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Innate responses to *Toxoplasma gondii* in mice and humans

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Abstract

Primary infection with *Toxoplasma gondii* stimulates production of high levels of IL-12 and IFN-γ by cells of the innate immune system. These two cytokines are central to resistance to *T. gondii*. Signaling through the Toll-like receptor (TLR) adaptor protein MyD88 is indispensible for activating early innate immune responses. Recent studies have established that TLR11 plays a dominant role in sensing *T. gondii*. At the same time, TLR11 is represented in humans only by a pseudogene, and the major question of how innate and adaptive immune responses occur in the absence of TLR11 remains unanswered. In this article similarities and differences in sensors and effector molecules that determine host resistance to the parasite in humans and mice are discussed.

MyD88-IL-12-IFN-γ axis in host defense against *Toxoplasma gondii*

Toxoplasma gondii is an intracellular protozoan parasite that infects at least one-third of the world's population. A majority of the seropositive humans display largely asymptomatic infection, with only a minor fraction of immunocompetent adults and children demonstrating non-specific clinical symptoms, predominantly limited to lymphadenopathy. In striking contrast, toxoplasmosis is a life-threatening disease in immunocompromised individuals, frequently manifesting in development of brain abscesses and encephalitis, indicating that an intact immune system is absolutely essential for controlling the parasite [1–2]. Experimental data and clinical observations strongly suggest that the innate immune recognition systems play a major role in predetermining susceptibility to the parasitic infection. As mice have become the major tool for identification of factors regulating host resistance to the parasite, we first focus on murine recognition systems and then discuss the similarities and differences in sensors and effector molecules that determine host resistance to this parasite in humans and mice.

Identification of IFN-γ as the major regulator of cell-mediated immunity to *T. gondii* in both mouse and human cells was a key discovery that served as a primer for enormous progress towards identification of major recognition and effector mechanisms essential for host resistance to *T. gondii* [3–4]. Lack of IFN-γ during initiation of immune responses to the parasite or blocking IFN-γ during chronic toxoplasmosis results in rapid mortality of infected animals, caused by uncontrolled parasite replication [4–7].

Studies during the last two decades have revealed that IL-12 plays a major role in regulation of IFN-γ production by NK and T cells (reviewed in [8–9]). In the absence of IL-12, mice

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are very susceptible to *T. gondii*, as blocking IL-12 during acute and chronic phases of infection results in uncontrolled parasite replication [10–13]. IL-12 can be produced by multiple innate immune cells, including dendritic cells (DC), macrophages, neutrophils, and monocytes [8, 14–17]. Each of these cell types plays an important role in host resistance. However, DC are crucial innate immune sensing cells responsible for the initial IL-12 responses and coordination of different cell type activation during acute responses to the parasite [18].

As discussed below, multiple mechanisms have been proposed to be involved in induction of IL-12 by DC. A groundbreaking observation revealed the TLR/IL-1R adaptor protein MyD88 as a major regulator of host defense to the parasite [19]. Because MyD88 can be activated by almost all TLRs, except TLR3 [20–21], major efforts were aimed at identifying TLRs responsible for the initial sensing of *T. gondii*.

TLR mediated recognition of *T. gondii*

TLR are a family of innate immune receptors involved in recognition of 'pathogen-associated molecular patterns' (PAMP), molecules that are essential for microorganisms and typically not expressed by host cells [22–24]. In the case of *T. gondii*, TLR11 is a major innate immune receptor that regulates IL-12 response to the parasite [25]. A defined ligand for TLR11 is an unconventional actin-binding protein profilin. Purified or recombinant *T. gondii* profilin elicits potent *in vitro* and *in vivo* IL-12 responses from DCs that are completely TLR11 and MyD88 dependent [25–26]. Formal evidence for profilin being the most robust parasitic protein capable of inducing IL-12 production was established by conditional inactivation of profilin in the parasite [26–27]. Both *in vitro* and *in vivo* studies demonstrate that abrogation of profilin expression completely diminishes IL-12 responses to the parasite by DC [26–27]. When combined with experiments involving TLR11-deficient animals or DC isolated from TLR11−/− or MyD88−/− mice, it can be concluded that *T. gondii* profilin is a major IL-12 inducing protein that elicits its immunological activity via TLR11-dependent activation of MyD88 [20]. Profilin fits to the *bona fide* definition of a PAMP [28–29] because it not only triggers TLR-dependent DC activation [25], but is also absolutely essential for the parasite biology and is a conserved motif across most apicomplexans [27]. Profilin-deficient *T. gondii* parasites are incapable of invading host cells and thus cannot survive without this molecule [27].

Despite major effects of TLR11 on induction of IL-12 responses to the parasite, the phenotype of infected TLR11−/− mice is distinct and less severe than that observed in MyD88−/− or IL-12−/− animals [12, 19–20, 30]. While in the absence of IL-12 (caused by IL-12p40 or IL-12p35 deficiency) or MyD88, mice are uniformly susceptible to parasitic infection, lack of TLR11 resulted in only partial mortality during the acute phase of the infection. TLR11−/− mice are also extremely vulnerable to the chronic phase of infection as measured by cyst burden in the brains of infected mice [25]. Nevertheless, results obtained with TLR11−/− mice indicate that additional factors should be able to trigger MyD88-dependent induction of IL-12 in response to the parasite *in vivo*. It was demonstrated that other TLRs, especially TLR2, can be activated in response to the parasite [31]. The defined TLR2 ligand, the parasitic GPI anchors, has been isolated and in addition to TLR2, *T. gondii* GPI can also activate TLR4 [32]. While *in vitro* experiments revealed that *T. gondii* GPI can trigger activation of both receptors, deficiency in TLR2 or TLR4 has little, if any, effect on IL-12 responses to the parasitic infection [19]. Instead, TLR2 seems to be involved in the regulation of TNF responses to the parasite while TLR4 plays no obvious role in regulation of cytokine production during experimental toxoplasmosis [19, 32–33].

A fundamental difference exists in the phenotypes of TLR11, TLR2, or TLR4-deficient mice infected with the parasite. TLR4−/− mice are practically indistinguishable from their wild type counterparts, indicating that this receptor displays no obvious function in host resistance to the parasite. Because TLR2 is involved in recognition of all pathogen groups including viruses, bacteria, and fungal pathogens [22], activation of TLR2 alone cannot elicit distinct parasite specific immune responses. Instead, this receptor may function as a danger-sensor, which can be activated by both microbial and self ligands. TLR2-deficient mice mount normal IL-12 responses to the parasite, but become susceptible to extremely high doses of infection [31]. Because a similar observation was made with other pathogens, where a function of TLR2 can be seen only with extremely high doses of pathogens [34], we hypothesize that enhanced susceptibility of TLR2 deficient mice to *T. gondii* is a result of compromised responses to both the microbial molecules and tissue damage caused by the infection. An alternate explanation stems from a hypothesis that a function of TLR2 is masked by the dominant effect of TLR11 on induction of IL-12 production by DC and other innate immune cells. This hypothesis can and needs to be addressed by careful analysis of TLR11- and TLR11xTLR2-doubly deficient animals. In either scenario, among identified innate sensors for *T. gondii*, TLR11 activation is unique in its ability to initiate a ‘parasite-specific‘ innate immune response. In contrast, because other TLRs, especially TLR2, are involved in recognition of multiple pathogens, their activation may coordinate, but would not be capable of inducing the highly specialized IL-12 dependent immune responses required for host defense against *T. gondii*.

In addition to TLRs, MyD88 is essential for signal transduction by IL-1R and IL-18R that, similar to TLRs, recruit adaptor proteins via their TIR-domains [35]. It is therefore possible that TLR-independent MyD88 activators play important roles in the regulation of host protection against *T. gondii*. Production of IL-1 and IL-18 requires a maturation step regulated by caspase-1 [36]. Because mice deficient in caspase-1 demonstrated no enhanced susceptibility to the parasitic infection, it was assumed that neither IL-1 nor IL-18 are important for immunity against *T. gondii* [37]. This conclusion is inconsistent, however, with the observations that IL-18 plays a crucial part in activation of NK cells, specifically, induction of IFN-γ production by these cells during acute toxoplasmosis [38]. Recent identification of mechanisms triggering maturation of IL-1 in the absence of caspase-1 may also be applicable to acute toxoplasmosis and thus explain a MyD88-dependent role for IL-18 in activation of NK cells during parasitic infection not seen in caspase-1 deficient mice [39].

Commensal gut bacteria drive indirect immune responses to *T. gondii*

The effects of TLR functions on the outcome of *T. gondii* infection are significantly influenced by the route of parasitic infection. For example, systemically (intraperitoneally) infected TLR9-deficient mice displayed no differences in the multiple parameters of host resistance to the parasite when compared with their wild type counterparts [20, 37]. Lack of TLR9 had no effects on the activation of innate or adaptive immune cells during acute and chronic phases of the infection. In contrast, TLR9 deficiency during the oral route of infection resulted in impaired DC maturation and migration to the draining lymph node, as well as reduced activation of CD8 and CD4 T cells [40–41]. As a consequence of impaired activation of both innate and adaptive arms of immunity against the parasite, TLR9−/− mice infected orally with *T. gondii* demonstrated enhanced susceptibility to the infection [40]. These results may imply that TLR9 is involved in sensing the parasite and is essential for coordinating both innate and adaptive immune responses against *T. gondii*. This interpretation is inconsistent with lack of detectable phenotype in TLR9−/− mice systemically infected with the parasite [37]. A similar discrepancy was observed in TLR2 or TLR4 deficient mice. Both TLR2 and TLR4 have profound roles in the regulation of

immunity to the parasite during mucosal immune responses, but play limited roles during the systemic responses to the parasites [41].

Recent data revealed that it is commensal gut bacteria, rather than the parasite itself, responsible for the TLR9 as well as TLR4 dependent IL-12 production following *T. gondii*-mediated intestinal damage [41–43]. A role for TLR2 is more complex. This receptor can be directly activated by *T. gondii* and commensal bacteria in addition to host-derived activators for this receptor [32, 44–45]. The impact of commensal bacteria on immunity to *T. gondii* was revealed by analysis of germ-free mice lacking intestinal microbiota in addition to TLR2, TLR4, and TLR9 deficient mice infected systemically or orally with *T. gondii* [41]. While orally infected TLR2- and TLR4-deficient mice demonstrate impaired IL-12 and IFN-γ responses to the parasite, the same animals infected systemically with the parasite have no detectable defects in the induction of IL-12 production and Th1 responses against *T. gondii*. Similarly, TLR9 plays a significant role during oral but not systemic immune responses to the parasite. These results demonstrate that TLR2, TLR4, and TLR9, which systemically play a limited role in the regulation of IL-12 production in response to *T. gondii*, are indispensible regulators of IFN-γ mediated immunity during peroral infection. We suggest that indirect activation of these TLRs by commensal bacteria is crucial in controlling immunity to *T. gondii* [41, 46–47]. This conclusion is in strong agreement with work from other laboratories demonstrating that oral infection with the parasite results in expansion of gram-negative bacteria thus triggering activation of TLR4 [42, 48]. While most studies of the immune response aim to understand the ways in which infectious pathogens are directly sensed by specific receptors, these results suggest that mucosal immune response to *T. gondii* is indirect and requires activation of ‘bacterial’ TLRs and MyD88 in response to gut commensal bacteria. Importantly, the effects of commensal bacteria on immunity to other pathogens were recently discovered [49–50], suggesting that commensal dependent immunity is a common feature of immune defense against various groups of pathogens.

Cell-specific regulation of immune defense to *T. gondii*

Despite conflicting results regarding specific TLRs involved in recognition of *T. gondii*, there is a consensus that MyD88 is indispensible for host resistance. Previous studies demonstrated that MyD88 activation is essential for protection against *T. gondii* and for the generation of effective T cell responses [20]. Complete deficiency in MyD88 prevents induction of pro-inflammatory cytokines and as a result, MyD88−/− mice fail to establish strong Th1 responses to the parasite [19, 51]. An essential cell-intrinsic role for MyD88 in activation of neutrophils, macrophages, DC, and even T cells during immune response to the parasite have been described [8]. It was demonstrated that MyD88 in neutrophils, macrophages and DC is essential for IL-12 cytokine production, and TLR signaling in DC have an additional role for activation of T cell responses to the parasite [14, 19, 26, 52]. A previously unknown T cell intrinsic function for MyD88 was revealed during the chronic phase of parasitic infection [53].

Analysis of tissue-specific MyD88-deficient mice unveiled that TLR signaling in DCs plays a critical role in the early restriction of *T. gondii* expansion prior to engagement of the T cell response [18]. Although MyD88 function in DCs is not required for recruitment of inflammatory cells into the peritoneum of *T. gondii*-infected mice, it was required for the vast majority of IL-12 production, which in turn was necessary for promoting IFN-γ production by NK cells and subsequent activation of the inflammatory monocytes in order to kill the parasites. Thus, the analysis of DC-specific MyD88-deficient mice revealed how innate cells cooperate *in vivo* to generate cell-mediated innate immunity to defend against a highly virulent intracellular pathogen [18]. These results illustrated in Figure 1 suggest that TLR activation in DC plays a central role in regulation of ‘type I innate immunity’, and that

innate recognition predetermines host resistance *versus* susceptibility to the parasitic infection. Further studies are needed to clarify a role for MyD88 in neutrophils, macrophages, and monocytes in host resistance. It is especially important during analysis of mucosal responses to the parasite, since in addition to leukocytes, epithelial cells are involved in sensing both the parasite and commensal bacteria, and their role in regulation of immunity and immunopathology is unknown.

MyD88-independent responses to *T. gondii*

The major role for MyD88 in TLR-dependent host resistance to the parasite is well established, but it is important to emphasize the existence of immune recognition and protective immunity in the absence of MyD88. *T. gondii*-infected macrophages were revealed to be capable of inducing IL-12 independently of MyD88 [54]. Furthermore, despite uniform mortality of MyD88−/− mice, adaptive immunity can be generated in the absence of MyD88 with the induction of impaired and delayed, but still highly significant numbers of, IFN-γ producing CD4+ T cells [55]. These results demonstrated that while TLR/MyD88 axis plays a major role in sensing the parasite, it is not the singular method of parasite detection. Furthermore, immunization with an avirulent uracil auxotrophic parasite strain cps-1 protects MyD88−/− mice from subsequent challenge with the highly virulent *T. gondii* strain RH [55]. This work, for the first time, demonstrated that MyD88-independent immunity can be fully sufficient for establishing protective immune responses against the parasite. Experiments with the temperature sensitive *T. gondii* strain ts-4 revealed that while IFN-γ-deficient animals are highly susceptible to the infection, lack of MyD88 or IL-12p40 had no effects on host resistance to the parasite (Figure 2). Because host resistance to the ts-4 strain of *T. gondii* is mediated by CD8 T cells [56], these results imply that although coordinated activation of NK and CD4+ T cells is regulated by MyD88, the TLR-recognition system is dispensable for activation of CD8 T cells, at least in the case of the attenuated strains of *T. gondii*. It is essential to identify other recognition systems that may selectively regulate IFN-γ dependent host resistance to the parasite in the absence of MyD88. Elegantly designed experiments utilizing genetically modified *T. gondii* strains indisputably demonstrated that direct infection of MHC class I expressing cells is essential for induction of CD8+ T cell responses [57–58], but the nature of the cytosolic receptors regulating CD8+ T cell immunity is unclear at present.

Species specific responses to *T. gondii*

Mouse models have been invaluable in deciphering cellular and molecular mechanisms of induction of innate and adaptive immune responses to *T. gondii*. Despite the crucial importance of TLR11 and MyD88 in induction of IL-12, human TLR11 is a non-functional pseudogene [59], and the importance of MyD88 in human immune responses to *T. gondii* is unapparent [60]. Furthermore, while in mice the major function for IL-12 is to initiate IFN-γ dependent induction of multiple immune related GTPases [61], this effector system is largely non-functional in humans and is limited to expression of only one protein (compared to 21 IRG genes in the C57/BL6 mouse) [62]. In our view, lack of both major recognition and effector systems are linked events that predetermine chief differences between human and mouse immune systems involved in detection and elimination of *T. gondii.*

TLR11 is unusual in its recognition of *T. gondii* because it senses the pathogen based on detection of released microbial profilin, rather than by direct interaction with the live parasite [63]. This recognition system allows innate sensing of the parasite in non-infected cells, which we termed ‘recognition at a distance’ strategy. This means of recognition assures that innate sensor cells, in particular DC, can initiate immune responses without the major immunosuppressive effects observed in infected macrophages and DC.

As mice are a natural host for *T. gondii*, both parasite virulence factors and host defenses have likely co-evolved. It is well established that murine antigen presenting cells infected with the parasite are largely compromised in their ability to upregulate co-stimulatory molecules and secrete pro-inflammatory cytokines [64–68]. Mice appear to have developed spatially separated detection and effector functions to combat *T. gondii*. Non-infected DCs are capable of sensing parasite via TLR11 and producing IL-12 which promotes IFN-γ dependent killing mechanisms within infected cells. Thus, potential virulence factors aimed at suppression of TLR signaling would be ineffective in non-infected DC while secreted rhoptry proteins capable of inhibiting IRG effectors are only effective in naïve but not IFN-γ primed macrophages [3, 69]. Therefore, by recognizing the parasite at a distance, mouse innate immune cells confer a selective advantage over the parasite by avoiding or at least delaying the potent immunosuppressive effects of the parasite seen in infected cells. Humans, not being a principal host of *T. gondii*, are likely not under selective pressure to maintain such elaborate defenses. This possibility is at least partially supported by observations that in mice, *T. gondii*-strain associated virulence and host susceptibility are closely linked [70–73]. While there may be an association between disease occurrences and virulence of the parasite in humans, this correlation is less obvious [74].

The significance of TLR11 deficiency in humans is less clear given that infected individuals are capable of developing protective immunity upon infection. One possible benefit of TLR11 deficiency in humans stems from recent analysis of wild type and TLR11−/− mice infected orally with the parasite [41]. While wild type animals are extremely sensitive to the parasite as a result of TLR11-driven intestinal immunopathology, TLR11−/− mice are fully protected from parasitic infection as a result of gut commensal driven immunity against *T. gondii*. Remarkably, TLR11−/− mice infected orally with the parasite were not only able to control the pathogen, but were largely free from the intestinal pathology seen in wild type animals [41]. For this reason, we speculate that a balanced immune response in humans can be achieved in the absence of TLR11 via other recognition systems that are able to either sense the parasite directly or rely on other indirect stimuli, including commensal bacteria. TLR11 mediated recognition of the parasite at a distance being an essential component of innate immunity in mice, may be too ‘dangerous’ in other species, including humans, that are accidental hosts of *T. gondii.*

A search for human innate receptors based on analysis of genetic association between congenital toxoplasmosis in humans revealed several candidate genes that may be involved in responses to *T. gondii*. Among the candidates are TLR9, the purinergic receptor P2X7, and a member of the NOD-like receptor family NALP1 [75–78].

Genetic association of NALP1 with congenital toxoplasmosis is particularly intriguing because the Nalp1 gene is also located within the rat genome susceptibility/resistance region (Toxo1 region) [79]. Experimental silencing of NAPL1 expression enhances proliferation of *T. gondii* in NALP1-deficient human cells [78]. These results strongly indicate that NALP1 may function as an intracellular sensor for the parasite. At present the molecular events responsible for activation of NALP1 in the cytosol of the infected cells are not known, but the identification of NALP1 as a sensor for *T. gondii* is an important discovery that may shed light on intracellular recognition systems for the parasite.

Another molecule that can be considered as a ‘danger’ sensor, purinergic receptor P2X7, has also been associated with congenital and ocular toxoplasmosis [77]. Recent results revealed that a polymorphism at P2RX7 (a gene encoding the P2X7 receptor) influences susceptibility to *T. gondii*. Experiments with the ATP treated mouse or human macrophages also suggest that P2X7 regulates ROS-mediated killing of the parasite [76, 80]. In addition, P2X7 may enhance lysosomal fusion with the parasitophorous vacuole in the infected cells

[80]. Similar to NALP1, activation of P2X7 should happen in conjunction with other innate immune receptors and their identification would be key to developing a better understanding of host–parasite interaction and generation of effective strategies for prevention and treatment of toxoplasmosis.

Future implications

Initial identification of MyD88 as a major regulator of resistance to *T. gondii* in mice precipitated many advances within the study of innate immunity to the pathogen. Both murine and human studies have contributed to identification of innate immune sensors that not only explain how mice and human develop immunity to the parasite but also revealed elements of parasite biology. These immunoparasitology approaches have uncovered a complex relationship between *T. gondii* and its hosts and shed light on fundamental aspects of host-parasite co-evolution. On a practical level, the identified molecules of interest will undoubtedly serve as a basis for design of vaccines targeting parasitic disease via selective activation of host immune system.

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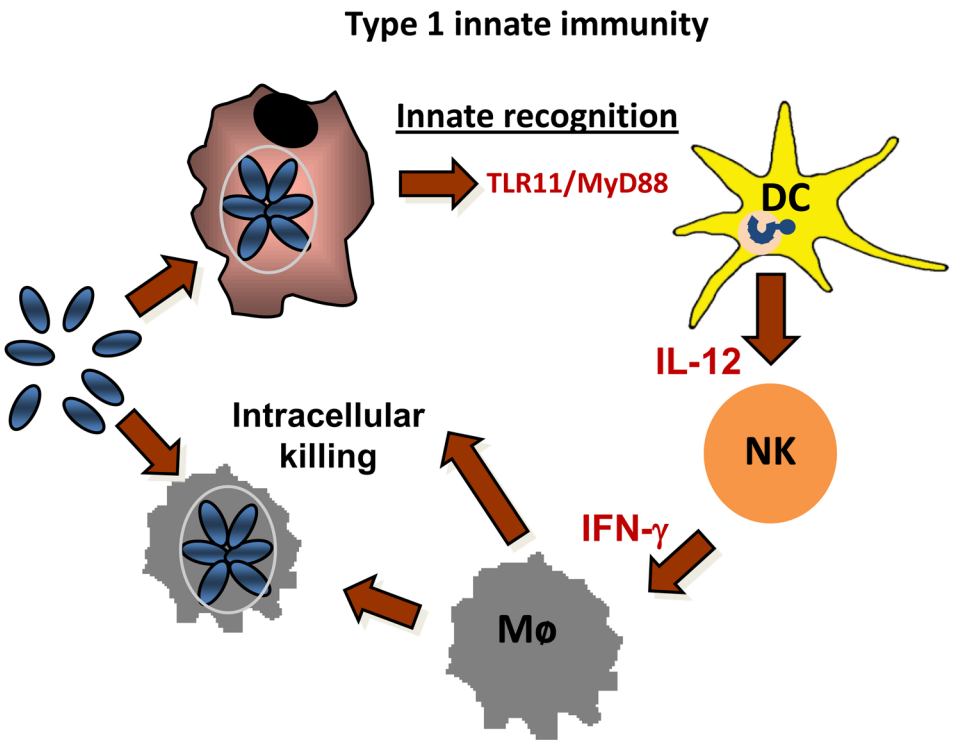

Figure 1. TLR activation in DC plays a central role in regulation of type I innate immunity

Recent results demonstrated that the crucial function of TLR/MyD88 signaling in DCs in defense against *T. gondii* infection was to activate NK cells and inflammatory monocytes [18]. Although MyD88 function in DCs was not required for the recruitment of inflammatory cells into the peritoneum of *T. gondii*-infected mice, it was required for the vast majority of IL-12 production, which in turn was required for promoting IFN-γ production by NK cells and subsequent activation of the inflammatory monocytes and macrophages to allow them to kill the parasites [18]. This model demonstrates how innate cells cooperate *in vivo* to generate cell-mediated innate immunity to defend against a highly virulent intracellular pathogen.

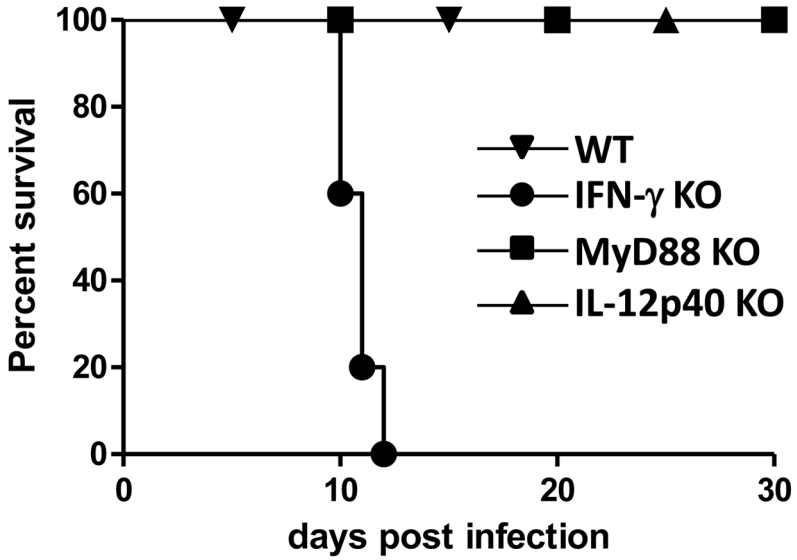

Figure 2. MyD88-independent responses to *T. gondii*

Effects of IL-12, MyD88, and IFN-γ deficiencies on survival of mice following ts-4 infection. WT, IL-12p40−/−, IFN-γ−/−, and MyD88−/− mice were infected intraperitoneally with 10^4 ts-4 tachyzoites and survival of mice was analyzed. Abbreviations: KO, knock out; WT, wild type.