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CUTTING EDGE

Cutting Edge: TLR9 and TLR2 Signaling Together Account for MyD88-Dependent Control of Parasitemia in *Trypanosoma cruzi* Infection[1]

Andre Bafica,[2] Helton Costa Santiago,† Romina Goldszmid,* Catherine Ropert,[2‡] Ricardo T. Gazzinelli,[2,3†‡] and Alan Sher[3]**

***Activation of innate immune cells by* Trypanosoma cruzi*-derived molecules such as GPI anchors and DNA induces proinflammatory cytokine production and host defense mechanisms. In this study, we demonstrate that DNA from* T. cruzi *stimulates cytokine production by APCs in a TLR9-dependent manner and synergizes with parasite-derived GPI anchor, a TLR2 agonist, in the induction of cytokines by macrophages. Compared with wild-type animals,* T. cruzi*-infected Tlr9$^{-/-}$ mice displayed elevated parasitemia and decreased survival. Strikingly, infected Tlr2$^{-/-}$Tlr9$^{-/-}$ mice developed a parasitemia equivalent to animals lacking MyD88, an essential signaling molecule for most TLR, but did not show the acute mortality displayed by MyD88$^{-/-}$ animals. The enhanced susceptibility of Tlr9$^{-/-}$ and Tlr2$^{-/-}$Tlr9$^{-/-}$ mice was associated with decreased in vivo IL-12/IFN-γ responses. Our results reveal that TLR2 and TLR9 cooperate in the control of parasite replication and that TLR9 has a primary role in the MyD88-dependent induction of IL-12/IFN-γ synthesis during infection with* T. cruzi. *The Journal of Immunology,* 2006, 177: 3515–3519.**

Mammalian TLR are a group of at least eleven structurally related signaling molecules that are thought to play a pivotal role in both innate immunity and the initiation of adaptive responses to pathogens (1–3). TLR are known to sense distinct molecular structures on microbes and trigger NF-κB-dependent gene functions leading to the production of proinflammatory cytokines, costimulatory molecules, and other mediators which both restrict early pathogen growth and promote the development of adaptive immunity (2, 3). Nevertheless, the mechanisms by which different pathogen-induced signals are orchestrated in the generation of host resistance are poorly understood.

Trypanosoma cruzi, the causative agent of Chagas disease, is an intracellular trypanosomatid protozoan. Host control of this parasite has been shown to depend on both humoral and cell-mediated adaptive responses as well as elements of the innate immune system (4). A role for TLR signaling in resistance to *T. cruzi* is supported by the observations that mice deficient in MyD88, an adaptor molecule required for signaling events by most TLR as well as IL-1R and IL-18R, show greatly enhanced susceptibility to infection with this protozoan parasite (5). So far, no single TLR has been shown to account for the acute susceptibility displayed by MyD88-deficient animals (5–9). For this reason, we hypothesized that more than one TLR might cooperate in controlling *T. cruzi* infection in vivo.

Mice lacking TLR2 have been shown to display slightly enhanced susceptibility to *T. cruzi* in vivo (5), whereas macrophages from the same animals present impaired proinflammatory cytokine production when exposed to the live pathogen in vitro (5). GPI anchors are a major class of *T. cruzi* molecules recognized by TLR2 (6). In addition, other parasite structures such as the *T. cruzi*-released protein Tc52 and a particular subset of free GPI anchors containing ceramide (GIPL-ceramide) have been shown to stimulate macrophage proinflammatory cytokine production via TLR2 (8) and TLR4 (7), respectively. DNA preparations from both *T. cruzi* and *Trypanosoma brucei* have been shown to stimulate cytokine responses from macrophages and dendritic cells (DCs) (9), suggesting that the genomes of these trypanosomatids contain sufficient CpG motifs to induce activation of host cells via TLR9 signaling as has been well documented in TLR recognition of bacteria. Indeed, DNA from *T. brucei* triggers macrophage cytokine production in a TLR9-dependent manner and mice deficient in this TLR display impaired resistance to *T. brucei* infection (10). Nevertheless, the role of TLR9 in host responses to *T. cruzi* infection and its possible interaction with the parasite-induced TLR2 signaling pathway described above has not been investigated.

In the present study, we show that mice lacking TLR2 and TLR9 display enhanced susceptibility to *T. cruzi* infection,

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which recapitulates the high blood parasitemia observed in mice deficient in MyD88 and correlates with defective Th1-like responses in vivo. Our data thus reveal a role for TLR9 in host resistance to *T. cruzi* and provide the first evidence for TLR cooperation in the control of protozoan infection in vivo.

Materials and Methods

Mice

C57BL/6 mice were purchased from Taconic Farms. Breeding pairs of B6.129P2-Myd88^{tmAki} (Myd88$^{-/-}$), B6.129P2-Tlr2^{tmAki} (Tlr2$^{-/-}$), and B6.129P2-Tlr9^{tmAki} (Tlr9$^{-/-}$) mice were obtained from Dr. S. Akira (Osaka University, Japan) via Dr. D. Golenbock (University of Massachusetts Medical School, MA) or Dr. R. Seder (Vaccine Research Center, National Institutes of Health). Tlr2$^{-/-}$Tlr9$^{-/-}$ mice were generated by mating TLR2$^{-/-}$ with TLR9-deficient animals as previously described (11). Animals were bred and maintained at an American Association of Laboratory Animal Care-accredited facility at the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Mice of both sexes between 8 and 14 wk old were used in all experiments.

T.cruzi *culture-derived trypomastigotes and* T.cruzi*-derived tGPI-mucin*

Trypomastigote forms of the Y strain of *T. cruzi* were grown in a human fibroblast cell line and purified by centrifugation as described previously (12). The tGPI-mucin was isolated from tissue culture trypomastigotes as described elsewhere (13).

TLR agonists and other reagents

Ultra-pure LPS (*Escherichia coli* 0111:B4) and DNA calf thymus were purchased from Invitrogen and Sigma-Aldrich, respectively. Purified genomic DNA from *T. cruzi* was purchased from American Type Culture Collection (30266D).

Cell populations

Bone marrow macrophages (BMM) and thioglycollate elicited peritoneal macrophages were obtained from C57BL/6 and knockout (KO) mice as previously described (6). Macrophages were cultured in supplemented DMEM (10^6 cells/ml) (Invitrogen Life Technologies) and stimulated with live trypomastigotes or TLR ligands for 24 h to evaluate TNF and IL-12p40 production by ELISA (R&D Systems).

Experimental infection with T. cruzi

Mice were i.p. infected with 1000 blood-trypomastigote forms of the Y strain of *T. cruzi* as previously described (5). Parasitemia levels were evaluated by counting parasites in 5 μl of blood from the tail vein, and mortality was assessed daily. Mice were bled on days 0 and 10 after infection, and the level of serum cytokine was evaluated by ELISA (1/2 sera dilution).

CD4$^+$ T cell recall response assay

Purified CD4$^+$ T spleen cells (10^6 cells/ml) from *T. cruzi*-infected wild-type (WT) or KO mice were cocultured with live trypomastigote-infected bone marrow-derived dendritic cell (BMDC) (5×10^5 cells/ml) from WT animals for 72 h (a protocol used elsewhere). IFN-γ (R&D Systems) levels in culture supernatants were then determined by ELISA.

Statistical analysis

ANOVA was used to analyze the significance of differences in means between multiple experimental groups. Survival curves were generated using the Kaplan-Meier method and the significance of differences was calculated by the log-rank test. Statistical significance was defined as $p < 0.05$.

Results and Discussion

DNA from T. cruzi *stimulates IL-12 and TNF in a TLR9-dependent manner and synergizes with GPI anchors for proinflammatory cytokine production*

Brown and colleagues (9) have demonstrated that *T. cruzi* DNA activates macrophages to produce cytokines and reactive nitrogen intermediates and stimulates B lymphocyte proliferation. These activities were destroyed with DNase treatment and were dependent on the presence of unmethylated CpG dinucleotides. As indicated in Fig. 1*A*, purified DNA from *T. cruzi* stimulated IL-12p40 as well as TNF (data not shown) production by BMM in a TLR9-dependent manner. Similarly, *T. cruzi* DNA induced IL-12p40 synthesis by CD11c$^+$ splenic DCs through a MyD88- and TLR9-dependent pathway (data not shown).

Because GPI anchors derived from *T. cruzi* activate TLR2 (6) and trigger the synthesis of various proinflammatory cytokines and reactive nitrogen intermediates (12–14), we next investigated whether GPI anchors and DNA derived from *T. cruzi* cooperate in the induction of cytokine synthesis by macrophages. As shown in Fig. 1, *B* and *C*, GPI and DNA from *T. cruzi* synergize in the induction of both IL-12p40 and TNF production by macrophages. Thus, simultaneous stimulation of TLR2 and TLR9 by *T. cruzi* components is important for the induction of optimal innate immune responses against the parasite.

Proinflammatory cytokine responses by APC exposed to live T. cruzi *in vitro are regulated by both TLR2 and TLR9*

We next investigated the role of TLR2 or TLR9 in *T. cruzi*-induced proinflammatory cytokine production by APC in

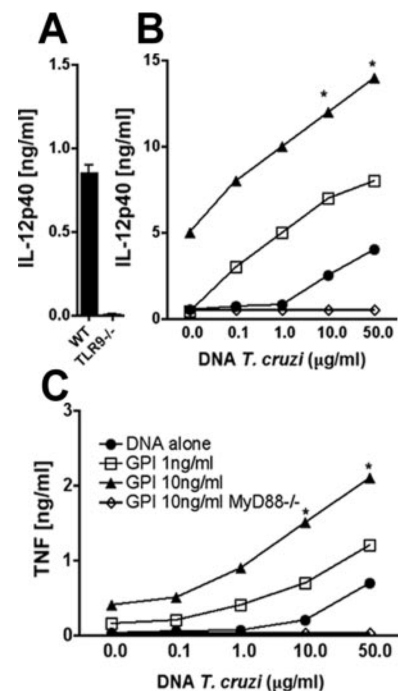

FIGURE 1. Effects of DNA and GPI-mucin from *T. cruzi* on proinflammatory cytokine responses by macrophages. *A*, BMM from WT or Tlr9$^{-/-}$ mice were exposed to purified DNA from [DNATc] (10 μg/ml), and 24 h later, IL-12p40 was assayed in supernatants by ELISA. *B* and *C*, Peritoneal macrophages from C57BL/6 or Myd88$^{-/-}$ (◇) mice were stimulated with different concentrations of DNATc (0.1–50 μg/ml), GPI (1 or 10 ng/ml), or both for 24 h. TNF (*A*) and IL-12p40 (*B*) production were measured in the culture supernatants by ELISA. Results are means ± SE of triplicate measurements. Experiments shown are representative of two performed. *, $p < 0.05$ between values from DNA plus GPI treatment vs DNA alone (10 or 50 μg/ml) or GPI alone (10 ng/ml). No synergy in proinflammatory cytokine production was observed when macrophages were treated with control calf thymus DNA (50 μg/ml) plus GPI (10 ng/ml) for 24 h.

[4] Abbreviations used in this paper: DC, dendritic cells, BMM, bone marrow-derived macrophages; BMDC, bone marrow-derived dendritic cell; KO, knockout; WT, wild type.

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vitro. *T. cruzi*-exposed peritoneal macrophages from $Tlr2^{-/-}$, $Tlr9^{-/-}$, or $Tlr2^{-/-}Tlr9^{-/-}$ mice displayed reduced IL-12p40 responses with double KO cells showing the greater defect (Fig. 2*A*), suggesting that TLR2 and TLR9 act in concert in triggering IL-12. In contrast, the TNF response appeared to be controlled primarily by TLR2 and not TLR9 (Fig. 2*B*).

TLR2 and TLR9 cooperate in the control of T. cruzi *infection*

Despite the fact that tGPI-mucins are potent TLR2 agonists, previous studies (5) have shown that infected $Tlr2^{-/-}$ mice mount an unimpaired proinflammatory cytokine response and display no loss in host resistance. To assess the possible role of TLR9 in host defense, WT and TLR-deficient animals were infected with 1000 trypomastigotes of the Y strain *T. cruzi* and followed for parasitemia as well as survival. As shown in Fig. 3*A*, $Tlr9^{-/-}$ animals presented elevated parasite numbers in the blood in contrast to $Tlr2^{-/-}$ mice, which failed to display a significant increase in parasitemia. Nevertheless, the $Tlr2^{-/-}$/$Tlr9^{-/-}$ mice were even more susceptible than the $Tlr9^{-/-}$ mice, indicating that although TLR9 may have a dominant role in innate recognition of the parasite, TLR2 also contributes to this process (Fig. 3*A*). The enhanced susceptibility to *T. cruzi* infection was associated with decreased serum levels of IL-12 as well as IFN-γ in the $Tlr9^{-/-}$ and $Tlr2^{-/-}Tlr9^{-/-}$ mice (Fig. 3*B*).

We have previously shown that $Myd88^{-/-}$ mice display a 4-fold enhancement in parasitemia and accelerated mortality

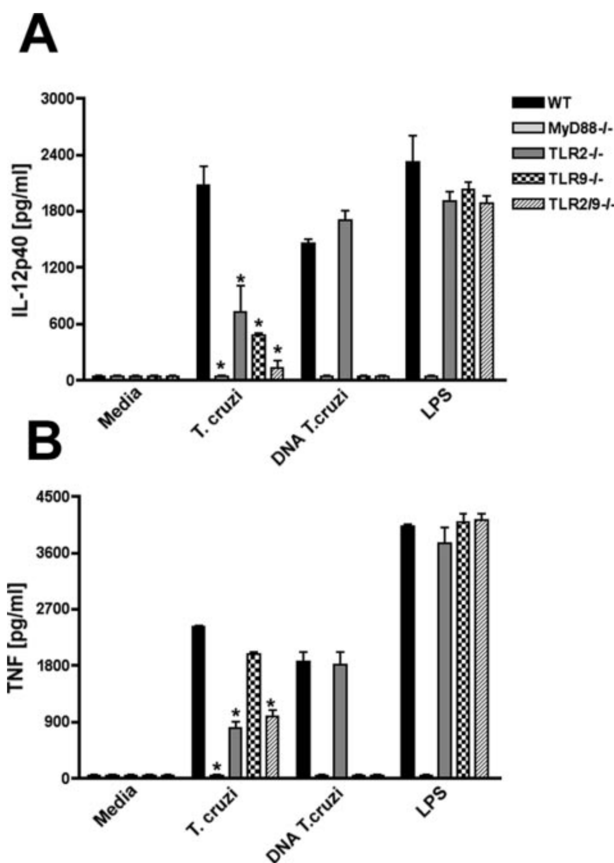

FIGURE 2. Role of TLR2 and TLR9 in the regulation of proinflammatory cytokine responses by *T. cruzi*-exposed macrophages in vitro. Peritoneal macrophages from WT, MyD88-, TLR2-, TLR9-, or TLR2/TLR9-deficient mice were exposed to live *T. cruzi* (1 parasite: 1 cell), DNA from *T. cruzi* (10 μg/ml), or LPS (1 μg/ml) for 20 h. IL-12p40 (*A*) and TNF (*B*) were measured in culture supernatants by ELISA. Results are means ± SE of triplicate measurements. Experiments shown are representative of two performed. *, $p < 0.05$ between WT vs KO values.

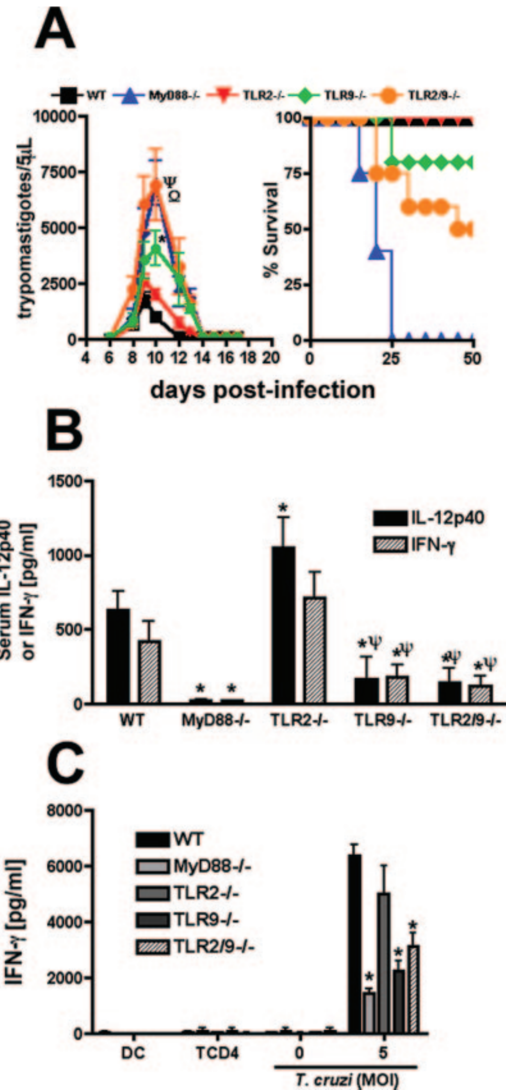

FIGURE 3. Interaction of TLR9 and TLR2 in host resistance to *T. cruzi* infection. *A*, WT, $Myd88^{-/-}$, $Tlr2^{-/-}$, $Tlr9^{-/-}$, and $Tlr2^{-/-}Tlr9^{-/-}$ mice were infected with 10^3 trypomastigotes of the Y strain of *T. cruzi,* parasitemia (*left panel*) was assessed daily and survival (*right panel*) was monitored. *Left panel*, Statistical analysis conducted at day 10 postinfection denotes differences in blood trypomastigotes between animal groups revealed statistically significant differences between Tlr9KO vs WT or Tlr2KO (*, $p < 0.05$). When comparing parasitemia from $Tlr2^{-/-}Tlr9^{-/-}$ (or $Myd88^{-/-}$) groups to WT (ψ) or $Tlr2^{-/-}$ mice (Ω), the symbols indicate that differences are statistically significant ($p < 0.05$). *Right panel*, Statistical analysis revealed that the MyD88- and TLR2/TLR9-deficient mice were significantly more susceptible ($p < 0.01$) than WT animals and that the mean survival curve of $Tlr2^{-/-}Tlr9^{-/-}$ mice is significantly different from that of the $Myd88^{-/-}$ ($p = 0.01$) animal group. The experiment shown is representative of two to three performed. *B*, Animals described in *A* were bled at 10 days postinfection and IL-12p40 (■) as well as IFN-γ (▨) levels were measured in sera by ELISA. *C*, Purified splenic $CD4^+$ T cells from mice as described in *A* were cocultured with syngeneic BMDC infected with *T. cruzi* (multiplicity of infection = 5) for 72 h. IFN-γ was assayed by ELISA in culture supernatants. The means ± SE of measurements from triplicate wells are presented. The experiment shown was performed twice with similar results. *, Values significantly different ($p < 0.05$) between WT and KO cells.

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when compared with WT mice challenged with *T. cruzi* (5). Importantly, Tlr2$^{-/-}$Tlr9$^{-/-}$ and Myd88$^{-/-}$ animals displayed similar numbers of circulating parasites in the blood, suggesting that together TLR2 and TLR9 account for most of the Myd88-dependent resistance to acute infection with *T. cruzi*. However, whereas Myd88$^{-/-}$ mice succumbed by day 30 postinfection, only 40–50% of the Tlr2$^{-/-}$Tlr9$^{-/-}$ animals died in a 50-day period (Fig. 3*A*), a finding that predicts the involvement of additional MyD88-dependent TLR/IL-1R family member(s) in the control of mortality.

In contrast to TLR2, TLR9 controls IFN-γ recall responses by CD4$^+$ T cells from T. cruzi*-infected mice*

Because IFN-γ is a major mediator of resistance to *T. cruzi* and our data indicate an important influence of TLR9 on parasite-induced IFN-γ in vivo, we asked whether defects in in vivo IFN-γ responses are also evident in Tlr9$^{-/-}$ as well as Tlr2$^{-/-}$Tlr9$^{-/-}$ mice. To do so, we tested the recall responses of splenic CD4$^+$ T cells from 10 day infected mice using BMDC from WT animals that had been infected in vitro with live *T. cruzi* as APCs. As shown in Fig. 3*C*, major defects in *T. cruzi*-specific IFN-γ responses were observed in Myd88$^{-/-}$, Tlr9$^{-/-}$, and Tlr2$^{-/-}$Tlr9$^{-/-}$ mice, but not in the Tlr2$^{-/-}$ animals.

It has been difficult to attribute the documented high susceptibility of Myd88$^{-/-}$ mice to *T. cruzi* infection to the role of a single TLR (5, 7). Two possible explanations are: 1) that an as yet-to-be-determined TLR accounts for the MyD88-dependent resistance; or 2) that different TLR act in concert in determining pathogen control. In this study, we have demonstrated that TLR9 is activated by *T. cruzi* genomic DNA and plays a major role in the early recognition of this parasite contributing to the activation of innate immune mechanisms such as DC and macrophage IL-12 production as well as the development of the adaptive immune response to the pathogen. In addition, we provide evidence for the cooperation of TLR9 with a second TLR (TLR2) in the induction of optimal host resistance to *T. cruzi*.

TLR9 is known to participate in the control of *T. brucei* infection and the Th1 responses it induces in mice (10). Nevertheless, the role of this receptor and its possible collaboration with other TLR in host resistance to *T. cruzi* have never been addressed. The findings presented herein confirm that *T. cruzi* DNA stimulates proinflammatory cytokine production through TLR9 and more importantly establish a role for this TLR in the IL-12 and IFN-γ/Th1 responses to live *T. cruzi* in vitro as well as in vivo. TLR9 deficiency was also associated with significantly increased parasitemia compared with WT animals and *T. cruzi*-infected Tlr9$^{-/-}$ but not Tlr2$^{-/-}$ mice showed impaired CD4$^+$ T cell IFN-γ recall responses. However, in contrast to Tlr2$^{-/-}$ macrophages, Tlr9$^{-/-}$ cells did not show defective TNF production in vitro, suggesting that TLR2 and TLR9 control distinct arms of the immune response to the parasite and that stimulation of both TLR2 and TLR9 in vivo (putatively by GPI and DNA) is required for optimal effector function and host resistance against *T. cruzi.* Because no defects in IL-10 production by spleen cells were noted in the *T. cruzi*-exposed TLR2$^{-/-}$ animals (data not shown), their resistance to infection cannot be attributed to the absence of TLR2-dependent triggering of this cytokine as described by others (15).

Although Tlr2$^{-/-}$Tlr9$^{-/-}$ and Myd88$^{-/-}$ animals displayed similar peripheral blood parasite counts, the former group did not show the acute mortality exhibited by Myd88$^{-/-}$ mice. This observation suggests that additional TLR/IL-1R family members are involved in the pathogenesis of *T. cruzi* infection in mice. A role for TLR4 has been suggested by the significantly enhanced susceptibility of mice deficient in this receptor to *T. cruzi* challenge (7). Therefore, it will be of interest to determine whether greater defects in resistance to *T. cruzi* will be evident in mice with the appropriate triple receptor (TLR2-TLR4-TLR9) deficiency. Although not evaluated in the present study, the contribution of MyD88-independent TLR-mediated mechanisms such as those involving the adaptor molecule TRIF should be considered. The participation of MyD88-independent signaling pathways in host resistance to *T. cruzi* is also suggested by the enhanced susceptibility to the parasite of IFN-γ$^{-/-}$ mice relative to MyD88-deficient animals (5).

Th1 responses have previously (1, 4) been demonstrated to play a major role in control of *T. cruzi* infection. Importantly, in the present study the decreased resistance of both Tlr9$^{-/-}$ and Tlr2$^{-/-}$Tlr9$^{-/-}$ mice was associated with impaired serum IFN-γ production as well as CD4$^+$/IFN-γ responses suggesting a specific effect on Th1 response generation. A similar effect of TLR9 deficiency on Th1 expression has been noted in recent studies in both *T. brucei* (10) and mycobacterial experimental infection models (11). Indeed, in the latter report TLR2 and TLR9 were observed to cooperate in resistance to *Mycobacterium tuberculosis* with TLR2 playing a major role in controlling TNF production by macrophages and lung pathology and TLR9 regulating IFN-γ synthesis by CD4$^+$ T cells (11). In the present experiments, we were unable to detect alterations in liver, spleen, or cardiac tissue pathology in Tlr2$^{-/-}$ mice (data not shown). Instead TLR2 function was primarily detected through its cooperation with TLR9 in control of pathogen load and role in regulating macrophage TNF and IL-12 responses in vitro. Because no effect of TLR2 deficiency on in vivo IL-12 or IFN-γ responses was detected and TNF is known to be crucial for NO synthase 2 expression and host resistance to *T. cruzi* (16–18) we speculate that TLR2 regulates control of the parasite through its influence on the latter effector arm. We propose that although asymptomatic in the single Tlr2$^{-/-}$ animals, the effects of this TNF response defect become accentuated in Tlr2$^{-/-}$Tlr9$^{-/-}$ mice because of the simultaneous reduction in TLR9-dependent IFN-γ response. Although TLR2/TLR9 interaction has now been shown to regulate host resistance to both *M. tuberculosis* and *T. cruzi*, the latter pathogen is distinct from the former in that it rapidly escapes host endocytic vacuoles and dwells in the cytoplasm (19), which is thought to be devoid of TLR9 (20, 21). Therefore, it is likely that TLR9 stimulation occurs either during the initial transient residence of the parasite within lysosome fused parasitophorus vacuoles following invasion or through phagocytosis of trypomastigotes already killed through action of other innate defense mechanisms. The cellular pathways leading to TLR9 activation in both *T. cruzi* and *M. tuberculosis* infections and the mechanisms underlying cooperation with TLR2 signaling are currently under study in our laboratories.

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Disclosures

The authors have no financial conflict of interest.

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