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TLR9 Is Required for the Gut-Associated Lymphoid Tissue Response following Oral Infection of *Toxoplasma gondii*

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TLRs expressed by a variety of cells, including epithelial cells, B cells, and dendritic cells, are important initiators of the immune response following stimulation with various microbial products. Several of the TLRs require the adaptor protein, MyD88, which is an important mediator for the immune response following *Toxoplasma gondii* infection. Previously, TLR9-mediated innate immune responses were predominantly associated with ligation of unmethylated bacterial CpG DNA. In this study, we show that TLR9 is required for the Th1-type inflammatory response that ensues following oral infection with *T. gondii*. After oral infection with *T. gondii*, susceptible wild-type (WT; C57BL/6) but not TLR9−/− (B6 background) mice develop a Th1-dependent acute lethal ileitis; TLR9−/− mice have higher parasite burdens than control WT mice, consistent with depressed IFN-γ-dependent parasite killing. A reduction in the total T cell and IFN-γ-producing T cell frequencies was observed in the lamina propria of the TLR9−/− parasite-infected mice. TLR9 and type I IFN production was observed by cells from infected intestines in WT mice. TLR9 expression by dendritic cell populations is essential for their expansion in the mesenteric lymph nodes of infected mice. Infection of chimeric mice deleted of TLR9 in either the hemopoietic or nonhemopoietic compartments demonstrated that TLR9 expression by cells from both compartments is important for efficient T cell responses to oral infection. These observations demonstrate that TLR9 mediates the innate response to oral parasite infection and is involved in the development of an effective Th1-type immune response. *The Journal of Immunology*, 2006, 176: 7589–7597.  

Immunology provides the first line of defense against invading pathogens and is essential for survival in the absence of adaptive immune responses. Innate immune recognition relies on a limited number of germline-encoded receptors, such as TLRs, that recognize pathogen-associated molecular patterns of microbial origin (1–6). To date, 13 members of the TLR family have been characterized in mice. Although TLRs are expressed in a broad range of tissues, the greatest variety of TLR mRNAs is found in professional APCs. A key role of TLRs in innate immunity is the development of the acquired immune response characterized by polarization of naïve CD4+ helper T cells toward the Th type 1 (Th1) or Th2 phenotype.  

The classically defined ligand for TLR9, bacterial unmethylated CpG DNA, has an immunomodulatory effect at the cellular level, and TLR9−/− mice are unresponsive to immunostimulatory CpG DNA (7–9). Human TLR9-conferred responsiveness to CpG-DNA occurs via the recognition of species-specific CpG motifs (10). The engagement of CpG DNA with TLR9 results in the activation of a wide range of transcription factors (i.e., NF-κB, AP-1, or IFN regulatory factor-7) by MyD88-dependent signaling pathways that ultimately leads to the expression of Th1 proinflammatory cytokines such as IL-12 and type I IFNs (11, 12). Whereas TLR1, TLR2, and TLR4 are cell surface expressed, current opinion is that TLR9 has a predominantly intracellular localization in macrophages and dendritic cells (DC), an advantageous position for the recognition of intracellular pathogens (13).  

Recent observations indicate that DNA is not the only ligand for TLR9. *Plasmodium falciparum* blood stage schizonts, soluble schizont extracts, and, most recently, hemozoin pigment activate plasmacytoid DCs and use the TLR9-MyD88 signaling pathway, indicating that TLR9 plays an essential role in recognizing Apicomplexa infections (14, 15).  

Oral infection with *Toxoplasma gondii* in certain strains of mice (C57BL/6) leads to an acute lethal ileitis within 7 days of infection. This experimental model of inflammatory bowel disease is similar to human ileitis with regard to disease localization, histological findings, and immunologic imbalance (16). The death is not related to an uncontrolled parasite replication but is due to an overwhelming Th1-like immune response. CD4+ T cells from the lamina propria secrete huge quantities of IFN-γ and TNF-α that are responsible for the inflammatory process (17). Mice genetically incapable of producing IFN-γ do not develop the lethal ileitis despite a high parasite replication. Indeed the Th1-like immune response, although deleterious if uncontrolled, is necessary to limit parasite replication.  

Defective APC responses were observed in MyD88−/− macrophages, neutrophils, and splenic DCs exposed to soluble *T. gondii* Ag, indicating the essential role of a MyD88-dependent TLR in responding to *T. gondii* infection (18). Additional reports show intact APC responses in TLR2- and TLR4-deficient mice, suggesting that neither of these MyD88-dependent TLRs are involved in systemic immune responses to *T. gondii* infection (19). Studies...  

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Abbreviations used in this paper: DC, dendritic cell; KO, knockout; WT, wild type; BM, bone marrow; IEC, intestinal epithelial cell; MLN, mesenteric lymph node; LPL, lamina propria lymphocyte; GALT, gut-associated lymphoid tissue; int, intermediate.
with TLR11 knockout (KO) mice demonstrate that after systemic infection with *T. gondii*, these mice have a persistently elevated serum titer of both IL-12 and IFN-γ, implying that other TLR pathways are involved in the host innate response to this parasite (20). Taken together, these observations indicate that *T. gondii* infection triggers an immune response that signals through a MyD88-dependent TLR, which may work independently or in concert with TLR11. In this study, we show for the first time that TLR9 expression by APCs from the hemopoietic and nonhemopoietic compartments is essential for initiating the innate immune response following oral infection with *T. gondii* and that TLR9 is required for an efficient T cell response in the small intestine.

**Materials and Methods**

**Mice and parasites**

Female 6- to 8-week-old C57BL/6 mice (wild-type; WT) obtained from The Jackson Laboratory and TLR9/−/− mice backcrossed onto a C57BL/6 background (provided by S. Akira, Department of Host Defense, Osaka University, Osaka, Japan; Ref. 9) were bred and housed under approved conditions at the Animal Research Facility at Dartmouth Medical School or at the Institut Pasteur (Paris, France). Bone marrow (BM) chimeric mice were used 7 wk after reconstitution. Mice were infected orally by intragastric gavage with 35 cysts of the 76K strain maintained through passage in CBA/J mice. After infection, mice were weighed and mortality was recorded daily. All experiments were performed with 4–6 mice/group and were repeated twice. Dithioerythritol was then added to the cells (1.6 mg/10 ml). Purification of lamina propria lymphocytes (LPL)

LPLs were purified as described previously (23). Briefly, fat and Peyer’s patches were removed from small intestines with increased blood flow and mild edema; 3, diffuse and massive infiltrating cells leading to disturbed mucosal architecture; 4, crypt abscess and superficial necrosis of the intestinal villi. 

**Intestine pieces**

Intestines were immediately fixed in 10% formalin overnight, embedded in paraffin, and sectioned. Sections were stained with H&E and photographed.

Histological inflammatory score from 0 to 4 was applied in a blinded fashion as described previously (21): 0, no inflammation; 1, slight infiltrating lymphocytes in the lamina propria with focal acute infiltration; 2, mild infiltrating cells in the lamina propria with increased blood flow and mild edema; 3, diffuse and massive infiltrating cells leading to disturbed mucosal architecture; 4, crypt abscess and superficial necrosis of the intestinal villi.

**Purification of intestinal epithelial cells (IEC)**

Small intestines were washed with PBS, and Peyer’s patches were removed. Intestines were opened longitudinally and cut into 1-cm-long samples. The pieces were incubated in PBS (Ca/Mg free)-EDTA 3 mM under agitation (210 rpm) and the supernatants containing the IECs were collected and washed in RPMI 1640 with 5% FBS. This process was repeated twice. Dithioerythritol was then added to the cells (1.6 mg/10 ml). After incubation (15 min, 37°C), cells were washed twice. Purified IECs were collected at the interface of a Percoll gradient of 60–30% (30 min, 2000 rpm) and washed with RPMI 1640 before use.

**Mesenteric lymph node (MLN) DC suspension**

DC suspensions from mice were performed as described previously (22). Briefly, MLNs and spleens were collected from mice, excess fat removed, and digested in 1.67 Wunsch U/ml Liberase Cl (Roche, Boehringer Mannheim) and 0.2 mg/ml DNasel (Sigma-Aldrich) in RPMI 1640 to form a single-cell suspension. Cells were then resuspended in RPMI 1640 10% FBS for further analysis.

**Purification of lamina propria lymphocytes (LPL)**

LPLs were purified as described previously (23). Briefly, fat and Peyer’s patches were removed from small intestines that were washed twice with PBS. Small intestines were opened longitudinally, cut into 1-cm pieces, and washed twice in 3 mM EDTA in Ca/Mg-free PBS for 10 min at 37°C. Intestine pieces were then washed twice in 1 mM EGTA, 1.5 mM MgCl<sub>2</sub> in RPMI 1640 1% FBS for 10 min at 37°C. Intestine pieces were then digested in Liberase (Roche) 0.14 Wunsch U/ml and DNase I (Sigma-Aldrich) at 5 U/ml in RPMI 1640 at 37°C for up to 1 h. Cell suspensions were washed twice in RPMI 1640 10% FBS then laid over Histopaque (density = 1.077) and centrifuged. Cells in the interphase were collected, washed, and used for further essays.

**Cell surface fluorescent-activated cell analysis**

Single-cell suspensions of typically 1×10<sup>6</sup> cells were stained using conventional methods in PBS 2% FBS with Fc Block (BD Pharmingen) using the following Abs: CD3FITC, CD3APC, CD4FITC, CD4APC, CD85RBPE, CD8βPE, CD8αPerCP, CD11cFITC, CD11cAPC, Gr-1PE, MHC class II PE, CD11bPE, B220APC, CD80FITC, CD86FITC, the appropriate isotype controls (BD Pharmingen), and mPDCA (Miltenyi Biotech); cells were analyzed for four-color staining on a BD FACSCalibur (BD Biosciences). Gating for analysis was determined using the appropriate isotype control.

**Intracellular cytokine staining**

Single-cell suspensions from the LPL or MLN were restimulated ex vivo with 50 ng/ml PMA (Sigma-Aldrich) and 300 ng/ml ionomycin (Sigma-Aldrich) for 2 h in RPMI 1640 10% FBS at 37°C followed by treatment with 10 μg/ml Brefeldin A (Sigma-Aldrich) for 2 h at 37°C. Following surface marker staining using conventional methods, cells were permeabilized using the BD Cytofix/Cytoperm kit (BD Pharmingen) and stained intracellularly with IFN-γ allophycocyanin or IFN-γPE (BD Pharmingen).

**Two-step SYBR green quantitative real-time PCR**

A total of 0.5 μg to 2.0 μg (within each experiment, the same quantity of mRNA was used) of QIAgen RNaseasy-purified (Qiagen) mRNA was reverse transcribed using SuperScript II RT (Invitrogen Life Technologies). A total of 200 ng of cDNA was amplified using the SYBR green Core reagents or the × 2 SYBR green mix (Applied Biosystems) on a Bio-Rad Cycler. Relative expression was normalized to β-actin and was expressed using the ΔC<sub>m</sub> method, where relative expression = 2<sup>−(exp−actin)</sup>×1000. The following primers were used: β-actin, forward AGAGGAAAATCCTGGCGTGCAC and reverse CAATAGTGAGACTGCCGT (27). For *T. gondii* B1 gene was amplified from 1 μg of total genomic DNA prepared using the DNAasy kit (Qiagen), and a standard curve for parasite equivalents was generated using a plasmid as described previously: QB1, forward CATCTTTCTAAAATTCTGGTGACCA and reverse TGGGAGTAGACAGCTACACC (26); IFN-γ, forward TCAAGTGTGACTAGTTGGAAAG and reverse TGTCCTGGCAGGATTCTCTAG (24); TNF-α, forward CATCTTTCTAAAATTCTGGTGACCA and reverse TGGGAGTAGACAGCTACACC (26). For *T. gondii* parasite burden, the B1 gene was amplified from 1 μg of total genomic DNA prepared using the DNAasy kit (Qiagen), and a standard curve for parasite equivalents was generated using a plasmid as described previously: QB1, forward GGAAGTGCAATGTCCAGTGA and reverse TCTTTAAGGCTTCATGTC (27).

**Statistical analyses**

Groups were compared using Prism Statistical Software’s unpaired t test; significance was expressed as *p* < 0.05 (∗), *p* < 0.01 (∗∗), or *p* < 0.001 (∗∗∗).

**Results**

**TLR9<sup>−/−</sup> mice are resistant to the ileitis following oral infection with *T. gondii***

We have reported previously that C57BL/6 mice die within 10 days following oral infection with *T. gondii*. These mice die of an acute ileitis that is associated with the complete, transmural destruction of the epithelial barrier. The entire ileum of the infected mice is disrupted with sparing of the colon. Microscopically, inflammation associated with large numbers of infiltrating cells and hemorrhages into the small intestine is observed. Histological examination of the small intestine from TLR9<sup>−/−</sup>-infected mice revealed the absence of inflammatory signs and showed intact integrity of the epithelial barrier similar to that of uninfected control mice (Fig. 1A). Histological scorings of ileum sections from infected mice revealed a significant (*p* < 0.001) reduction in inflammation (Fig. 1B) as compared with the WT mice. Additionally, TLR9<sup>−/−</sup> mice had a significantly lower weight loss at day 8 (*p* < 0.001) and 100% survival by day 15 postinfection compared with the WT-infected controls that had rapid weight loss and succumbed to the inflammation brought on by the infection by day 9.
TLR9−/− mice failed to develop ileitis through the completion of these studies, indicating a reduced capacity to mount an inflammatory response following infection (Fig. 1C). To confirm that the absence of inflammation in TLR9−/− mice was not due to decreased infectivity of the mice, parasite burden was assessed using real-time quantitative PCR. TLR9−/− mice had higher parasite burden in the intestine and spleen compared with WT controls at day 3 postinfection (Fig. 1D) that continued until the WT controls succumbed to the inflammation (data not shown).

TLR9−/− mice have reduced T cell responses in the lamina propria and impaired Th1 cytokine responses in the small intestine

To characterize the immune response in the small intestines of TLR9−/− mice following oral infection with T. gondii, cytokine mRNA levels were compared using quantitative real-time PCR. TNF-α levels appeared reduced at days 3 and 7 postinfection; however, the reduction was not significant (Fig. 2A). There was a significant difference in IFN-γ mRNA expression, the hallmark of the Th1-like immune response at day 7 postinfection (p < 0.01) between the WT and TLR9−/− mice (Fig. 2B).

To determine whether T cell responses were altered as a result of the TLR9 deletion following oral infection with T. gondii, the frequency of CD3+ CD4+ and CD3+ CD8+ T cells from the lamina propria was measured by FACS analysis. As expected, by day 7 postinfection there was a significant increase of CD3+ CD4+ T cells in the lamina propria of both WT and TLR9−/− mice compared with days 0 and 3 (Fig. 2C). Although there was an overall increase in CD4+ T cells in the lamina propria, TLR9−/− mice exhibited a reduced frequency of CD3+ CD4+ T cells compared with the WT controls at day 7 (p < 0.001). Moreover, there was an overall decrease in the percentage of IFN-γ-producing T cells in the lamina propria by both the CD8+ population as well as CD4+ by day 7 postinfection (Fig. 2D). There was no demonstrable change in either IL-4 or IL-10 expression (both by intracellular FACS analysis of cells from the lamina propria and by quantitative real-time PCR of infected small intestines), confirming that the decrease in Th1 cytokine production was not associated with a Th2 shift (data not shown). Analysis of the CD4+ CD25+ CD45RBlow population in the lamina propria and the MLNs failed to demonstrate a shift in the CD45RBlow T cell population from TLR9−/− mice as compared with control mice (data not shown). These data as well as the observed decrease in total small intestine T cell population from TLR9−/− mice compared with control mice (data not shown). These data as well as the observed decrease in total small intestine T cell population from TLR9−/− mice as compared with control mice (data not shown).

TLR9 and type I IFN-β are expressed in the small intestine by IECs and by cells from the lamina propria in response to T. gondii infection

The small intestine constantly samples a variety of microbial and food Ags. TLR9 is expressed by nonprofessional APC such as

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**FIGURE 1.** TLR9−/− mice are resistant to T. gondii-induced ileitis and exhibit higher parasite burdens compared with WT controls. WT (C57BL/6) and TLR9−/− mice were orally infected with 35 cysts of T. gondii, and histological evidence of inflammation was noted at day 7 postinfection. Histological analysis of day 7 postinfection small intestines from TLR9−/− mice showed an absence of inflammation following oral infection with T. gondii (A). Histological scoring revealed a significant decrease in intestinal inflammation in TLR9−/− mice compared with WT controls (B). Following oral infection with 35 T. gondii cysts, TLR9−/− mice were resistant to T. gondii-induced weight loss compared with WT controls; 100% mortality at day 9 in WT mice compared with 100% survival at day 15 in TLR9−/− mice (C). Parasite burden as determined by quantitative real-time PCR of the T. gondii B1 gene measured at day 3 postinfection was significantly higher in TLR9−/− mice in the gut and spleen compared with WT controls (D). Data shown are one representative experiment of three independent experiments with at least 5 mice/group.
epithelial cells (28), and more specifically by the IECs (29). In addition to TLR9 mRNA levels, previous studies have shown that TLR9 engagement can be measured in terms of type I IFN expression (14, 30, 31). Exposure to type I IFNs (α or β) results in the phenotypic maturation of DCs (32). Quantitative real-time PCR was performed for IFN-β and TLR9 to determine their expression over the course of infection. At early time points, such as 12 h postinfection, elevated mRNA for TLR9 and IFN-β was observed in the small intestine. TLR9 and IFN-β mRNA appeared to peak at day 3 postinfection in this oral infection model (Fig. 3A). IECs isolated from mice at serial time points postinfection expressed a basal level of TLR9 and IFN-β before dropping between 24 h and 3 days postinfection (Fig. 3B). The peak of TLR9 and IFN-β was again observed at day 4 postinfection. A cyclic pattern in the kinetics of TLR9 expression was also observed from cells in the lamina propria (Fig. 3C). These results are consistent with successive stages of the parasite life cycle of invasion/replication/cysts, and intestines were measured at day 3 and day 7 postinfection for inflammatory cytokines.

**FIGURE 2.** Infected TLR9−/− mice have a decreased Th1 response in the small intestine and in the lamina propria. WT and TLR9−/− mice were orally infected with 35 T. gondii cysts, and intestines were measured at day 3 and day 7 postinfection for inflammatory cytokines. SYBR green quantitative real-time PCR of small intestines from infected mice showed a reduction in TNF-α mRNA and a significant decrease in IFN-γ mRNA (day 7), measured at days 3 and 7 postinfection (A and B). Lymphocytes from the lamina propria were isolated, and the frequency of CD3+CD8+ T cells and CD3+CD4+ T cells were determined by FACS analysis (lamina propria preparations from six mice with three pooled intestines stained and measured three times per independent experiment). An overall increase of CD3+CD4+ cells was observed at day 7 compared with naive controls (day 0) and day 3 postinfection (C). A significant decrease in the CD3+CD4+ population in the lamina propria of TLR9−/− mice was observed at day 7 postinfection compared with the WT controls (C). Cells purified from the lamina propria were restimulated ex vivo with PMA and ionomycin and stained intracellularly with IFN-γ. Following restimulation, TLR9−/− mice displayed a 2-fold reduction of IFN-γ-producing T cells (D), compared with WT controls. Data shown are representative of three independent experiments with 4–6 mice/group.

DC populations in the MLNs of TLR9−/− mice are reduced compared with B6 mice after infection

The decrease of the inflammatory response in TLR9−/− mice might result from a reduced capacity of DCs to help with the induction of a Th1-like immune response. Because DCs are essential for the switch of the immune response toward a Th1 or Th2 profile, they were more thoroughly examined. TLR9 is expressed by a wide variety of APCs including DCs.

Gut-associated lymphoid tissue (GALT) DCs are distinct from peripheral DCs in terms of subset representation, phenotype, and function (33–37); MLN DCs can be characterized based on their expression of CD11c and CD8α. Previous studies have shown that MLN plasmacytoid-like DC can be CD11clow, CD8α+, B220+, GR-1−, class IIlow, CD80low, and CD86low (34). Separate studies suggest that CD11c+CD8α+CD11b+ cells could also be considered plasmacytoid DC (33). MLN CD11c+CD8α− DCs can be either nonplasmacytoid or myeloid DCs (36). mPDCA is expressed on peripheral plasmacytoid DCs (38); its expression on GALT DCs had not been described previously. In humans, only plasmacytoid DCs are known to express TLR9, whereas myeloid, plasmacytoid, and conventional DC (pDC) subsets (CD11c+) express TLR9 in mice (8) (39).

Four populations of MLN DCs were analyzed, CD11clow CD8α− (CD11clow, CD8α− DCs), CD8α− CD11clow (CD8α− DCs), CD8α− CD11clow (CD8α− DCs), and CD8α− CD11clow (CD8α− DCs) (34). These subsets were phenotyped for the expression of B220, CD11b, GR-1, mPDCA, MHC class II, CD80, and CD86 (Fig. 4B). Both naive B6 and TLR9−/− mice had similar DC populations in terms of frequency (Fig. 4A) and phenotype (Fig. 4B). The
expression of B220 and GR-1, mPDCA⁺, CD11bʰ, MHC class IIʰ, CD8⁰, and CD8⁶, suggestive of pDCs. Naïve CD8αʰ DCs were B220ʰ, mostly CD11bʰ, GR-1ʰ, mPDCAʰ, MHC class IIʰ, CD8⁰, and CD8⁶, also suggestive of a pDC population.

MLNs from infected WT mice showed higher frequencies of all DC populations compared with the infected TLR9⁻/⁻ mice (Fig. 4A). Of the DCs from infected mice, the most striking increase was in the CD8αʰ population in the B6 mice; these cells were B220ʰ, CD11bʰ, GR-1ʰ, mPDCAʰ, MHC class IIʰ, suggesting an expansion or migration of a different set of DCs than were present before infection. This population differs significantly from the CD8αʰ cells from the TLR9⁻/⁻ mice that expressed B220ʰ, mPDCAʰ, and MHC class IIʰ (Fig. 4B), similar to the CD8αʰ DCs present in naïve mice. These results suggest that this subset of DCs may require TLR9 for their expansion and/or migration into the MLN of parasite-infected mice. In both WT and TLR9⁻/⁻ mice, after infection the CD11cʰ subsets expressed mPDCA and expression of costimulatory molecules CD80 and CD86 (Fig. 4B) and a modest increase in IL-12(p40/p70) expression (data not shown). Both intracellular and extracellular TLR9 were detected in B6 mice on DCs from the MLN (data not shown).

TLR9 expressed by the cells from both the hematopoietic and nonhemopoietic compartments is essential for the immune response following oral infection of *T. gondii*

TLR9 chimeric mice were generated to identify whether nonhemopoietic cells, such as epithelial cells, or the hematopoietic cells were responsible for initiating the T cell response following oral infection with *T. gondii*. Our studies focused on the CD4⁺ T cells from the lamina propria because we showed in this study as well as previously (17, 40, 41), that these CD4⁺ T cells infiltrated the lamina propria and produced copious amounts of IFN-γ following oral infection. We observed a reduced frequency of IFN-γ-producing CD4⁺ cells from the lamina propria in irradiated WT mice that were reconstituted with BM from TLR9⁻/⁻ mice (Fig. 5A) (the nonhemopoietic cells from these TLR9⁻/⁻, whereas the hematopoietic cells are TLR9⁻/⁻). These results were similar to the observed reduction of IFN-γ-producing CD4⁺ in TLR9⁻/⁻ mice (Fig. 5A). In chimeric mice where the nonhemopoietic system contained a TLR9⁻/⁻ phenotype and the hematopoietic system a WT one (irradiated TLR9⁻/⁻ mice reconstituted with BM from WT mice), there was also a reduction of IFN-γ-producing CD4⁺ cells from the lamina propria (Fig. 5A), similar to what was observed in TLR9⁻/⁻ mice. WT mice reconstituted with WT BM were also used as controls and responded similarly to the WT mice (data not shown). These results indicate that there is no endogenous defect in T cells from TLR9⁻/⁻ mice and that the TLR9-dependent Ag responses in the nonhemopoietic as well as the hematopoietic compartments are essential for the initiation of both the innate and adaptive immune response following oral infection with *T. gondii*. Histological analyses of the chimeric mouse intestines revealed a mixed phenotype in the TLR9⁻/⁻ mice reconstituted with WT BM and in the WT mice reconstituted with TLR9⁻/⁻ BM (Fig. 5B), further implicating a role for APCs from both the hemopoietic and nonhemopoietic compartments.

**Discussion**

TLRs expressed by cells of the mucosal epithelium and associated lymphoid tissue play an essential role in the defense against microbes by recognizing conserved bacterial molecules. Previous studies as well as unpublished observations in our laboratory indicate that neither TLR4 nor TLR2 participate in the innate mucosal response to this parasite, although signaling through the
MyD88 pathway is essential. We report that TLR9 expression in susceptible WT mice is critical to the initiation of innate immune response and, subsequently, the adaptive immune response to this obligate intracellular pathogen and that both the hemopoietic and nonhemopoietic compartments are involved in Ag presentation. TLR9 expression on mucosal tissues and its role in the protection against various microbes has been well characterized. It is now appreciated that TLR9 can engage a wide range of microbial infections including bacteria (42, 43), viral (44, 45), and parasitic (8, 9, 15). TLR9 has been localized to several tissues and cell types, including the gastric mucosa, tonsils (29, 46), IECs, Paneth cells in the gut mucosa, B cells, and several DC populations (2, 15, 39, 47–49).

TLR9 expression has been previously described on several cell types including epithelial cells, B cells, and professional APC. Small intestine, ex vivo-purified IECs, and cells from the lamina propria exhibit an early up-regulation of TLR9 and IFN-β (a measure of TLR9 activation) mRNA, followed by a second up-regulation by day 3 postinfection with T. gondii. Type I IFNs are thought to be important in the phenotypic maturation of DCs, where they may work in an autocrine manner (32). These data indicate that TLR9 is expressed on cells from the nonhemopoietic compartment (i.e., the Paneth cells and/or enterocytes) in response to oral T. gondii infection.

TLR9 may exert different downstream effects depending on the TLR9-ligand interaction (50–53). TLR9 was shown to respond to non-CpG DNA resulting in a Th2 type instead of a Th1 type immune response (53), further indicating that TLR9 is important in polarizing the immune system to different antigenic stimuli. These different functions of activation might contribute to microbial clearance but also could lead to an increase in autoreactive B or T cell stimulation potentially enhancing the inflammatory processes that contribute to the immunopathology of several diseases, including acute ileitis. We examined whether TLR9−/− mice displayed a Th2 profile following oral infection with T. gondii. mRNA analyses of IL-4, IL-10, and IL-13 show no significant increase in Th2 cytokines compared with WT controls (data not shown). Additional analyses of T cells using intracellular cytokine
staining confirmed mRNA analyses, adding further support that TLR9/−/− mice are deficient in inducing a Th1 response as opposed to shifting toward a Th2 response.

DC responses, in terms of IL-12p40 production from soluble T. gondii Ag, were shown to be associated with the newly identified MyD88-dependent TLR11; however, a small amount of IL-12 and IFN-γ were still present in the serum of infected TLR11/−/− mice (20), indicating that another MyD88-dependent TLR must be involved in the immune response to T. gondii. Although multiple TLRs are up-regulated in the intestine of T. gondii-infected mice, mice lacking the expression of TLR4 or TLR2 were still susceptible to the development of ileitis and died within a week of severe ileitis, (our unpublished observation), indicating a comparable immune response to orally infected WT mice. Taken together, these studies indicate that T. gondii infection triggers a MyD88-dependent TLR that may act independently or in concert with TLR11 to induce the IFN-γ response by T cells from the lamina propria.

Systemically and in the GALT, the balance between mounting an effective immune response vs a tolerogenic effect to several Ags depends largely upon the APC presenting the Ag and the T cells that respond (54). DCs help the immune system to maintain a tenuous yet crucial balance between attacking pathogens and sparing the body’s own tissue through educating T cells as to the appropriate targets. Different GALT DC subsets may accomplish these different functions (54, 55). GALT DCs are distinct from peripheral DCs; our data indicate that in the MLN, CD11clow of variable CD8 expression might be plasmacytoid-like DCs. Although an increase in the CD11clow populations was observed after infection, the most pronounced increase was present in the CD8αint population. The increase of CD8αint cells and also the up-regulation of costimulatory molecules CD80 and CD86 provides sufficient evidence to suggest that these DCs are important for initiating the inflammation in B6 mice after oral T. gondii infection. Our data is consistent with previous studies using an oral cholera toxin model of inflammation; in this model, CD8αint DCs both accumulate in the MLNs following cholera toxin treatment and are immunostimulatory to naive CD4+ T cells (33).

CD4 T cells (CD45RBhigh, CD25−), isolated from the lamina propria, were identified as a major player in the inflammatory process (41). Following infection, CD4+ T cells migrate into the lamina propria, where they act as critical effector cells secreting copious amounts of Th1-type cytokines such as IFN-γ, which we and others have identified as being critical in the development of the mucosal inflammatory process (16, 17). By day 7 postinfection, we observed a modest reduction in the overall numbers of CD3+ T cells (both the CD8+ and CD4+) in TLR9−/− mice compared with WT controls. Quantification of these lamina propria T cells shows that T cells of a regulatory phenotype (CD4+CD25+CD45RBlow) are not involved in the lack of inflammation observed in TLR9−/− mice (data not shown). TLR9−/− mice display decreased IFN-γ levels both in the small intestine and by the CD4+ T cells isolated from the lamina propria of TLR9−/− mice compared with WT controls. The reduction of IFN-γ-producing T cells in the lamina propria following oral infection does not appear to be
due to an endogenous defect in the ability of TLR9<sup>−/−</sup> T cells to mount an IFN-γ response because TLR9<sup>−/−</sup>-irradiated mice reconstituted with WT BM also have reduced IFN-γ production.

mRNA analysis of purified B cell populations from the GALT reveals that TLR9 expression is not dramatically affected in this compartment during infection (data not shown). Double chimeric mice (WT B6 mice reconstituted with BM cells from B cell KO mice (50%) and from TLR9 KO mice (50%)) display no reduction in IFN-γ expression by the B cell population.

In summary, this study identifies TLR9 as a critical receptor for the parasite and essential for the development of an efficient T cell response in the small intestine.

Disclosures
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References


