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TLR-Dependent Induction of IFN- β Mediates Host Defense against *Trypanosoma cruzi*¹

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Host resistance to the intracellular protozoan parasite *Trypanosoma cruzi* depends on IFN- γ production by T cells and NK cells. However, the involvement of innate immunity in host resistance to *T. cruzi* remains unclear. In the present study, we investigated host defense against *T. cruzi* by focusing on innate immunity. Macrophages and dendritic cells (DCs) from MyD88^{-/-}TRIF^{-/-} mice, in which TLR-dependent activation of innate immunity was abolished, were defective in the clearance of *T. cruzi* and showed impaired induction of IFN- β during *T. cruzi* infection. Neutralization of IFN- β in MyD88^{-/-} macrophages led to enhanced *T. cruzi* growth. Cells from MyD88^{-/-}IFNAR1^{-/-} mice also showed impaired *T. cruzi* clearance. Furthermore, both MyD88^{-/-}TRIF^{-/-} and MyD88^{-/-}IFNAR1^{-/-} mice were highly susceptible to in vivo *T. cruzi* infection, highlighting the involvement of innate immune responses in *T. cruzi* infection. We further analyzed the molecular mechanisms for the IFN- β -mediated antitrypanosomal innate immune responses. MyD88^{-/-}TRIF^{-/-} and MyD88^{-/-}IFNAR1^{-/-} macrophages and DCs exhibited defective induction of the GTPase IFN-inducible p47 (IRG47) after *T. cruzi* infection. RNA interference-mediated reduction of IRG47 expression in MyD88^{-/-} macrophages resulted in increased intracellular growth of *T. cruzi*. These findings suggest that TLR-dependent expression of IFN- β is involved in resistance to *T. cruzi* infection through the induction of IRG47. *The Journal of Immunology*, 2006, 177: 7059–7066.

The parasite *Trypanosoma cruzi* is an intracellular protozoan that causes Chagas' disease, a chronic systemic disorder affecting nearly 20 million people in Central and South America. Host defense against *T. cruzi* depends on a variety of cell populations, including NK, CD4⁺ T cells, CD8⁺ T cells, and Ig-producing B cells (1–3). In addition, macrophages and dendritic cells (DCs)³ produce proinflammatory cytokines, such as IL-12, in response to invasion by *T. cruzi* (4–6). IL-12 induces IFN- γ production by NK, CD4⁺ T cells, and CD8⁺ T cells. In turn, IFN- γ induces NO production by macrophages and mediates the killing of *T. cruzi* (7, 8). This cytokine milieu is therefore responsible for host resistance to *T. cruzi* infection in vivo. However, it remains uncertain how innate immune cells, such as macrophages and DCs, mediate *T. cruzi*-induced immune responses during the early phase of infection. In addition, *T. cruzi* infection induces the

production of type I IFNs ($\alpha\beta$ IFN), which possess antiviral activities (9, 10). However, the nature of the involvement of type I IFNs in response to *T. cruzi* infection remains controversial (11).

A family of TLRs has been identified that recognize specific components of various microorganisms, including bacteria, viruses, fungi, and protozoan parasites (12). Recognition of microbial components by TLRs triggers the activation of innate immunity and the subsequent development of Ag-specific adaptive immunity. TLR-mediated signaling pathways originate from the cytoplasmic Toll/IL-1R (TIR) domains, which are conserved among all family members. A group of TIR domain-containing adaptors (MyD88, Toll/IL-1R domain-containing adaptor protein, TIR domain-containing adaptor-inducing IFN- β (TRIF), and TRIF-related adaptor molecule) have been shown to be integral to these TLR signaling pathways (13). The TLR signaling pathways consist of two cascades: a MyD88-dependent pathway and a TRIF-dependent (MyD88-independent) pathway. The MyD88-dependent pathway mediates all TLR-induced productions of proinflammatory cytokines, including IL-12p40, whereas the TRIF-dependent pathway is indispensable for the induction of type I IFNs through TLR3 and TLR4.

Previous studies have analyzed the involvement of TLR-dependent activation of innate immunity in *T. cruzi* infection. TLR2, TLR4, and TLR9 have been implicated in the recognition of *T. cruzi*-derived components (6, 14–16), whereas mice lacking MyD88 were found to be susceptible to the acute phase of *T. cruzi* infection accompanied by defective proinflammatory cytokine production (17). However, even in MyD88-deficient mice, significant IFN- γ production was still observed, indicating the presence of MyD88-independent immune responses. Thus, the nature of the involvement of innate immunity in *T. cruzi* infection still remains to be precisely characterized.

In the present study, we analyzed the involvement of innate immune cells in *T. cruzi* infection using mice lacking both

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³ Abbreviations used in this paper: DC, dendritic cell; TIR, Toll/IL-1R; TRIF, TIR domain-containing adaptor-inducing IFN- β ; WT, wild type; siRNA, small interfering RNA; EF-1 α , elongation factor-1 α .

MyD88 and TRIF, in which all of the previously described TLR-mediated activation mechanisms of innate immunity are totally abolished.

Materials and Methods

Mice

MyD88^{-/-} and TRIF^{-/-} mice were generated as previously described (18, 19). Type I IFN receptor (IFNAR1)^{-/-} mice were purchased from B & K Universal (20). Each mouse strain was backcrossed to C57BL/6 for at least five generations, and then used to generate double-mutant mice. MyD88^{-/-}TRIF^{-/-} mice were generated by crossing MyD88^{+/-}TRIF^{+/-} mice. Littermate wild-type (WT) (MyD88^{+/-}TRIF^{+/-}), MyD88^{-/-} (MyD88^{-/-}TRIF^{+/-}), and TRIF^{-/-} (MyD88^{+/-}TRIF^{-/-}) mice were used for the experiments. MyD88^{-/-}IFNAR1^{-/-} mice were generated by crossing MyD88^{+/-}IFNAR1^{+/-} mice, and used for the experiments at 8–10 wk of age. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University.

Preparation of macrophages and DCs

To isolate peritoneal macrophages, mice were i.p. injected with 2 ml of 4% thioglycolate medium (Sigma-Aldrich), and peritoneal exudate cells were isolated from the peritoneal cavity at 3 days postinjection. The cells were incubated for 2 h and washed three times with HBSS. The remaining adherent cells were used as peritoneal macrophages in experiments. To prepare bone marrow-derived DCs or macrophages, bone marrow cells were prepared from the femur and tibia, passed through a nylon mesh and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 mM 2-ME, and 10 ng/ml GM-CSF (PeproTech) or 30% L cell culture supernatant. After 6 days, the cells were used as DCs or macrophages in experiments.

Parasites and experimental infection

The *T. cruzi* Tulahuén strain was maintained in vivo in IFN- γ R^{-/-} mice by passages every other week (21). For in vitro experiments, macrophages or DCs (5×10^6) were infected with 5×10^4 trypomastigotes. After 6 h of infection, the cells were washed twice with PBS to remove the extracellular parasites and cultured in RPMI 1640 supplemented with 10% FBS for the indicated time periods. Trypomastigotes in the culture supernatants were counted microscopically. Alternatively, the cells were pulsed with 1 μ Ci of [³H]uracil and cultured for 72 h. The cells were then harvested on glass fiber filters and the incorporated uracil was measured using a liquid scintillation counter. The net cpm was calculated by subtracting the background cpm in uninfected cultures from the cpm of the infected cultures. In some experiments, macrophages were infected with *T. cruzi* in the absence or presence of 10 ng/ml of an anti-IFN- β neutralizing Ab (YAMASA) for 6 h, washed and then further cultured with or without the anti-IFN- β Ab.

In other experiments, extracellular parasites were removed by repeated washing after 6h of infection, and the cells were incubated for a further 48 h. Subsequently, the cells were washed, fixed and stained using a Diff-Quik kit (Sysmex). The intracellular parasite numbers in 250 macrophages were counted under a light microscope. Counting was performed in a blinded manner by two independent investigators.

For in vivo experiments, mice were i.p. injected with plasma containing 2×10^3 or 1×10^4 trypomastigotes as indicated. The number of parasites in the blood of each animal was then counted microscopically using 5 μ l of blood taken from the tail. Statistical significance was determined using a paired Student's *t* test. Differences were considered to be statistically significant at *p* < 0.05.

Measurement of cytokine production

Peritoneal macrophages or DCs (5×10^4) were infected with 5×10^4 *T. cruzi* for 6 h, extensively washed and cultured for 24 h. The culture supernatants were collected and analyzed for their levels of TNF- α by ELISA (Genzyme Techne) and NO using the Griess reagent (Dojindo Laboratories).

Quantitative real-time RT-PCR

Total RNA was isolated with an RNeasy mini kit (Qiagen), and 2 μ g of the RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega) and oligo(dT) primers (Toyobo) after treatment with RQ1 DNase I (Promega). Quantitative real-time PCR was performed in an ABI 7000 (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). All data were normalized to the corresponding level of elon-

gation factor-1 α (EF-1 α) expression, and the fold difference relative to the EF-1 α level was calculated. The amplification conditions were: 50°C (2 min), 95°C (10 min), and 40 cycles of 95°C (15 s), and 60°C (60 s). Each experiment was performed independently at least three times, and the results of one representative experiment are shown. All primers were purchased from Assay on Demand (Applied Biosystems).

RNA interference

For small interfering RNA (siRNA) experiments, dsRNA duplexes targeting the coding region of the GTPase IFN-inducible p47 (IRG47) (5'-GGUGGAUAGAGACUUAUUt-3') were synthesized by Ambion. Bone marrow cells were cultured in the presence of 30% L cell culture supernatant for 6 days. The differentiated bone marrow macrophages were then harvested by 5 mM EDTA treatment and transfected with 1.5 μ g of the siRNA using Nucleofector and a Mouse Macrophage Nucleofector kit (Amaya Biosystems) according to the manufacturer's instructions. The cells were immediately transferred to culture medium and cultured for 18 h. Next, cells were infected with *T. cruzi* for 48 h, and parasite growth was analyzed. To determine the efficiency of gene silencing, cells were infected with *T. cruzi* for 6 h, and the expression of IRG47 mRNA was analyzed by quantitative real-time RT-PCR.

Results

Increased growth of *T. cruzi* in MyD88^{-/-}TRIF^{-/-} DCs and macrophages

To study the direct involvement of innate immunity in *T. cruzi* infection, bone marrow-derived DCs prepared from WT, MyD88^{-/-}, TRIF^{-/-}, or MyD88^{-/-}TRIF^{-/-} mice were infected with *T. cruzi*. After 6 h of *T. cruzi* infection, the cells were extensively washed and changed to fresh medium. After culture periods of 4, 5, and 7 days, the number of trypomastigotes released into the culture supernatants were counted (Fig. 1A). The culture supernatant of TRIF^{-/-} DCs contained a similar number of trypomastigotes to that of WT DCs. For MyD88^{-/-} DCs, the number of trypomastigotes increased after 5 and 7 days of infection. Furthermore, for MyD88^{-/-}TRIF^{-/-} DCs, the number of trypomastigotes increased considerably. Next, peritoneal macrophages were infected with *T. cruzi* (Fig. 1B). The number of trypomastigotes in the culture supernatant of MyD88^{-/-} macrophages was slightly increased compared with those of WT or TRIF^{-/-} cells after 5 and 7 days of infection. For MyD88^{-/-}TRIF^{-/-} macrophages, larger numbers of trypomastigotes were observed compared with the other cell genotypes after 7 days of infection. Next, replication of *T. cruzi* within macrophages was assessed based on [³H]uracil incorporation (Fig. 1C). Intracellular growth of *T. cruzi* was slightly increased in MyD88^{-/-} macrophages, and markedly increased in MyD88^{-/-}TRIF^{-/-} cells compared with WT cells. Bone marrow-derived macrophages were also infected with *T. cruzi* and cultured for 48 h, before the number of intracellular parasites was counted. The number of infected cells did not differ among the genotypes (data not shown). However, infected MyD88^{-/-}TRIF^{-/-} macrophages contained an increased number of parasites after 48 h of infection (Fig. 1, D and E). Thus MyD88^{-/-} DCs and macrophages showed a slight increase in *T. cruzi* growth, whereas MyD88^{-/-}TRIF^{-/-} cells showed a marked increase in growth, indicating that MyD88^{-/-}TRIF^{-/-} DCs and macrophages were defective in the clearance of *T. cruzi*.

Defective *T. cruzi* induction of proinflammatory mediators in MyD88^{-/-} macrophages and DCs

The killing of parasites by macrophages has been shown to be mediated by TNF- α and NO (22–25). Therefore, we next analyzed the production of TNF- α and NO by *T. cruzi*-infected peritoneal macrophages (Fig. 2). Both WT and TRIF^{-/-} macrophages secreted TNF- α and NO in response to *T. cruzi* infection. In contrast,

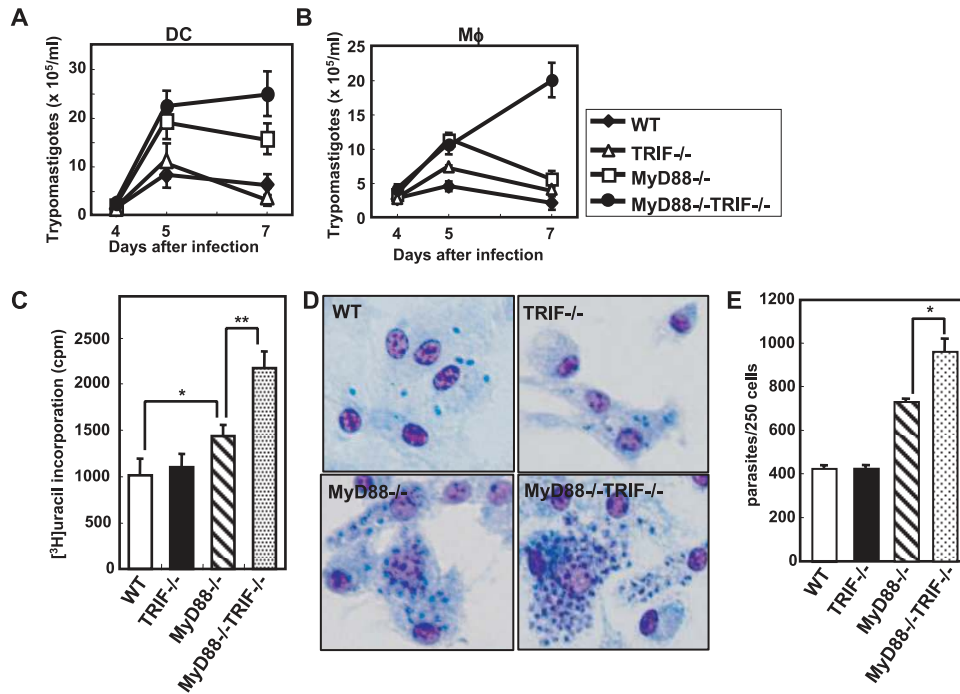


FIGURE 1. Defective *T. cruzi* clearance in MyD88^{-/-}TRIF^{-/-} DCs and macrophages. Bone marrow-derived DCs (A) or peritoneal macrophages (Mφ) (B) from WT, TRIF^{-/-}, MyD88^{-/-}, or MyD88^{-/-}TRIF^{-/-} mice were seeded onto 96-well plates, and infected with *T. cruzi* for 6 h. The cells were then washed to remove the extracellular parasites and cultured for the indicated periods, before the numbers of trypanostigotes in the culture supernatants were counted. Data are representative of four independent experiments. C, Peritoneal macrophages were infected with *T. cruzi*, washed and cultured in the presence of [³H]uracil for 72 h, before the [³H]uracil incorporation was measured. *, *p* < 0.01; **, *p* < 0.005. D and E, Bone marrow-derived macrophages were infected with *T. cruzi*, washed, and cultured for 48 h. The cells were then fixed, stained, and analyzed by microscopy. Representative stained cells from three independent experiments are shown. Magnification, ×400. The intracellular parasites were counted, and the data represent the mean + SD of the number of parasites per 250 macrophages. *, *p* < 0.02.

secretion of these mediators was severely reduced in both MyD88^{-/-} and MyD88^{-/-}TRIF^{-/-} macrophages, and no significant differences were observed between the two genotypes. These findings indicate that *T. cruzi*-induced production of TNF-α and NO was dependent on MyD88, but that the higher susceptibility to *T. cruzi* infection of MyD88^{-/-}TRIF^{-/-} macrophages was not due to defective induction of these mediators.

Defective *T. cruzi* induction of IFN-inducible genes in MyD88^{-/-}TRIF^{-/-} macrophages and DCs

Next, we tried to identify which genes were selectively less active in *T. cruzi*-infected MyD88^{-/-}TRIF^{-/-} DCs. *T. cruzi* infection has been shown to induce IFN-β (9, 10). Therefore, we analyzed *T. cruzi*-induced gene expression focusing on IFN-β and IFN-inducible chemokines as well as proinflammatory cytokines in peritoneal macrophages and DCs from WT, TRIF^{-/-}, MyD88^{-/-}, and MyD88^{-/-}TRIF^{-/-} mice by quantitative real-time RT-PCR. In WT and TRIF^{-/-} macrophages, *T. cruzi* infection led to robust induction of TNF-α and IL-12p40 mRNAs (Fig. 3A). In contrast, both MyD88^{-/-} and MyD88^{-/-}TRIF^{-/-} macrophages showed defective induction of TNF-α and IL-12p40. Expression of the mRNAs for IFN-β and IFN-inducible genes, such as *Ccl2* (MCP-1), *Ccl5* (RANTES), and *Cxcl10* (IP-10) was induced in *T. cruzi*-infected WT DCs (Fig. 3B). In contrast, *T. cruzi*-induced expression of IFN-α4 mRNA was not observed in any of the macrophage and DC genotypes (data not shown). In MyD88^{-/-} DCs, *T. cruzi*-induced expression of *Ccl2*, *Ccl5*, and *Cxcl10* was only slightly reduced. However, DCs from TRIF^{-/-} and MyD88^{-/-}TRIF^{-/-} mice showed severely impaired induction of these genes after *T. cruzi* infection. Peritoneal macrophages from each genotype showed similar patterns of *T. cruzi*-induced gene expression (Fig.

3C). Thus, MyD88^{-/-} macrophages and DCs showed defective induction of proinflammatory cytokine genes during *T. cruzi* infection, whereas TRIF^{-/-} cells showed defective induction of IFN-β and IFN-inducible genes during the infection. Furthermore, MyD88^{-/-}TRIF^{-/-} cells displayed defective expression of all these genes.

IFN-β-mediated inhibition of *T. cruzi* growth in MyD88^{-/-} macrophages

MyD88^{-/-}TRIF^{-/-} macrophages and DCs displayed defective clearance of *T. cruzi* with impaired expression of IFN-β and IFN-inducible genes. Therefore, we next addressed whether IFN-β is involved in the resistance to *T. cruzi* infection in MyD88^{-/-} macrophages. Peritoneal macrophages from WT and MyD88^{-/-} mice were infected with *T. cruzi* in the presence of an anti-IFN-β neutralizing Ab, and intracellular growth of *T. cruzi* was measured (Fig. 4). In WT macrophages, *T. cruzi* growth remained unaltered by the addition of the anti-IFN-β Ab. In contrast, anti-IFN-β Ab addition dramatically increased the intracellular growth of *T. cruzi* in MyD88^{-/-} macrophages. These findings indicate the possible involvement of IFN-β in resistance to *T. cruzi* infection in the absence of MyD88.

High-sensitivity to *T. cruzi* infection in MyD88^{-/-}IFNAR1^{-/-} macrophages

To further address whether IFN-β is involved in the resistance to *T. cruzi* infection, we generated mice lacking both MyD88 and the IFNAR1 subunit of the αβ IFN receptor complex (MyD88^{-/-}IFNAR1^{-/-} mice). Bone marrow-derived macrophages were infected with *T. cruzi*, washed, and cultured. After culture periods of 4, 5, and 7 days, the numbers of trypanostigotes in the culture

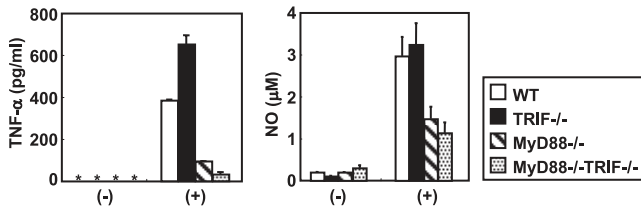


FIGURE 2. Defective production of TNF- α and NO in *T. cruzi*-infected MyD88^{-/-} macrophages. Peritoneal macrophages from WT, TRIF^{-/-}, MyD88^{-/-}, or MyD88^{-/-}TRIF^{-/-} mice were infected with (+) or without (-) *T. cruzi* for 6 h, washed to remove the extracellular parasites, and cultured for 24 h. The levels of TNF- α and NO in the culture supernatants were measured. *, Not detected.

supernatants were counted (Fig. 5A). As mentioned, the culture supernatant of MyD88^{-/-} macrophages contained a larger number of trypomastigotes than that of WT macrophages. In the supernatant of IFNAR1^{-/-} macrophages, a slight increase in the number of trypomastigotes was observed compared with WT cells. Furthermore, the number of trypomastigotes in the culture supernatant of MyD88^{-/-}IFNAR1^{-/-} macrophages was considerably increased. Next, intracellular replication of *T. cruzi* was assessed by counting [³H]uracil incorporation (Fig. 5B). MyD88^{-/-} and IFNAR1^{-/-} macrophages showed slightly increased growth rates of *T. cruzi*. However, MyD88^{-/-}IFNAR1^{-/-} macrophages showed markedly increased growth rates of *T. cruzi* compared with MyD88^{-/-} or IFNAR1^{-/-} cells. Furthermore, at 48 h after the *T. cruzi* infection, increased numbers of parasites were observed in MyD88^{-/-}IFNAR1^{-/-} macrophages (Fig. 5, C and D). Thus, IFNAR1^{-/-} macrophages displayed a slightly increased sensitivity to *T. cruzi* infection, whereas MyD88^{-/-}IFNAR1^{-/-} macrophages displayed a higher sensitivity to the infection. These findings suggest that IFN- β is responsible for resistance to *T. cruzi* infection and that this responsibility becomes evident in the absence of MyD88.

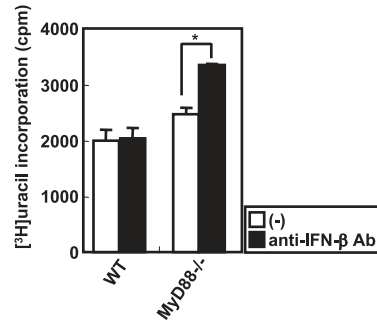
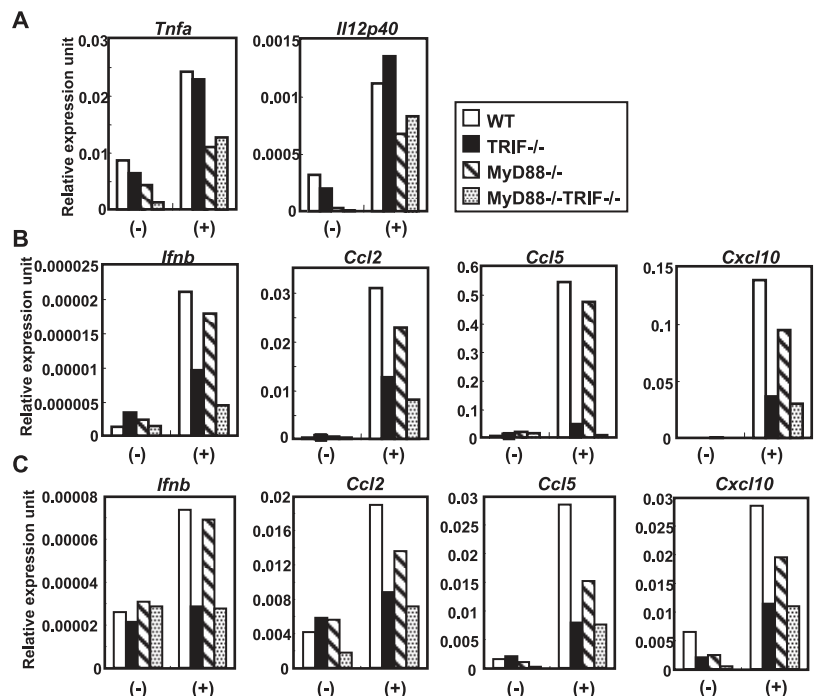


FIGURE 4. Effect of an anti-IFN- β neutralizing Ab on *T. cruzi* growth in macrophages. Peritoneal macrophages from WT or MyD88^{-/-} mice were infected with *T. cruzi* for 6 h in the presence or absence of an anti-IFN- β neutralizing Ab, washed, and cultured in the presence of [³H]uracil for 72 h. The [³H]uracil incorporation was then measured. *, $p < 0.005$.

High-sensitivity MyD88^{-/-}IFNAR1^{-/-} mice to *T. cruzi* infection

Macrophages are the primary site of *T. cruzi* replication, and thus act as the major cell population for controlling the infection in vivo, especially for reticulotropic strains such as the Tulahuén strain used in the present study (21, 26). Therefore, we next addressed whether IFN- β mediates antitrypanosomal responses in vivo. Mice were i.p. infected with *T. cruzi*, and the parasitemia was monitored (Fig. 6A). In WT and TRIF^{-/-} mice, the trypomastigote counts in the sera peaked by day 13 of the infection, and subsequently decreased. In IFNAR1^{-/-} mice, serum trypomastigotes were slightly increased compared with WT or TRIF^{-/-} mice, and peaked around 11–13 days of infection. In MyD88^{-/-} mice, the parasite counts were increased at 13 days of infection. In MyD88^{-/-}TRIF^{-/-} mice, the serum parasite counts continued to increase, and these mice showed much higher levels of parasitemia by day 15 of infection than levels found in MyD88^{-/-} mice. In MyD88^{-/-}IFNAR1^{-/-} mice, the parasite counts increased in a similar manner

FIGURE 3. *T. cruzi*-induced expression of inflammatory genes in macrophages and DCs. A, Peritoneal macrophages from WT, TRIF^{-/-}, MyD88^{-/-}, or MyD88^{-/-}TRIF^{-/-} mice were cultured in the presence (+) or absence (-) of *T. cruzi* for 6 h. Total RNA was then extracted and analyzed for the expressions of *Tnfa* or *Il12p40* by quantitative real-time RT-PCR. The data are shown as the relative mRNA levels normalized by the corresponding EF-1 α mRNA level. Bone marrow-derived DCs (B) or peritoneal macrophages (C) from WT, TRIF^{-/-}, MyD88^{-/-}, or MyD88^{-/-}TRIF^{-/-} mice were cultured in the presence (+) or absence (-) of *T. cruzi* for 6 h. Total RNA was then extracted and analyzed for the expressions of *Ifnb*, *Ccl2*, *Ccl5*, and *Cxcl10* by quantitative real-time RT-PCR. Data are presented in relative expression units and have been normalized to the corresponding EF-1 α mRNA level.



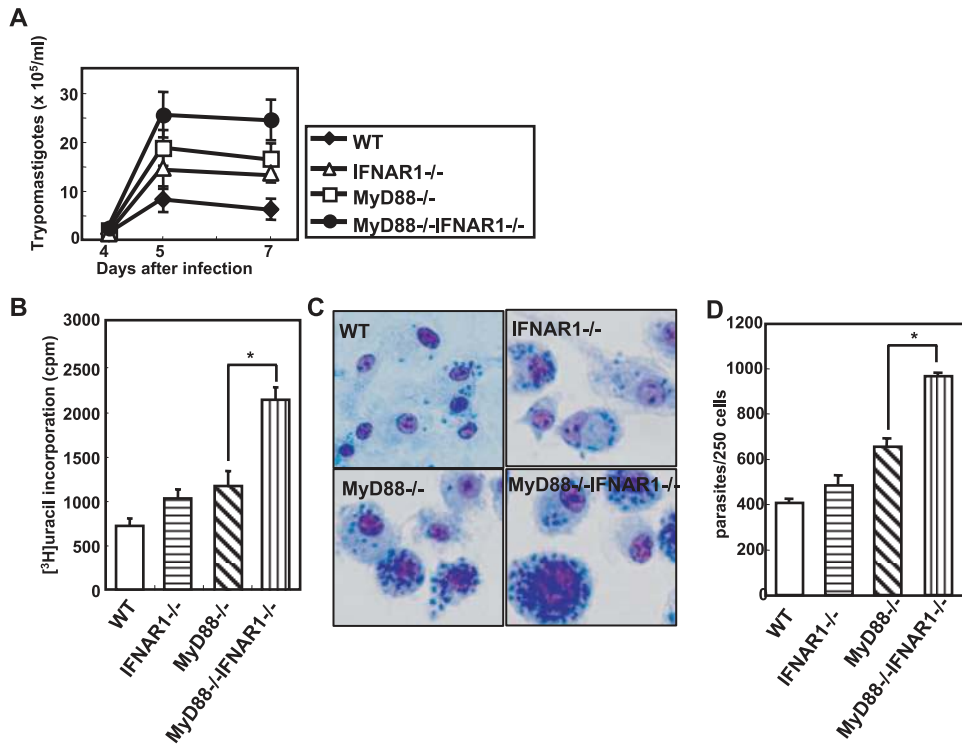


FIGURE 5. Increased *T. cruzi* growth in MyD88^{-/-}IFNAR1^{-/-} macrophages. **A**, Bone marrow-derived macrophages from WT, MyD88^{-/-}, IFNAR1^{-/-}, or MyD88^{-/-}IFNAR1^{-/-} mice were infected with *T. cruzi* for 6 h, washed to remove the extracellular parasites, and cultured for the indicated periods. The trypomastigotes in the culture supernatants were counted. Data are representative of four independent experiments. **B**, Peritoneal macrophages were infected with *T. cruzi*, washed, and cultured in the presence of [³H]juracil for 72 h. The [³H]juracil incorporation was then measured. *, *p* < 0.0001. **C** and **D**, Bone marrow-derived macrophages from each genotype were infected with *T. cruzi*, washed, and cultured for 48 h. The cells were then fixed, stained, and analyzed by microscopy. Representative stained cells from three independent experiments are shown. Magnification, ×400. Intracellular parasites were counted, and the data represent the mean + SD of the number of parasites per 250 macrophages. *, *p* < 0.02.

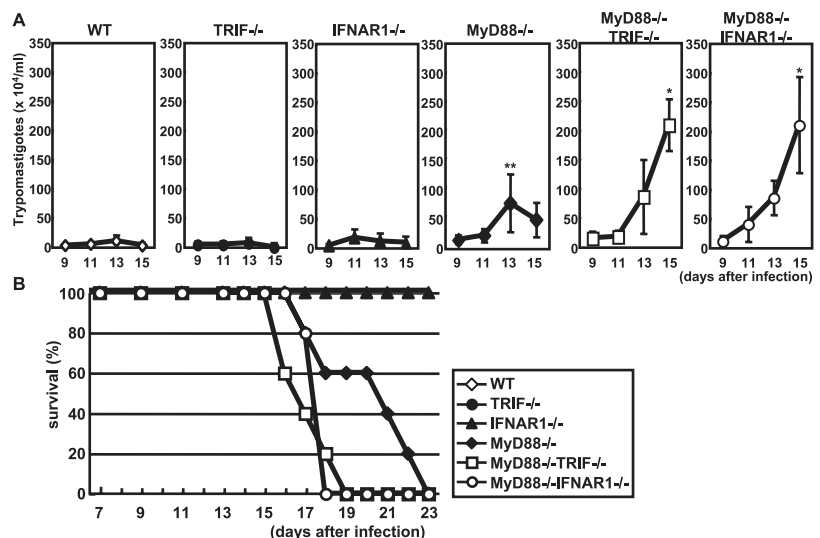
to those in MyD88^{-/-}TRIF^{-/-} mice. We further monitored the mortality of the mice after *T. cruzi* infection (Fig. 6B). WT, TRIF^{-/-}, and IFNAR1^{-/-} mice were resistant to *T. cruzi* infection, and all the mice survived for more than 19 days after the infection, whereas MyD88^{-/-} mice started to die around 15 days after the infection, and about half of the mice had died within 19 days. In contrast, all the MyD88^{-/-}TRIF^{-/-} and MyD88^{-/-}IFNAR1^{-/-} mice died within 19 days of the infection. Thus, MyD88^{-/-}TRIF^{-/-} and MyD88^{-/-}IFNAR1^{-/-} mice were more sensitive to in vivo *T. cruzi* infection than MyD88^{-/-} mice, suggesting that IFN-β mediates in vivo resis-

tance to *T. cruzi* infection, and this effect becomes evident in the absence of MyD88.

Involvement of IFN-β-inducible IRG47 in resistance to T. cruzi infection

Next, we addressed the molecular mechanisms of the IFN-β-mediated resistance to *T. cruzi* infection in innate immune cells. The family of p47 GTPases has been shown to control innate immune responses to intracellular pathogens, including protozoan parasites (27, 28). In addition, expression of p47 GTPases, such as LRG47

FIGURE 6. High-sensitivity MyD88^{-/-}IFNAR1^{-/-} mice to *T. cruzi* infection. WT (*n* = 9), TRIF^{-/-} (*n* = 10), IFNAR1^{-/-} (*n* = 10), MyD88^{-/-} (*n* = 5), MyD88^{-/-}TRIF^{-/-} (*n* = 5), and MyD88^{-/-}IFNAR1^{-/-} (*n* = 5) mice were i.p. infected with 1 × 10⁴ *T. cruzi*. Parasitemia (**A**) and mortality (**B**) were monitored at the indicated times after infection. *, *p* < 0.001 compared with MyD88^{-/-} mice and **, *p* < 0.005 compared with control mice.



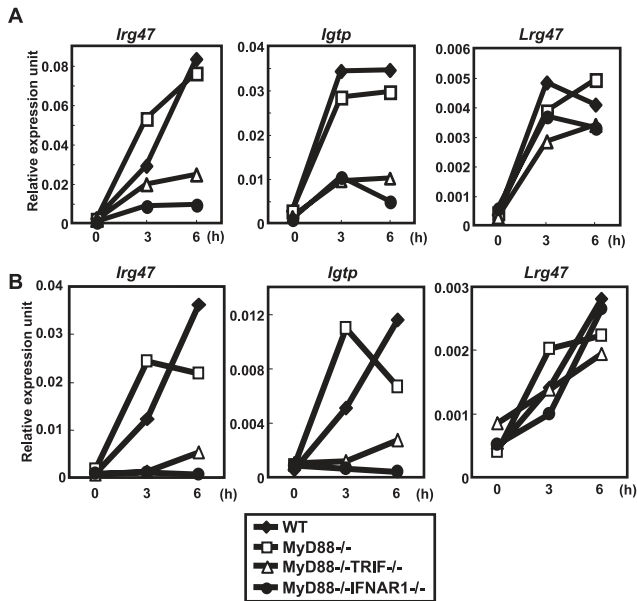


FIGURE 7. Impaired expression of IRG47 in *T. cruzi*-infected MyD88^{-/-}TRIF^{-/-} mice. Bone marrow-derived macrophages (A) or DCs (B) from WT, MyD88^{-/-}, MyD88^{-/-}TRIF^{-/-}, or MyD88^{-/-}IFNAR1^{-/-} mice were infected with *T. cruzi* for 3 or 6 h. Next, total RNA was extracted and analyzed for the expressions of *Irg47*, *Igtp*, and *Lrg47* by quantitative real-time RT-PCR. Data are shown as the relative mRNA levels normalized to the corresponding EF-1 α mRNA level.

and IRG47, and inducibly expressed GTPase (IGTP), has been shown to be induced through activation of TLR and IFN signaling pathways during infection with intracellular pathogens (27, 28). Therefore, we analyzed the expression levels of these p47 GTPases in *T. cruzi*-infected DCs and macrophages. Bone marrow-derived macrophages or DCs from WT, MyD88^{-/-}, MyD88^{-/-}TRIF^{-/-}, and MyD88^{-/-}IFNAR1^{-/-} mice were infected with *T. cruzi* for 3 or 6 h, and the expression of LRG47, IRG47, and IGTP mRNAs was analyzed (Fig. 7, A and B). In WT and MyD88^{-/-} macrophages and DCs, *T. cruzi* infection resulted in robust mRNA expressions of all these p47 GTPases. Even in MyD88^{-/-}TRIF^{-/-} and MyD88^{-/-}IFNAR1^{-/-} cells, almost normal *T. cruzi*-induced expression of LRG47 mRNA was observed. However, *T. cruzi*-

induced expression of IRG47 and IGTP mRNAs was severely impaired in MyD88^{-/-}TRIF^{-/-} and MyD88^{-/-}IFNAR1^{-/-} macrophages and DCs. Although IGTP was previously shown to have a minor role in *T. cruzi* infection, the involvement of IRG47 in *T. cruzi* infection is less well defined (29). Therefore, we next analyzed whether IRG47 is responsible for antitrypanosomal responses in the absence of MyD88. To complete this analysis, siRNA-mediated knockdown of IRG47 was performed in MyD88^{-/-} macrophages. We transfected an IRG47 or control siRNA into bone marrow-derived macrophages and extracted the total RNA after 18 h for analysis of the IRG47 expression (Fig. 8A). Introduction of the IRG47 siRNA into bone marrow-derived macrophages from MyD88^{-/-} mice resulted in an effective (81%) reduction in IRG47 mRNA expression. MyD88^{-/-} macrophages transfected with the IRG47 or control siRNA were further infected with *T. cruzi*, and the intracellular parasites were visualized and counted (Fig. 8, B and C). In MyD88^{-/-} macrophages, siRNA-mediated knockdown of IRG47 led to increased numbers of intracellular *T. cruzi*. These results indicate that IRG47 is involved in resistance to *T. cruzi* infection in innate immune cells.

Discussion

In the present study, we analyzed innate immune responses to the intracellular protozoan parasite *T. cruzi* using MyD88^{-/-}TRIF^{-/-} mice, in which TLR-dependent activation of innate immunity is not induced. Macrophages and DCs derived from MyD88^{-/-}TRIF^{-/-} mice showed impaired clearance of *T. cruzi*. Analysis of the gene expression profiles of *T. cruzi*-infected MyD88^{-/-}TRIF^{-/-} DCs revealed that IFN- β was induced in a TRIF-dependent manner during *T. cruzi* infection, whereas analyses with an anti-IFN- β neutralizing Ab and MyD88^{-/-}IFNAR1^{-/-} cells demonstrated that IFN- β mediated antitrypanosomal innate immune responses. Furthermore, both MyD88^{-/-}TRIF^{-/-} and MyD88^{-/-}IFNAR1^{-/-} mice were highly sensitive to in vivo *T. cruzi* infection. These findings indicate that MyD88-dependent induction of proinflammatory cytokines and TRIF-dependent induction of IFN- β both contribute to innate immune responses to *T. cruzi* infection. We further showed that the p47 GTPase IRG47 is responsible for the resistance to *T. cruzi* infection in MyD88^{-/-} macrophages.

Type I IFNs are well-known cytokines that exhibit antiviral activities (30). However, a large body of evidence has demonstrated

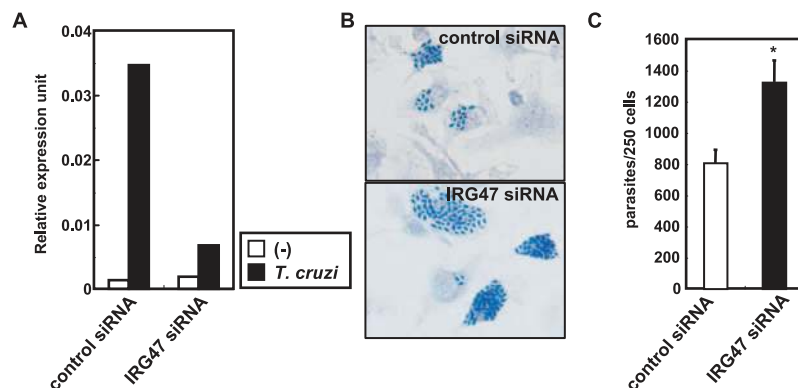


FIGURE 8. IRG47 mediates antitrypanosomal activity in MyD88^{-/-} mice. A, Bone marrow-derived macrophages were transfected with IRG47 or control siRNA and cultured for 18 h. The cells were then infected with *T. cruzi* for 6 h and analyzed for the expression of IRG47 mRNA by quantitative real-time RT-PCR. Data are shown as the relative mRNA levels normalized to the corresponding EF-1 α mRNA level. B and C, Bone marrow-derived macrophages transfected with an IRG47 or control siRNA were infected with *T. cruzi*, washed, and cultured for 48 h. The cells were then fixed, stained, and analyzed by microscopy. Representative stained cells from three independent experiments are shown. Magnification, $\times 400$. Intracellular parasites were counted, and the data represent the mean + SD of the number of parasites per 250 macrophages. *, $p < 0.02$ compared with control siRNA-transfected cells.

that type I IFNs are also induced by nonviral pathogens, such as bacteria, mycobacteria, and protozoan parasites (11, 31). In the case of bacterial infection, type I IFNs seem to have opposing effects depending on the type of bacteria (31). Although exogenous type I IFNs show protective actions in response to infection with *Salmonella typhimurium* or *Shigella flexneri*, the protective effects of endogenous type I IFNs remain unclear (32, 33). In contrast, endogenous type I IFNs reduce resistance to *Listeria monocytogenes* infection (34–36). During infection with the protozoan parasite *Leishmania major*, these exogenous IFNs presumably have a protective effect through the induction of inducible NO synthase, although the involvement of endogenous type I IFNs in antileishmanial immunity is less clear (37, 38). Following infection with *T. cruzi*, administration of exogenous $\alpha\beta$ IFN was reported to reduce the number of serum parasites (10). However, a subsequent study showed that IFNAR1^{-/-} mice were not susceptible to the infection, indicating that endogenous $\alpha\beta$ IFN do not contribute to the host defense against *T. cruzi* (39). Thus, the possible roles of type I IFNs in antitrypanosomal immune responses remain controversial. In the present study, we have clearly established that IFN- β produced by DCs and macrophages contributes to host defense against *T. cruzi*. Thus, endogenous type I IFNs produced during *T. cruzi* infection are responsible for antitrypanosomal immune responses, although the MyD88-dependent production of proinflammatory cytokines overshadows the effects of type I IFNs in normal mice. In the future, it will be interesting to investigate whether this mechanism also applies to immune responses to other protozoan parasites, such as *L. major* and *Toxoplasma gondii*.

We further analyzed the mechanisms by which IFN- β exerts antitrypanosomal responses. The p47 GTPase family members control innate immune responses to intracellular pathogens, including protozoan parasites (27, 28). Expression of p47 GTPases, such as LRG47 and IRG47, and of IGTP is induced through the activation of TLR and IFN signaling pathways during infection with intracellular pathogens. Mice lacking LRG47, IRG47, or IGTP have been shown to become sensitive to infection with *L. major* and *T. gondii*, indicating the possible involvement of these GTPases in *T. cruzi* infection (27, 40, 41). Indeed, LRG47-deficient mice have recently been shown to be sensitive to *T. cruzi* infection (42). We found that induction of IRG47 was impaired in *T. cruzi*-infected cells from MyD88^{-/-}TRIF^{-/-} and MyD88^{-/-}IFNAR1^{-/-} mice. Knockdown of IRG47 in MyD88^{-/-} macrophages led to increased intracellular parasites. Thus, TLR-dependent expression of IFN- β probably mediates antitrypanosomal responses through the induction of IRG47.

Recently, MyD88^{-/-}TRIF^{-/-} macrophages have been shown to produce IFN- β when infected with intracellular pathogens that escape into the cytosol, such as *L. monocytogenes* and *Legionella pneumophila* (43). In contrast, *T. cruzi*-induced IFN- β production was not observed in MyD88^{-/-}TRIF^{-/-} macrophages, although this parasite also invades the cytosol (44). In the case of the cytosolic escape of *Listeria* or *Legionella*, dsDNA from the bacteria is responsible for the induction of IFN- β (43, 45). In contrast to these prokaryotic bacteria, *T. cruzi* is a eukaryote. Therefore, it seems less likely that trypanosomal DNA within the nucleus is exposed to the host cell cytosol, which may lead to the observed absence of TLR-independent induction of IFN- β . Thus, recognition of *T. cruzi* invasion is mainly dependent on TLR systems, possibly at the plasma membrane or in the phagolysosome. However, even in MyD88^{-/-}TRIF^{-/-} macrophages, the gene encoding LRG47 was induced after *T. cruzi* infection, indicating the presence of TLR-independent mechanisms for gene expression. The mechanisms for the TLR-independent induction of this p47 GTPase are currently under investigation.

To date, TLR2, TLR4, and TLR9 have been implicated in the recognition of *T. cruzi*-derived components (6, 14–16). TLR2 recognizes GPI-anchored mucin-like proteins and the *T. cruzi*-released protein Tc52 (6, 46, 47), whereas TLR4 is responsible for the recognition of glycoinositolphospholipids (15). TLR9 is also involved in the recognition of the CpG motif present in *T. cruzi* DNA (14). Among these *T. cruzi*-derived components, glycoinositolphospholipids can activate the TRIF-dependent pathway to induce IFN- β via TLR4. It is also possible that currently unknown components are recognized by TLR4 or TLR3, both of which use the TRIF-dependent pathway. Identification of such components responsible for the induction of IFN- β would provide important insights toward understanding innate immune responses to *T. cruzi* infection.

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Disclosures

The authors have no financial conflict of interest.

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