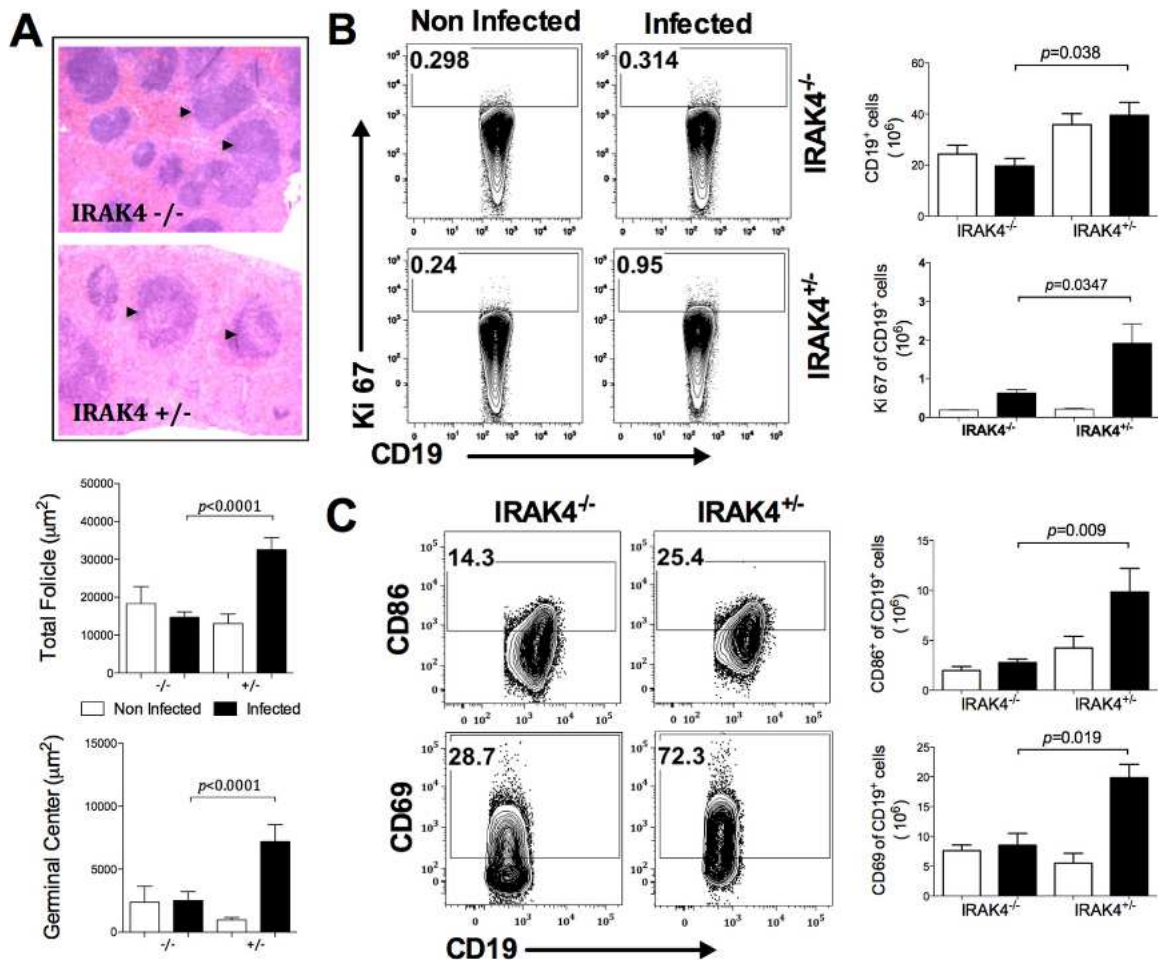
**Genotyping of human IRAK4.** Peripheral blood mononuclear cells (PBMCs) from 179 children and their parent or parents were cryopreserved in liquid nitrogen at  $-135^{\circ}\text{C}$  until placed in buffer for DNA extraction (38). This DNA was genotyped for 7 tag-SNPs distributed throughout the *IRAK4* gene. The tag-SNPs were selected from the Hap-Map project (<http://www.hapmap.org>) using a 10-kb flanking sequence on each side of the *IRAK4* gene, a minor allele frequency (MAF) cutoff of 5% in the European cohort (CEU), and an  $r^2$  threshold of 0.8. The Tagger tool within Haploview (<http://www.broad.mit.edu/mpg/haploview>) was used to select tag-SNPs. Allelic association analyses were performed for the 124 children with confirmed clinical findings in the eye and/or brain using UNPHASED (<http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased>).

**Statistical analyses.** For the studies of mice, the cumulative mortality rate in the experimental groups was analyzed using the log rank test. The differences between groups were verified using the  $t$  test or the Mann-Whitney test for parametric or nonparametric data, respectively. All tests were performed using GraphPad Prism (v 5.0) (GraphPad Software Inc.,

La Jolla, CA), and differences were considered statistically significant when the  $P$  values were  $< 0.05$ . For genetic studies of humans, allelic association analyses were performed using a conventional transmission disequilibrium test (TDT). Linkage disequilibrium (LD) is the nonrandom association of alleles at two or more loci. It is an approximation for the existence of historical recombination between two loci. The  $P$  values were calculated using Haploview (<http://www.broadinstitute.org/haploview>). For association with disease,  $P$  values of 0.05 or less were considered significant.

## RESULTS

**Functional IRAK4 mediates the control of parasite replication during primary infection in mice.** To evaluate the role of IRAK4 during *T. gondii* infection, IRAK4<sup>-/-</sup>, IRAK4<sup>+/-</sup>, and wild-type (WT) 129 mice were orally infected with 20 cysts of the ME49 strain. Animals were evaluated for up to 30 days postinfection. Mortality of the infected IRAK4<sup>-/-</sup> mice was detected as early as 10 days, reaching 100% by 30 days postinfection. In contrast, no death was observed in either the heterozygous or 129 animals (Fig. 1A). Heterozygote and WT mice showed signs of morbidity classified as mild to moderate, which resolved by 2 to 3 weeks of infection. In contrast, IRAK4<sup>-/-</sup> mice infected with *T. gondii* showed severe signs of morbidity, including neurological symptoms indicating cerebral commitment and weight loss (Fig. 1B and C). Since the IRAK4<sup>-/-</sup> mice are in the C57BL/6 and 129/Ola genetic background and not fully backcrossed, we used IRAK4<sup>+/-</sup> littermates as controls in most experiments, as indicated in the figure legends. Importantly, for all experiments in which we used



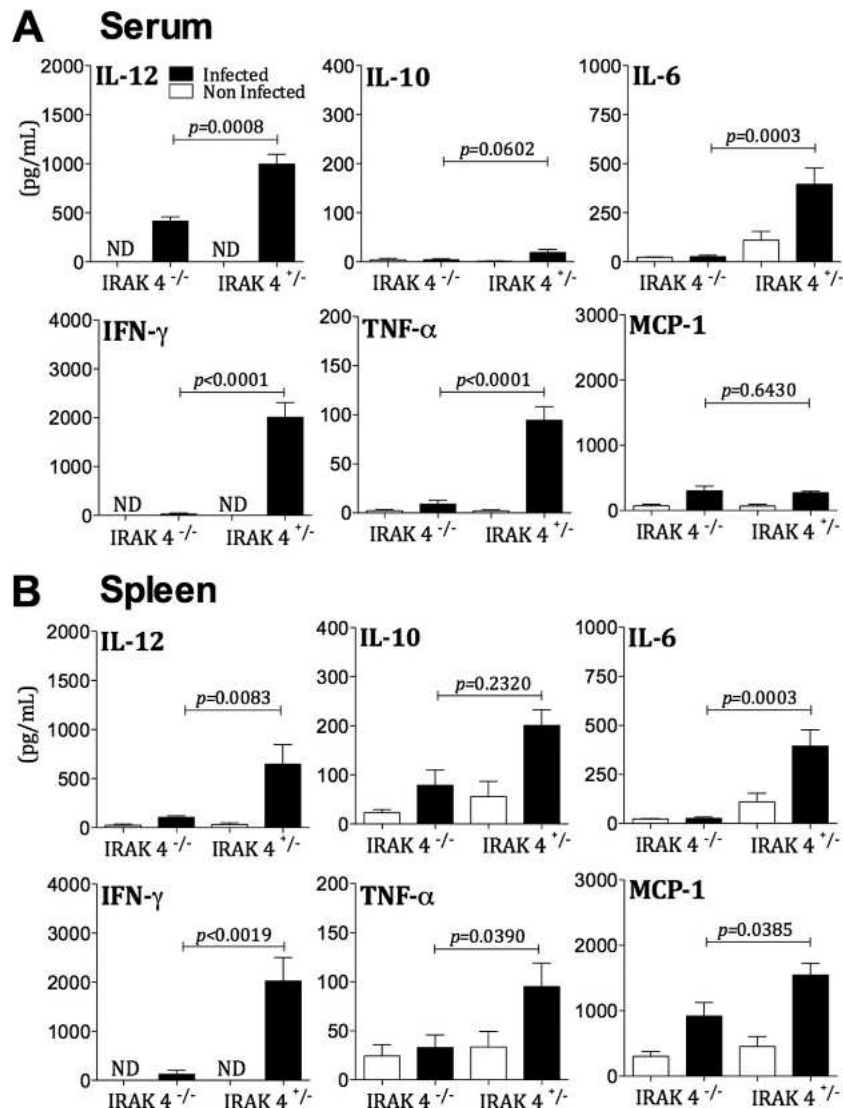
**FIG 2** Altered structures of follicles and germinal centers in the spleens of IRAK4<sup>-/-</sup> mice infected with *T. gondii*. (A) Spleens from mice at 7 days postinfection were removed and stained with hematoxylin and eosin for histological evaluation. Black arrowheads indicate activated and nonactivated germinal centers in spleens from IRAK4<sup>-/-</sup> (upper panel) and IRAK4<sup>+/-</sup> (bottom panel) mice, respectively. Bar graphs show the quantification of follicles and germinal center areas in spleens from IRAK4<sup>-/-</sup> and IRAK4<sup>+/-</sup> mice. (B) Dot plot graphs (frequency) and bar graphs (absolute number) show B cells in proliferation (CD19<sup>+</sup> Ki67<sup>+</sup>) from IRAK4<sup>+/-</sup> and IRAK4<sup>-/-</sup> mice (left and bottom right graphs). (C) The contour plots on the left and the graphs show, respectively, the frequency and total numbers of activated (i.e., CD86<sup>+</sup> or CD69<sup>+</sup>) B lymphocytes (CD19<sup>+</sup>). The results from uninfected and infected mice are represented with white and black bars, respectively. The presented results are an average of data from four mice per group, and the *P* values were calculated for a 95% confidence interval. The experiments were repeated three (A) or two (B and C) times with similar results.

two parallel groups, the parasitological and immunological tests were similar when we compared 129 and IRAK4<sup>+/-</sup> mice infected with *T. gondii* (data not shown).

Additionally, to assess whether the clinical signs of diseases and lethality in IRAK4<sup>-/-</sup> mice were due to an uncontrolled parasite replication, we analyzed the tissue parasitism in IRAK4<sup>-/-</sup>, IRAK4<sup>+/-</sup>, and WT mice. Brains of animals infected for more than 20 days were harvested, tissue sections were stained with Giemsa, and cysts were quantified. The brains of IRAK4<sup>-/-</sup> mice infected with *T. gondii* showed significantly higher cyst counts than those of the control groups (IRAK4<sup>+/-</sup> and 129 mice) (Fig. 1D and E). Additionally, IRAK4<sup>-/-</sup> mice showed numerous foci of necrosis in the central nervous system (CNS), always associated with parasitic cysts, while the IRAK4<sup>+/-</sup> and 129 mice had few necrotic foci, which were not associated with parasites. Nevertheless, heterozygote and 129 animals showed moderate inflammatory infiltrates with foci of perivascular inflammation, suggesting an effective inflammatory response, which effectively controlled parasite expansion in the CNS of both IRAK4<sup>+/-</sup> and WT mice.

Spleens and livers were harvested 7 and 9 days postinfection for parasitism evaluation by real-time PCR (RT-PCR). *T. gondii* DNA levels were greater in both the spleens and the livers of IRAK4<sup>-/-</sup> mice than in heterozygote mice (Fig. 1E). These results indicate that control of tachyzoite replication and tissue parasitism during primary infection, and therefore host resistance to *T. gondii* infection, is dependent on functional IRAK4.

**Defective expansion of splenic B lymphocytes in IRAK4-deficient mice.** When examined at 7 days after infection, the spleens from IRAK4<sup>-/-</sup> mice showed an altered structure. The areas of follicles and germinal centers from infected IRAK4<sup>-/-</sup> mice, but not in uninfected animals, were relatively smaller than in IRAK4<sup>+/-</sup> mice, with little cellular infiltration and scant lymphocytic halo (Fig. 2A), suggesting that both lymphoid and myeloid cells were less active in this organ during *T. gondii* infection. As expected, the total number of B cells was significantly lower in IRAK4<sup>-/-</sup> mice than in IRAK4<sup>+/-</sup> mice (Fig. 2B, bar graphs). The frequency of activated B cells, as assessed by CD86 and CD69 expression, was consistently significantly decreased in IRAK4<sup>-/-</sup>



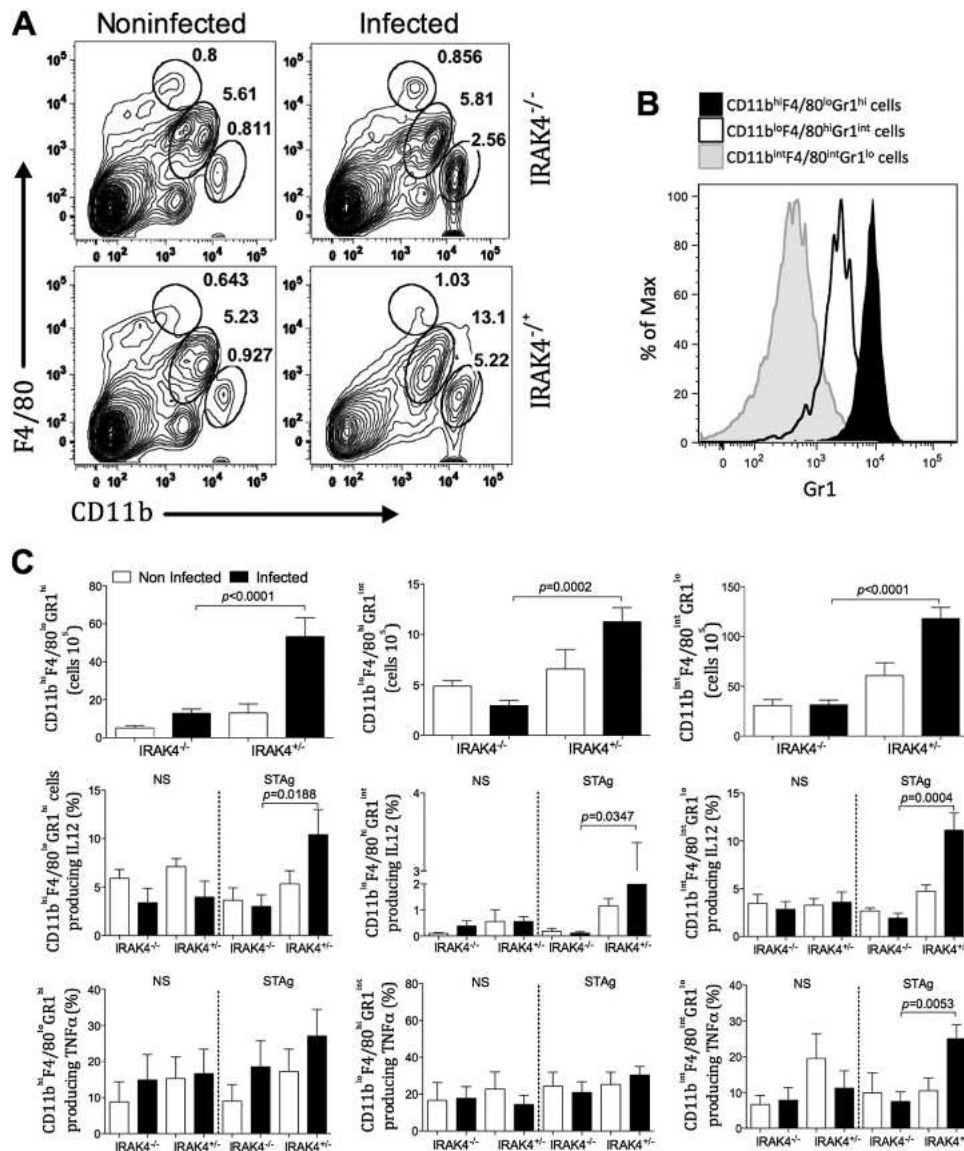
**FIG 3** Impaired cytokine response in IRAK4<sup>-/-</sup> mice infected with *T. gondii*. IRAK<sup>-/-</sup> and IRAK<sup>+/-</sup> mice were orally infected with the ME49 strain of *T. gondii*. (A) The levels of cytokines in serum were evaluated at 7 days postinfection by enzyme-linked immunosorbent assay (ELISA) and cytometric bead array (CBA). The *P* values were calculated for a 95% confidence interval. (B) Splenocytes from IRAK<sup>-/-</sup> and IRAK<sup>+/-</sup> mice were harvested at 7 days postinfection and cultured for 48 h without the addition of exogenous stimuli. Cytokine levels were measured in the supernatants by ELISA and CBA. The results of uninfected and infected mice are represented in white and black, respectively. The presented results are an average of data from eight mice per group, and the *P* values were calculated for a 95% confidence interval. The experiments were repeated three times with similar results.

mice compared to that in the heterozygote mice infected with *T. gondii* (Fig. 2C). To confirm that the observed changes in the germinal centers were, at least in part, due to the altered proliferation of B cells, we also analyzed the expression of the cell cycle marker Ki67 (interphase stage). We observed that upon infection with *T. gondii*, the number of Ki67-expressing B lymphocytes was dramatically increased in IRAK4<sup>+/-</sup> mice but not in IRAK4<sup>-/-</sup> mice (Fig. 2B).

**Impaired production of proinflammatory cytokines in IRAK4-deficient mice infected with *T. gondii*.** Cytokines such as IL-12, IFN-γ, and TNF-α play an important role in host resistance to *T. gondii* infection. Thus, we assessed the cytokine production by cells from IRAK4<sup>-/-</sup> mice infected with *T. gondii*. Sera were collected from mice at 7 days postinfection. Spleens were also harvested, and splenocytes were cultured for 48 h. IRAK4<sup>-/-</sup> an-

imals infected with *T. gondii* displayed impaired IL-12, IFN-γ, and TNF-α production. These lower levels of cytokines were observed in both the sera (Fig. 3A) and supernatants of splenocyte cultures (Fig. 3B). The levels of IL-10 and MCP-1 (CCL2) were also significantly lower in the supernatant of cultures of spleen cells derived from infected IRAK4<sup>-/-</sup> mice.

**Altered distribution and impaired cytokine production by myeloid cells from IRAK4-deficient mice challenged with *T. gondii*.** We next asked whether the deficiency of IRAK4 is associated with changes in myeloid effector cell compartments. To investigate this, we assessed monocyte/macrophage subpopulations and DCs in the spleens of infected mice. Monocyte/macrophage subpopulations were defined by the expression of F4/80, which is a specific marker for monocytes/macrophages, and CD11b and GR1 (Fig. 4A and B). Moreover, all these subpopulations express

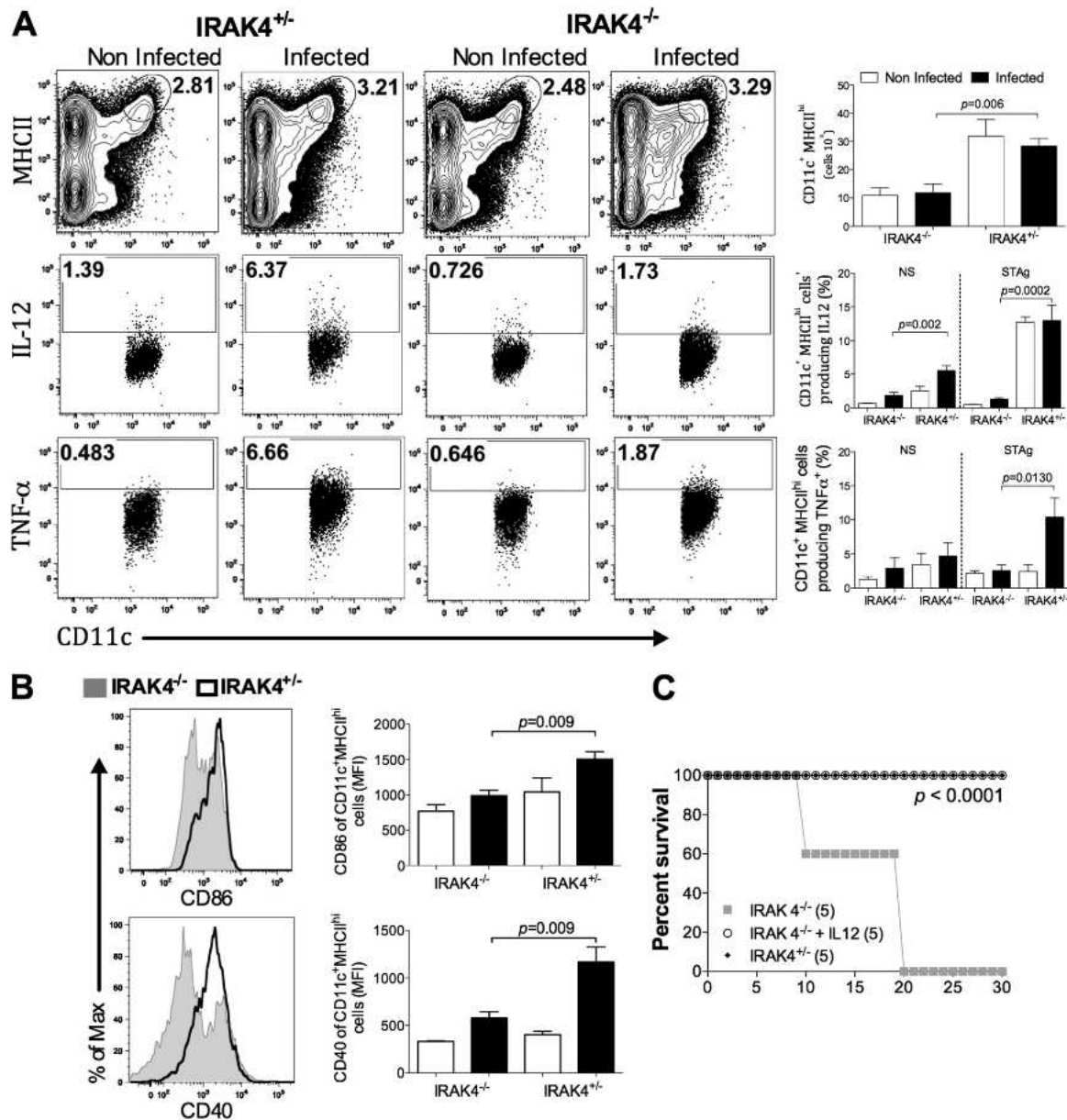


**FIG 4** IRAK4-dependent IL-12 and TNF- $\alpha$  production by macrophages from mice infected with *T. gondii*. Macrophages of IRAK4<sup>+/-</sup> and IRAK4<sup>-/-</sup> mice were analyzed at 7 days after infection with *T. gondii*. Representative dot plots of the frequency (A) and bar graphs (C, top graphs) showing the average number of different subsets of myeloid cells in spleens from IRAK4<sup>+/-</sup> and IRAK4<sup>-/-</sup> mice, based on the levels of F4/80, CD11b, and Gr1 expression (B). The percentages of CD11b<sup>hi</sup> F4/80<sup>lo</sup> Gr1<sup>hi</sup>, CD11b<sup>lo</sup> F4/80<sup>hi</sup> Gr1<sup>int</sup>, and CD11b<sup>int</sup> F4/80<sup>int</sup> Gr1<sup>lo</sup> cells producing IL-12 and TNF- $\alpha$  are shown in panel C (middle and bottom graphs, respectively). The results of uninfected and infected mice are represented by white and black bars, respectively. The presented results are an average of data from four mice per group, and the *P* values were calculated for a 95% confidence interval. The experiments were repeated three times with similar results. NS, not stimulated.

major histocompatibility complex (MHC) class II, which is not expressed by neutrophils. A very small population (less than 1%) of neutrophils (F4/80<sup>-</sup> CD11b<sup>+</sup> Gr1<sup>+</sup> MHCII<sup>-</sup>) is seen in spleens from mice infected with *T. gondii*. This was also confirmed by histological analysis of the spleens from heterozygote and IRAK4 knockout mice (data not shown). Notably, IRAK4<sup>-/-</sup> mice displayed a lower frequency of F4/80<sup>+</sup> cells than the IRAK4<sup>+/-</sup> mice. The absolute number of all three different macrophage/monocyte subsets (F4/80<sup>hi</sup> CD11b<sup>lo</sup> Gr1<sup>lo</sup>, F4/80<sup>int</sup> CD11b<sup>int</sup> Gr1<sup>lo</sup>, and F4/80<sup>lo</sup> CD11b<sup>hi</sup> Gr1<sup>hi</sup> cells, where the superscript “hi,” “lo,” and “int” indicate high, low, and intermediate levels of expression, respectively) were decreased in the IRAK4<sup>-/-</sup>

mice compared with the IRAK4<sup>+/-</sup> mice infected with *T. gondii* (Fig. 4C). However, the frequency of monocytes/macrophages producing IL-12 or TNF- $\alpha$  when stimulated with STAg was higher in IRAK4<sup>+/-</sup> mice than in the IRAK4<sup>-/-</sup> animals (Fig. 4C). Among the macrophages/monocytes, the F4/80<sup>int</sup> CD11b<sup>int</sup> Gr1<sup>lo</sup> cells were the main source of IL-12, followed by the F4/80<sup>hi</sup> CD11b<sup>lo</sup> Gr1<sup>hi</sup> subset, whereas the production of TNF- $\alpha$  was induced in only the F4/80<sup>int</sup> CD11b<sup>int</sup> Gr1<sup>lo</sup> subpopulation in the IRAK4<sup>+/-</sup> mice compared to uninfected controls (Fig. 4C).

Higher absolute numbers of CD11c<sup>+</sup> MHCII<sup>hi</sup> cells were found in IRAK4<sup>+/-</sup> mice than in IRAK4<sup>-/-</sup> mice. Upon *T. gondii* infection, IRAK4<sup>-/-</sup> mice displayed a lower frequency of IL-12-

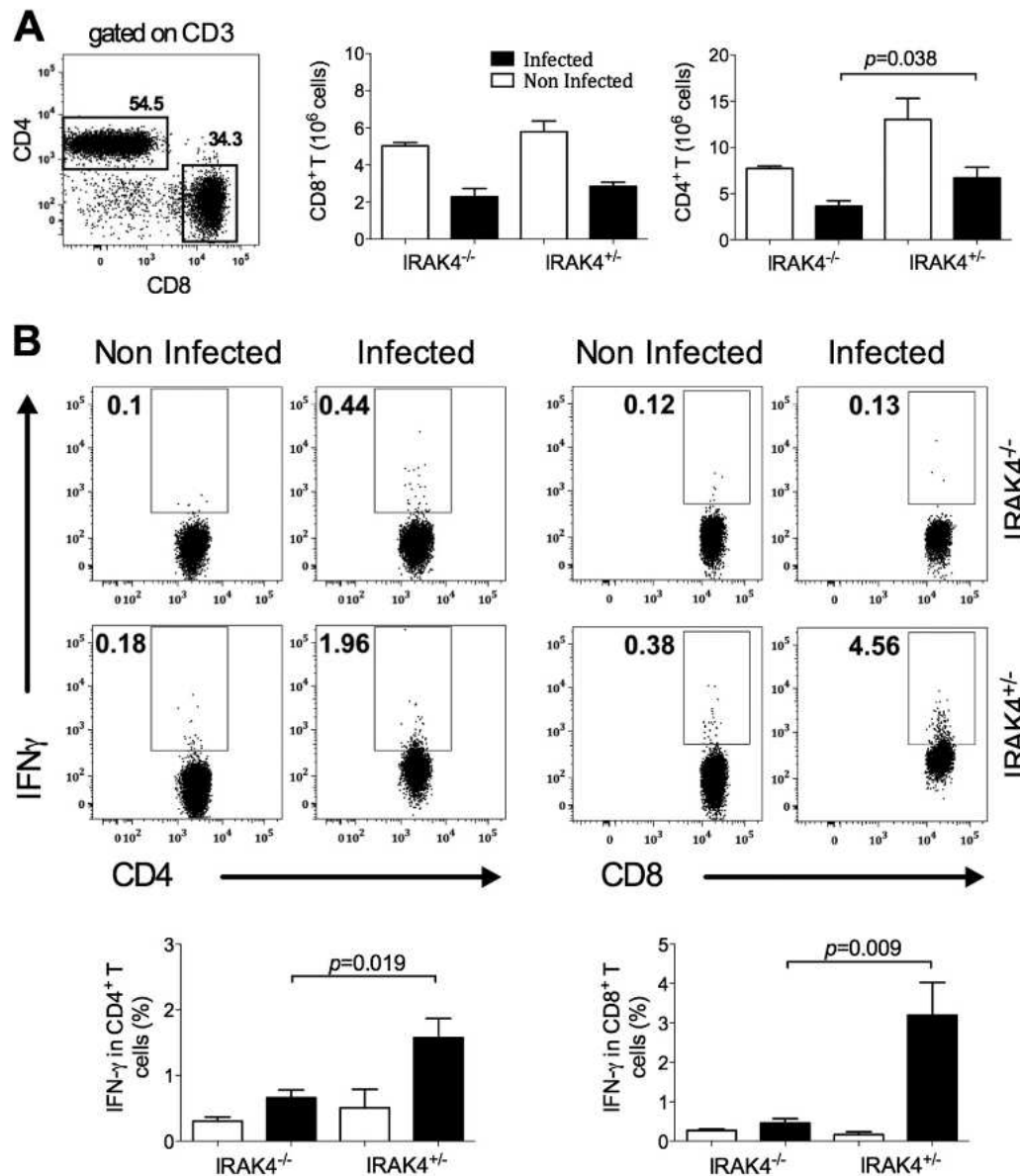


**FIG 5** IRAK4-dependent production of IL-12 by dendritic cells from mice infected with *T. gondii*. Dendritic cells (DCs) were analyzed 7 days after infection with *T. gondii*. (A) Representative dot plots (top graphs) show the frequency of DCs (CD11<sup>+</sup>, MHC-II<sup>hi</sup>) in spleens from IRAK4<sup>+/-</sup> and IRAK4<sup>-/-</sup> mice. Representative dot plots of the frequency of DCs producing IL-12 or TNF- $\alpha$  are shown in the middle and bottom graphs. Bar graphs showing the average number of DCs (CD11<sup>+</sup>, MHC-II<sup>hi</sup>) (top right graph) and the frequency of DCs producing IL-12 (middle right graph) or TNF- $\alpha$  (bottom-right graph) in spleens from IRAK4<sup>+/-</sup> and IRAK4<sup>-/-</sup> mice. (B) The expression (median fluorescence intensity [MFI]) of activation of the markers CD86 and CD40. (C) Survival curve of the deficient mice treated with recombinant IL-12 or not treated during 7 days of *T. gondii* infection. The results for uninfected and infected mice are represented by the white and black bars, respectively. The presented results are an average of data from four mice per group, and the *P* values were calculated for a 95% confidence interval. The experiments were repeated three (A and B) or two (C) times with similar results.

producing DCs than IRAK4<sup>+/-</sup> mice even in the absence of STAg stimulation. Production of both IL-12 and TNF- $\alpha$  by DCs was enhanced upon STAg stimulation (Fig. 5A). In addition, when the expression of CD86 and CD40 was assessed, the IRAK4-deficient mice showed lower levels of both activation markers on DCs than heterozygote mice (Fig. 5B). Hence, our results indicate a failure of activation of the DCs from IRAK4<sup>-/-</sup> mice infected with *T. gondii* (Fig. 5B). To evaluate the importance of the impairment of IL-12 production by DCs, IRAK4<sup>-/-</sup> mice infected with *T. gondii*

were treated daily beginning at day 1 postinfection. As expected, IRAK4<sup>-/-</sup> mice treated with rIL-12 became resistant to infection (Fig. 5C), and all mice survived over 30 days after challenge with the ME49 strain of *T. gondii*.

**Impaired Th1-mediated immune responses in IRAK4-deficient mice infected with *T. gondii*.** Since the activation of antigen-presenting cells was diminished in IRAK4-deficient mice, we next asked whether the T cell responses were also impaired. Our results showed that the total number of CD4<sup>+</sup>, but not



**FIG 6** Impairment of the cell number and IFN- $\gamma$ -producing T cells in IRAK4-deficient mice. (A) Representative dot plot on the left shows the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, within the CD3<sup>+</sup> lymphocytes. Bar graphs on the right show the absolute number of CD4<sup>+</sup> T as well as CD8<sup>+</sup> T lymphocytes from IRAK4<sup>+/-</sup> and IRAK4<sup>-/-</sup> mice infected or not infected with *T. gondii*. (B) Dot plots illustrating the IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes stimulated with STAg from IRAK4<sup>-/-</sup> mice (lower panels) compared to IRAK4<sup>+/-</sup> mice (upper panels) infected with *T. gondii*. Bar graphs show the frequency of IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes cultured with STAg. The presented results are an average of data from four mice per group, and the *P* values were calculated for a 95% confidence interval. The experiments were repeated three times with similar results.

CD8<sup>+</sup> T lymphocytes was significantly lower in the knockout mice than in the IRAK4<sup>+/-</sup> mice (Fig. 6A). Importantly, the frequencies of IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were decreased in IRAK4<sup>-/-</sup> mice compared to IRAK4<sup>+/-</sup> mice upon STAg stimulation (Fig. 6B). Altogether, our results indicate that the decreased production of IL-12 by macrophages and DCs leads to a consequent impairment in the IFN- $\gamma$  production by T cells, and it is likely to be the primary mechanism responsible for enhanced susceptibility of IRAK4<sup>-/-</sup> mice to *T. gondii* infection.

**Human congenital toxoplasmosis associates with IRAK4 SNPs.** In humans, congenital toxoplasmosis is a serious disease

which threatens sight, cognition, and life. It was therefore of interest to study whether there is an association of IRAK4 with congenital toxoplasmosis. Seven tag-SNPs in the *IRAK4* gene were studied. The 7 SNPs were rs4251545, rs1057190, rs4251580, rs4251513, rs1461567, rs4251520, and rs17121283. The frequency of the SNP variants were tested in the North American cohort of patient parent trios for 124 congenitally infected persons. We used an *r*<sup>2</sup> threshold of 0.8. SNPs with a call rate of >90% and in Hardy-Weinberg equilibrium (HWE) in parents were kept for further analysis. Among the tested SNPs, rs1461567 and rs4251513 were associated with congenital toxoplasmosis (*P* < 0.023 and *P* < 0.045, respectively) (Fig. 7). An etiological variant of SNPs in



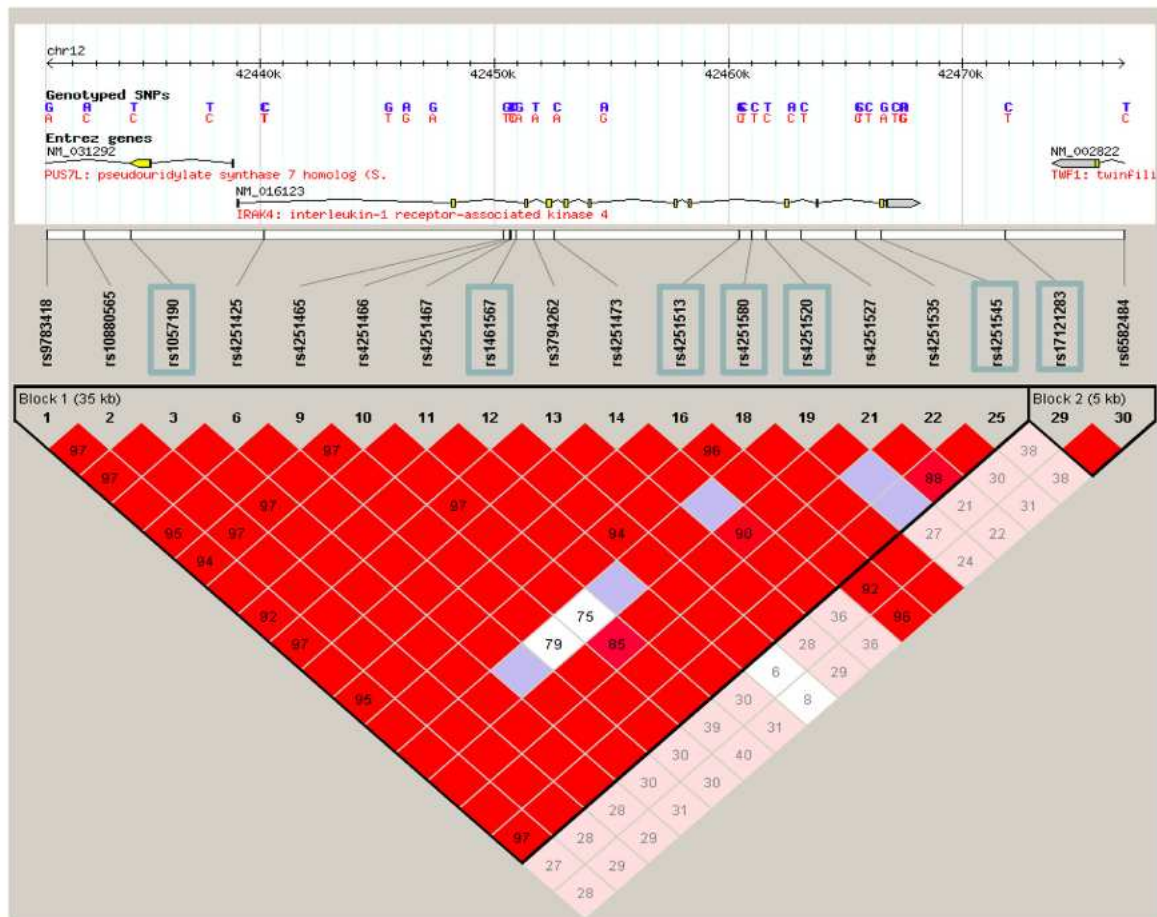


FIG 7 Association of SNPs in the *IRAK4* gene with human congenital toxoplasmosis. The upper diagram shows the positions of genotyped SNPs relative to the intron/exon structure of the gene. The lower diagram shows the LD plots generated in Haploview using *IRAK4* gene data from our North American patient cohort. LD values ( $D' \times 100$ ) between markers are indicated at the intersection of the 2 markers on the matrix. Where there is no value,  $D' = 1$  (i.e., 100). In outlining gene SNP association with susceptibility to congenital toxoplasmosis,  $D'$  values between loci were calculated and displayed using Haploview. When there is high confidence in the value of  $D'$  (logarithm of the odds score,  $>2$ ), pink and red are used. If the confidence is lower (logarithm of the odds score,  $<2$ ), blue shading ( $D' = 1$ ) or no shading ( $D' < 1$ ) is used.

strong LD with these markers could account for observed associations with susceptibility to this congenital disease.

## DISCUSSION

The key features of infection with *T. gondii* are the strong activation of innate immunity and the development of a solid antigen-specific T cell-mediated immune response (10). Among the innate immune receptors, TLRs are prominent in the recognition of protozoan molecular patterns (14). The activation of all TLRs (except TLR3) sequentially triggers the adaptor molecule MyD88, the downstream kinase IRAK4, and the transcription factor NF- $\kappa$ B, culminating in the production of proinflammatory cytokines (46) that are essential for the development of acquired immunity and host resistance to *T. gondii*. Hence, the strongest data suggesting that TLRs are important in host resistance to toxoplasmosis are those obtained in studies demonstrating that MyD88<sup>-/-</sup> mice have an impaired production of IL-12 and IFN- $\gamma$ , enhanced parasitism, and accelerated mortality (7, 9, 31, 43, 45).

Similar to the MyD88<sup>-/-</sup> mice, IRAK4-deficient mice had a dramatic increase of parasitism in the spleen, liver, and brain, as well as accelerated mortality, that was associated with impaired production

of the proinflammatory cytokines IL-12, TNF- $\alpha$ , and IFN- $\gamma$ . IRAK4 also mediates the function of the IL-1 and IL-18 receptors. However, the *IL-1R* and the *IL-18* knockout mice are not more susceptible to *T. gondii* infection (19). Thus, the most likely explanation for the impaired response of IRAK4<sup>-/-</sup> macrophages is the disrupted signaling triggered by TLRs exposed to *T. gondii* components. Indeed, a defective release of nitrogen radical intermediates by IRAK4<sup>-/-</sup> macrophages exposed to *T. gondii*-derived heat shock protein 70 (HSP70), which is a TLR4 agonist, has been reported (34). In addition, studies performed in our laboratory have shown that glycosylphosphatidylinositol anchors extracted from *T. gondii* tachyzoites activate TLR2 and TLR4 (9). However, deficiencies in TLR2 and TLR4 had no major impact on mouse resistance to *T. gondii* infection (9). On the other hand, TLR11, the counterpart receptor for a profilin-like protein from *T. gondii*, was shown to mediate IL-12 production by DCs, and its deficiency results in enhanced susceptibility to infection (41, 55). TLR9 was also shown to mediate inflammation in peroral infection with *T. gondii* (33). Notably, we reported that 3d mice are extremely susceptible to infection with *T. gondii* (31). The 3d mice have a missense allele in the *UNC93B1* gene, which results in nonfunctional TLR3, TLR7, TLR9, and TLR11 (6, 50). Thus, our results indicate that

in addition of TLR11, the nucleic acid-sensing TLRs have a fundamental role in host resistance to *Toxoplasma* infection.

The activation of DCs through TLRs not only induces cytokine secretion but also enhances their capacity to prime naïve pathogen-specific T cells, providing signals (costimulatory molecules) that are important for the development of Th1 cells during protozoan infection (14, 51). It has been consistently shown that, as opposed to DCs from infected heterozygote mice, the expression of costimulatory molecules (i.e., CD86 and CD40) as well as IL-12 was not augmented in DCs from IRAK4<sup>-/-</sup> mice. Our *in vivo* results contrast with the ones obtained *in vitro* by Aosai and colleagues (2), who reported that DC maturation induced by *T. gondii*-derived Hsp70 was mediated by TLR4 but independent of the MyD88 pathway. The defective DCs in infected IRAK4<sup>-/-</sup> mice are of special interest, because impaired IL-12 production results in diminished IFN- $\gamma$  production by T lymphocytes and enhanced susceptibility to *T. gondii* (16, 25, 26, 43, 48, 54). Indeed, treatment with rIL-12 rescued resistance of IRAK4-deficient mice to experimental infection with *T. gondii*.

It was also noticeable that the frequency of monocytes/macrophages (CD11b<sup>hi</sup> F4/80<sup>lo</sup> GR1<sup>hi</sup>) that is increased in control mice infected with *T. gondii* was not observed in the spleens from IRAK4<sup>-/-</sup> mice. In addition to contributing as a source of IL-12, inflammatory monocytes are essential as effector cells for controlling parasite replication *in vivo* (12). The decreased frequencies of monocytes/macrophages observed in IRAK4<sup>-/-</sup> mice infected with *T. gondii* can be explained based on the impaired production of MCP-1 (CCL2) by splenocytes from IRAK4-deficient mice, as this chemokine is of critical importance for the recruitment of inflammatory monocytes (1, 11).

IRAK4 deficiency is also associated with an altered function of T and B lymphocytes (21, 39). As previously reported, upon infection with *T. gondii*, the total number of T cell subsets was decreased (3, 17, 24). This was observed in both heterozygote and IRAK4<sup>-/-</sup> mice. Nevertheless, a major defect was observed in terms of IFN- $\gamma$  production by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells from IRAK4-deficient mice. A likely explanation for these findings is the impaired expression of costimulatory molecules and IL-12 by dendritic cells from IRAK4<sup>-/-</sup> mice, which culminates in less-effective priming and differentiation of CD4<sup>+</sup> T lymphocytes. We also found a major defect in the activation and expansion of B lymphocytes. To begin with, both the total areas of the follicles and the sizes of germinal centers were dramatically decreased in the spleens of infected IRAK4<sup>-/-</sup> mice. This was associated with a significant decrease in the number of activated B cells expressing CD86 as well as CD69. TLR activation, either directly on B lymphocytes (20) or on follicular dendritic cells (13), is critical for the activity of germinal centers. This defective activation and expansion of B lymphocytes can be also explained by the impaired CD4<sup>+</sup> T cell responses observed in the IRAK4-deficient mice.

Importantly, the expression of nonfunctional IRAK4 in humans results in recurrent life-threatening infections (5, 30). Mutations found in the *IRAK4* gene impair the innate and acquired immune responses to microbial TLR ligands and various pathogens of clinical relevance (8, 21, 39). Recent studies have associated the presence of clinical outcomes of congenital and/or acquired toxoplasmosis, in particular the development of retinochoroiditis, with genes related to the innate immunity. Thus, we also investigated whether polymorphisms in the *IRAK4* gene would associate with the sequelae of congenital toxoplasmosis in humans. In fact, we found evidence of the

association of two tag-SNPs (i.e., rs1461567 and rs4251513) distributed in the *IRAK4* gene with brain and ocular alterations in congenital toxoplasmosis. Taken together, the associations of SNPs in *TLR9* with retinochoroiditis (37) and in *IRAK4* with congenital toxoplasmosis support the hypothesis that TLRs are critical in mediating resistance to *T. gondii* infection in humans.

In conclusion, innate immunity plays a major role in host resistance to infection with *T. gondii* in naïve hosts. This is highly relevant to congenital toxoplasmosis, which is mainly transmitted from nonimmune pregnant women to the fetus during primary infection. The results presented here indicate that IRAK4 plays critical roles in the activation and function of splenic dendritic cells and macrophages. As a consequence, it is essential for the development of an acquired immunity; thus, it is an important determinant for host resistance to primary infection with *T. gondii* and the clinical outcome of congenital disease in humans.

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