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Cutting Edge: MyD88 Is Required for Resistance to *Toxoplasma gondii* Infection and Regulates Parasite-Induced IL-12 Production by Dendritic Cells

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Cutting Edge: MyD88 Is Required for Resistance to *Toxoplasma gondii* Infection and Regulates Parasite-Induced IL-12 Production by Dendritic Cells¹

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Host resistance to the intracellular protozoan *Toxoplasma gondii* is highly dependent on early IL-12 production by APC. We demonstrate here that both host resistance and *T. gondii*-induced IL-12 production are dramatically reduced in mice lacking the adaptor molecule MyD88, an important signaling element used by Toll-like receptor (TLR) family members. Infection of MyD88-deficient mice with *T. gondii* resulted in uncontrolled parasite replication and greatly reduced plasma IL-12 levels. Defective IL-12 responses to *T. gondii* Ags (soluble tachyzoite Ag (STAg)) were observed in MyD88^{-/-} peritoneal macrophages, neutrophils, and splenic dendritic cells (DC). In contrast, DC from TLR2- or TLR4-deficient animals developed normal IL-12 responses to STAg. In vivo treatment with pertussis toxin abolished the residual IL-12 response displayed by STAg-stimulated DC from MyD88^{-/-} mice. Taken together, these data suggest that the induction of IL-12 by *T. gondii* depends on a unique mechanism involving both MyD88 and G protein-coupled signaling pathways. *The Journal of Immunology*, 2002, 168: 5997–6001.

Innate immunity to pathogens is thought to be triggered by pattern recognition receptors on APC that detect and respond to conserved structural motifs on invading microorganisms (1, 2). An important class of pattern recognition receptors are the Toll-like receptors (TLR),³ a family of evolutionarily conserved germline-encoded transmembrane receptors that have been shown to stimulate APC function in response to defined lipid, protein, and

nucleic acid structures present on bacterial, fungal, and viral pathogens (2). Recently, it has been shown that triggering of the TLR family member TLR2 plays a role in the initiation of cytokine responses to a protozoan pathogen, *Trypanosoma cruzi*, suggesting that TLR may be also involved in the innate recognition of this group of eukaryotic microorganisms (3).

In the present study, we have further examined the possible involvement of TLR in innate immunity to protozoa, focusing on the induction of host resistance to the apicomplexan parasite *Toxoplasma gondii*. During early infection, this parasite stimulates a potent IL-12 response that leads to IFN- γ -dependent control of its replication (4). In turn, a series of in vitro and in vivo studies have demonstrated that dendritic cells (DC), as well as neutrophils and IFN- γ -primed macrophages, produce IL-12 in response to parasite stimulation (5–7). More recently, the chemokine receptor CCR5 has been shown to play a major role in *T. gondii*-induced IL-12 production by DC (8). Correlating with this observation, CCR5-deficient animals exhibit increased susceptibility to the parasite (8), succumbing by 20 days postinfection (J. Aliberti and A. Sher, unpublished data).

To determine the possible function of TLR in the induction of IL-12-dependent host resistance to *T. gondii*, we tested the requirement for the TLR-associated adapter protein MyD88 in control of parasite infection. MyD88 has been shown to be a critical signaling element for most TLR as well as IL-1R family-triggered responses (9), and APC from MyD88-deficient mice have been shown to mount defective IL-12 responses to a number of microbial stimuli (10–12). As reported here, MyD88-deficient mice infected with *T. gondii* displayed a complete loss in acute resistance to infection, a defect associated with impaired IL-12 production by DC, macrophages, and neutrophils. These findings implicate TLR triggering as a critical step in the initiation of innate immunity to *T. gondii* and in the case of DC argue that full induction of IL-12 by this pathogen involves both G protein-coupled and MyD88-dependent signaling pathways.

Materials and Methods

Experimental animals

MyD88^{-/-}, TLR2^{-/-}, and TLR4^{-/-} mice (13–15) on a partially backcrossed 129/Ola \times C57/Bl6 background were kindly provided by Drs. S. Akira (Osaka University, Osaka, Japan) and D. T. Golenbock (University of Massachusetts, Worcester, MA). These animals, along with CCR5^{-/-} mice (16), were bred and maintained at an American Association of Laboratory Animal Care-accredited National Institute of Allergy and Infectious Diseases animal facility. Wild-type control mice (129/Ola \times

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³ Abbreviations used in this paper: TLR, Toll-like receptor; STAg, soluble tachyzoite Ag; DC, dendritic cell; PEC, peritoneal exudate cells, PTx, pertussin toxin.

C57BL/6) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice of both sexes between 5 and 12 wk old were used for experiments.

Parasites, experimental infection, and treatments

Mice were infected with 20 cysts of the avirulent ME49 *T. gondii* strain by i.p. inoculation and cumulative mortality was determined (17). In vivo tachyzoite growth was assessed at 5 days after infection by microscopic examination of Diff-Quik (Dade Behring, Newark, DE)-stained cytocentrifuge smears of peritoneal exudate cells (PEC) (17). RH strain *T. gondii* tachyzoites were maintained in tissue culture as previously described (6) and used to prepare soluble tachyzoite Ag (STAg) (18). For in vivo stimulation of DC, mice were i.p. injected with 5 μ g STAg or with PBS alone, and spleens were harvested 6 h later (19). In some experiments, mice received pertussis toxin (PTx, 400 ng i.v.) 30 min before STAg or PBS injection (20).

Cell isolation and in vitro stimulation

For measurement of ex vivo cytokine production, single-cell suspensions were prepared from spleens and PEC 5 days after infection. PEC were cultured at 4×10^5 cells/well in 200 μ l and spleen cells at 4×10^6 cells/well in 1 ml medium in the presence or absence of STAg (5 μ g/ml) and supernatants were collected 3 days later. In some wells, 20 μ g/ml blocking mAb to CD4 (GK1.5) and CD8 (2.43) were added to assess the contribution of these T cell subsets as previously described (21).

Neutrophils were isolated from bone marrow of noninfected mice as described elsewhere (22) and plated at $2\text{--}2.5 \times 10^6$ cells/well. Cells were 95% neutrophils based on microscopic examination of stained preparations. Peritoneal macrophages were prepared from thioglycolate-elicited PEC (17) by overnight plating at 4×10^5 cells/well in the presence or absence of 100 U/ml murine rIFN- γ (Genentech, South San Francisco, CA) as a priming signal followed by removal of nonadherent cells. DC were isolated by low-density separation of collagenase-treated spleen (5) and plated at 1×10^6 cells/well.

For in vitro stimulation, macrophages and DC were exposed to STAg (20 μ g/ml) and neutrophils to either STAg (100 μ g/ml) or live RH tachyzoites (1×10^6 /well). Supernatants were collected at 16 h for cytokine measurements.

IL-12 and IFN- γ measurements

IL-12p40 and IFN- γ levels were assayed by ELISA as previously described (17). IL-12p70 was measured using a commercial ELISA kit (R&D Systems, Minneapolis, MN).

Statistical analyses

The statistical significance of differences in data means was analyzed using an unpaired, two-tailed Student's *t* test.

Results and Discussion

MyD88-deficient mice fail to control *T. gondii* infection

To evaluate the role of MyD88 in host resistance to *T. gondii*, MyD88^{-/-} and control mice were infected with cysts of the avirulent ME49 parasite strain and survival of the animals was monitored. In contrast to control mice that survived for >60 days, MyD88-deficient animals succumbed to acute infection, dying between 13 and 16 days after parasite inoculation (Fig. 1). The latter mortality kinetics closely resemble those observed for similarly infected IL-12p40-deficient mice (Fig. 1). Since mortality during acute *T. gondii* infection can result either from uncontrolled inflammation (23) or increased parasite replication (17), we also assessed parasite burden in the same animals by enumerating parasitized PEC 5 days postinoculation. A dramatic increase in parasite load was seen in the MyD88^{-/-} vs control mice which was comparable to, if not exceeding, that observed in the IL-12p40-deficient mice (Fig. 1, inset). Thus, MyD88 plays an essential role in host resistance by influencing the control of *T. gondii* replication.

MyD88-deficient mice mount defective parasite-induced IL-12/IFN- γ responses

Host resistance to *T. gondii* in the murine model is mediated by IL-12-dependent IFN- γ production (24). To determine whether

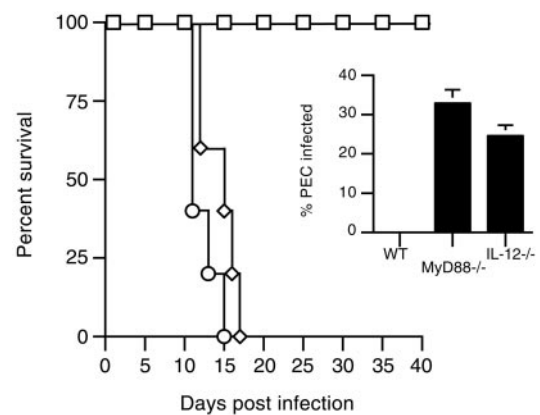


FIGURE 1. MyD88^{-/-} mice fail to control acute *T. gondii* infection. Control mice (□), MyD88^{-/-} mice (◇), and IL-12p40^{-/-} mice (○) were infected i.p. with 20 cysts of the ME49 strain of *T. gondii* and survival was monitored. Data are representative of two experiments performed, each with five to eight mice per group. Inset, Percentage (mean \pm SD, *n* = 5 mice/group) of tachyzoite-infected PEC from the above animal groups at day 5 after infection.

impaired resistance of MyD88^{-/-} mice stems from a defect in this pathway, we measured IL-12 and IFN- γ in plasma from 5-day infected mice. The Myd88^{-/-} mice displayed greatly reduced levels of plasma IL-12p40 (Fig. 2A) and IL-12p70 (data not shown). Plasma IFN- γ levels in MyD88-deficient mice also were much lower relative to control animals (Fig. 2B). Nevertheless, IFN- γ production in the MyD88^{-/-} mice was significantly greater than that observed in IL-12p40-deficient animals (Fig. 2B). To confirm this defect at the cellular level, we examined IFN- γ production by spleen cells from the same animals after in vitro restimulation with STAg. MyD88-deficient animals showed dramatically reduced IFN- γ production, again slightly elevated over that displayed by IL-12p40-deficient mice (Fig. 2C). Experiments adding blocking Abs to CD4 and/or CD8 to these cultures indicated that the IFN- γ response of control mice is only partially dependent on T cells (Fig. 2C), consistent with the previously described role of NK cells as a major source of this cytokine during acute infection (4, 7). In contrast, the residual response seen in the MyD88^{-/-} mice was found to be totally CD4⁺ T lymphocyte dependent (Fig. 2C).

MyD88-dependent signaling regulates *T. gondii*-induced IL-12 production in DC, macrophages, and neutrophils

Previous studies have demonstrated that macrophages, DC, as well as neutrophils produce IL-12 in response to stimulation with *T. gondii* in vitro (7, 8, 25) and, in the case of DC and neutrophils, in vivo (6, 8). To examine whether MyD88 controls IL-12 responsiveness in one or more of these cell types, highly enriched bone marrow-derived neutrophils, splenic DC, and thioglycolate-elicited peritoneal macrophages were stimulated in vitro with STAg or live tachyzoites and IL-12p40 production was measured by ELISA. The STAg-induced IL-12 response observed in IFN- γ -primed macrophages from control mice was completely absent in comparable cell populations from MyD88-deficient mice (Fig. 3A). IL-12 responsiveness was also greatly impaired in neutrophils from MyD88^{-/-} mice stimulated in vitro with either live tachyzoites or with STAg (Fig. 3B). Finally, the IL-12 response of splenic DC was highly reduced in the absence of MyD88 (Fig. 3C) but not to the extent seen in MyD88^{-/-} macrophages and neutrophils. A similar reduction in the DC cytokine response was observed when IL-12p70 was measured rather than p40 (Fig. 3D).

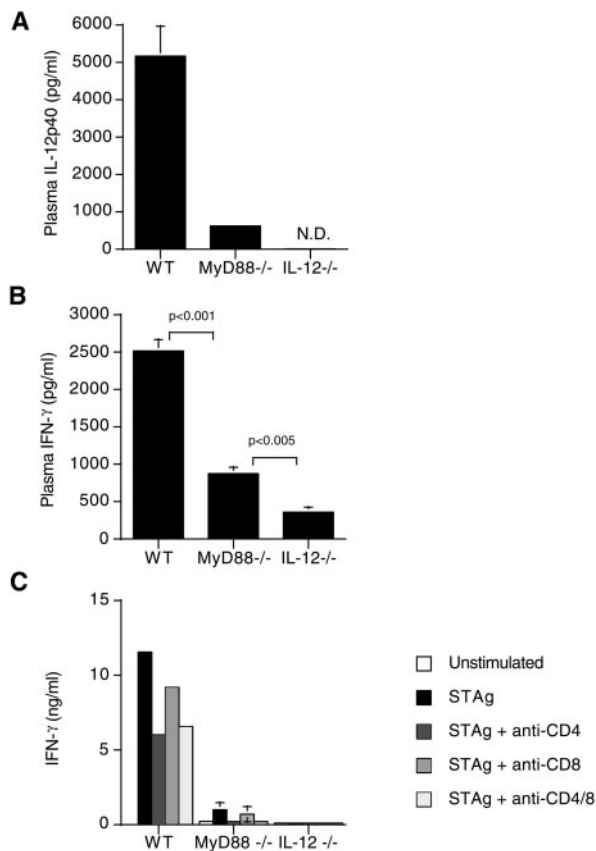


FIGURE 2. IL-12 and IFN- γ responses to *T. gondii* infection are reduced, but not abolished, in MyD88 $^{-/-}$ mice. *A*, Plasma IL-12p40 levels in control, MyD88 $^{-/-}$, and IL-12p40 $^{-/-}$ mice measured by ELISA 5 days after infection. ND, None detected. *B*, IFN- γ levels in the same plasma. *C*, Splenocytes from the above animals were incubated in medium alone (□) or in medium containing STAg (■) for 72 h. Blocking anti-CD4 (dark gray bar), anti-CD8 (medium gray bar) or both Abs (light gray bar) were added in parallel cultures at 20 μ g/ml. IFN- γ levels in the supernatants were determined by ELISA. Anti-CD3 stimulation induced high levels of IFN- γ (>20 ng/ml) in splenocyte cultures from each of the three mouse strains (data not shown). Bars represent means \pm SD of ELISA values performed on five mice per group.

Effect of MyD88 on T. gondii-induced DC IL-12 production is not the result of impaired TLR2 or TLR4 signaling

Since DC are thought to represent an important cell population in the initiation of cell-mediated immunity to *T. gondii* (5), we further investigated the effect of MyD88 in a STAg-induced IL-12 response in these cells. We have previously shown that splenic DC recovered from STAg-injected mice produce high levels of IL-12 ex vivo (5). As shown in Fig. 4A, splenic DC from STAg-injected MyD88 $^{-/-}$ mice showed a highly impaired IL-12p70 response compared with that displayed by DC from comparably injected control mice and indicate that the defect in MyD88-deficient DC function occurs in vivo and in vitro.

MyD88 is an important adapter molecule required for most TLR-dependent signaling responses (2). TLR2 and TLR4 are two TLR previously implicated in antimicrobial responses in vivo (1). Animals deficient in either of these receptors nevertheless mounted normal DC IL-12 responses to injected STAg (Fig. 4A), indicating that the MyD88 defect in *T. gondii*-induced IL-12 production does not involve either of these TLR.

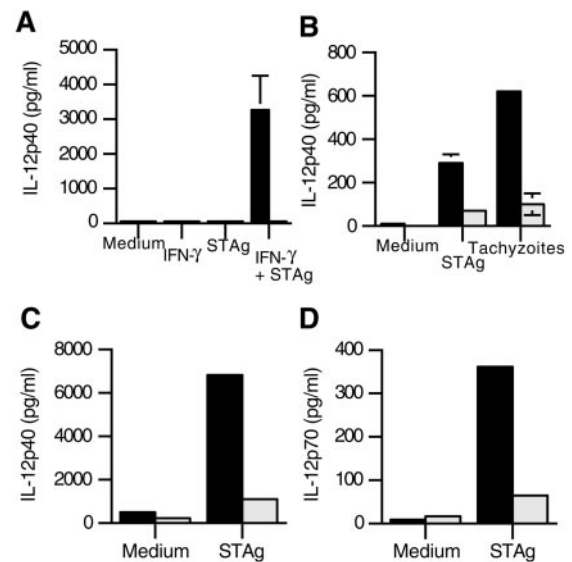


FIGURE 3. Peritoneal macrophages, neutrophils, and DC from MyD88 $^{-/-}$ mice show impaired *T. gondii*-induced IL-12 responses in vitro. Purified thioglycolate-elicited peritoneal macrophages (*A*), bone marrow-derived neutrophils (*B*), or splenic DC (*C* and *D*) from control (■) or MyD88 $^{-/-}$ (□) mice were stimulated in vitro with STAg (*A–D*) or live tachyzoites (*B*) and IL-12p40 (*A–C*) or IL-12p70 (*D*) measured in the supernatant by ELISA after overnight incubation. In the case of the peritoneal macrophages, some of the cultures were primed with IFN- γ overnight before STAg stimulation. Bars are means \pm SD of measurements from quadruplicate wells. Each of the experiments shown was performed at least twice with similar results.

MyD88 and CCR5 influence T. gondii-induced IL-12 production by DC through independent pathways

Previous studies have demonstrated that the IL-12 response triggered by *T. gondii* in DC is highly dependent on the chemokine receptor CCR5 (8). The discovery that the same response also partially depends on MyD88 raised the question of whether CCR5 and MyD88 are components of distinct or common pathways involved in signaling for IL-12 production. A side-by-side comparison revealed that MyD88 $^{-/-}$ and CCR5 $^{-/-}$ mice display quantitatively comparable defects in the IL-12p70 response to injected STAg (Fig. 4B). To analyze the interdependence of the two defects, we treated mice with PTx, an agent that uncouples the G protein signaling pathway utilized by CCR5 and other chemokine receptors (26). As expected, in vivo PTx treatment reduced the STAg-triggered IL-12 response of control mice to a level comparable to that of CCR5 $^{-/-}$ animals (Fig. 4B), suggesting that the residual response in both cases is due to a non-G protein-dependent signaling mechanism. At the same time, PTx treatment completely abolished the residual response of MyD88 $^{-/-}$ animals (Fig. 4B), arguing that it is the product of a G protein signaling pathway. Together, these two observations are consistent with a model in which potent *T. gondii*-induced IL-12 production stems from two independently triggered pathways that either overlap or augment one another.

Our findings establish a major role for MyD88 in IL-12-dependent resistance to *T. gondii* as well as in parasite-induced IL-12 production by the three principal cell types known to mount this response. Since we were unable to simultaneously identify the involvement of a specific or combination of specific TLR in the pathway leading to parasite-triggered IL-12 synthesis, a role for TLR in innate recognition of *T. gondii* in this system, although likely, remains to be formally demonstrated. Such a role for TLR

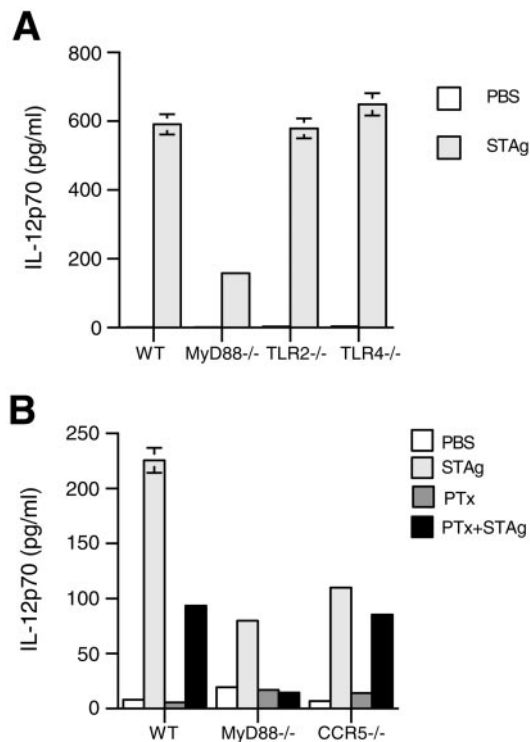


FIGURE 4. STAg-induced IL-12 production by DC is TLR2 and TLR4 independent and requires both CCR5- and MyD88-dependent signals. *A*, Control mice or mice deficient in MyD88, TLR2, or TLR4 were injected with either PBS (□) or STAg (▤) and splenic DC were purified 6 h later, plated, and IL-12p70 was measured in the supernatant after overnight incubation. *B*, MyD88^{-/-}, CCR5^{-/-} mice and control (wild type (WT)) mice were pretreated with either PBS (open bars and light gray bars) or PTx (dark gray and black bars) for 30 min and then injected with either PBS (open and dark gray bars) or STAg (light gray and black bars). After 6 h, splenic DC were isolated, plated, and incubated overnight. IL-12p70 was then measured in the supernatant. Bars represent means \pm SD of measurements from quadruplicate wells. The experiments shown are representative of two performed.

would be consistent with previous studies demonstrating the function of TLR2 in the induction of cytokines by the intracellular protozoa *T. cruzi* (3), although our data argue against the involvement of this TLR (as well as TLR4) in the response to *T. gondii*. The alternate hypothesis that MyD88 may regulate IL-12 production through its participation in IL-18 and IL-1R signaling at present cannot be ruled out but appears unlikely based on previous evidence implicating a limited role for these cytokines in host resistance to *T. gondii* (27, 28). Additionally, although IL-1 β recently has been reported to induce IL-12p70 in human DC, this response requires costimulation of the cells with CD40 ligand or IFN- γ (29), a feature not shared by the murine DC and neutrophil responses studied here.

Interestingly, while MyD88 signaling usually involves NF- κ B-dependent downstream events, IL-12 production by *T. gondii* has been shown not to require the major NF- κ B transcription factors NF- κ B₁, NF- κ B₂, relB, or c-rel (30), suggesting the existence of a unique pathway for MyD88 triggering of cytokine production by *T. gondii*. Although apparently NF- κ B independent, *T. gondii*-induced IL-12 synthesis is known to require the IFN regulatory factor family member IFN consensus sequence-binding protein (31), raising the possibility that MyD88 triggers IL-12 production through an alternative pathway involving this transcription factor.

T. gondii is a particularly potent stimulator of IL-12 production by DC, a response previously shown to be highly dependent on G

protein-coupled signaling mediated by the chemokine receptor CCR5 (8). The data presented here establish a dual role for CCR5 and MyD88 in *T. gondii*-induced IL-12 synthesis by DC and suggest that these elements signal through distinct pathways. The mechanism by which CCR5 and MyD88 interact to provide high level induction of IL-12 in the response of DC to *T. gondii* is currently under investigation.

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