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Requirement of UNC93B1 Reveals a Critical Role for TLR7 in Host Resistance to Primary Infection with Trypanosoma cruzi

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UNC93B1 associates with TLR3, 7, and 9, mediating their translocation from the endoplasmic reticulum to the endolysosome, thus allowing proper activation by microbial nucleic acids. We found that the triple-deficient 3d mice, which lack functional UNC93B1 as well as functional endosomal TLRs, are highly susceptible to infection with Trypanosoma cruzi. The enhanced parasitemia and mortality in 3d animals were associated with impaired proinflammatory response, including reduced levels of IL-12p40 and IFN-γ. Importantly, the phenotype of 3d mice was intermediary between MyD88−/− (highly susceptible) and TLR9−/− (moderately susceptible), indicating the involvement of an additional UNC93B1-dependent TLR(s) on host resistance to T. cruzi. Hence, our experiments also revealed that TLR7 is a critical innate immune receptor involved in recognition of parasite RNA, induction of IL-12p40 by dendritic cells, and consequent IFN-γ by T lymphocytes. Furthermore, we show that upon T. cruzi infection, triple TLR3/7/9−/− mice had similar phenotype than 3d mice. These data imply that the nucleic acid-sensing TLRs are critical determinants of host resistance to primary infection with T. cruzi. The Journal of Immunology, 2011, 187: 000–000.

Trypanosoma cruzi, the causative agent of Chagas disease, is an intracellular protozoan parasite (1). Control of T. cruzi replication during early stages of infection in mice is highly dependent on both innate and acquired T cell-mediated immune responses (2, 3). The mammalian TLRs sense conserved molecules from all classes of microorganisms, including those from protozoan parasites (4). The activation of the innate immune system by microbial products leads to the induction of antimicrobial effector mechanisms, and gives way, over time, to the development of Th1 lymphocytes (4). Importantly, mice deficient in MyD88, an adapter molecule required for signaling events by most TLRs, except TLR3, show greatly enhanced susceptibility to infection with different protozoan parasites (3, 5–7), including T. cruzi (8).

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Abbreviations used in this article: AHADH2, aromatic l-α-hydroxy acid dehydrogenase; DC, dendritic cell; i.p., intraperitoneal; MASP, mucin-associated surface protein; TLA, T. cruzi total lysate Ag; WT, wild-type.

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GI anchors have been previously defined as a major class of T. cruzi glycolipids that are recognized by TLRs. Purified GI anchors derived from mucin-like glycoproteins of trypomastigotes contain unsaturated fatty acid chains and are potent agonists of TLR2 (9). In addition, a particular subset of free glycosphospholipid-containing ceramides stimulates the production of proinflammatory cytokines by macrophages via TLR4 (10). Until recently, dogma in the field suggested that recognition of parasite surface GI anchors was the critical component of the host innate immune response, analogous to the role of surface LPS in the genesis of fever and inflammation in Gram-negative sepsis. However, more recent studies showed that T. cruzi genomic DNA contains abundant oligodeoxynucleotide unmethylated CpG motifs (11) that promote host cell activation via TLR9 and stimulate cytokine response from macrophages and dendritic cells (DCs), triggering effector mechanisms that are essential for protection against acute infection (12, 13). Whereas TLR9 has been shown to be involved in host resistance to infection with T. cruzi, the contribution of other nucleotide-sensing TLRs, that is, TLR3, TLR7, and TLR8 (14–16), has not been explored.

Tabeta et al. (17) identified a mutant mouse line by forward genetic screening that is unresponsive to TLR3, TLR7, and TLR9 ligands (there is no known agonist for mouse TLR8). These animals, named 3d after their triple defect in TLR response, have altered function of UNC93B1, an endoplasmic reticulum resident protein that mediates the translocation of the nucleotide-sensing TLRs from the endoplasmic reticulum to the endolysosomes, allowing their proper activation by microbial RNA and DNA (18, 19). The 3d mouse has a point mutation in a transmembrane domain of UNC93B1, which renders the protein incapable of interaction with and translocation of intracellular TLRs. Consequently, 3d animals are unable to respond to nucleic acids of pathogens and to produce proinflammatory cytokines postinfection, which culminates with enhanced susceptibility to many intracellular agents (17).
In this study, we show that the 3d mice are extremely susceptible to infection with *T. cruzi*. We provide evidence for a critical role of UNC93B1 in mediating IL-12p40 as well as early IFN-γ production during acute infection with *T. cruzi*. Furthermore, we provide evidence indicating that TLR7 is critical on recognition of parasite RNA, induction of early cytokine response, and host resistance to primary *T. cruzi* infection. Altogether, our experiments reveal that UNC93B1 is an essential element in host resistance to *T. cruzi* infection, by mediating the translocation and subsequent activation of TLR7 and TLR9 by parasite nucleic acids in the endolysosomal subcellular compartment.

**Materials and Methods**

**Ethics statement**

Experiments involving animals were performed in accordance to guidelines set forth by the Institutional Animal Care and Use Committee and the Department of Animal Medicine from the University of Massachusetts Medical School (Institutional Animal Care and Use Committee Protocol A-1817-09).

**Reagents**

Cell culture reagents were obtained from Mediatech (Manassas, VA). Chicken egg albumin (OVA) and LPS derived from *Escherichia coli* strain 0111:B4 were purchased from Sigma-Aldrich (Saint Louis, MO). LPS was re-purified by phenol chloroform to remove lipopolysaccharides, as described (20). Imiquimod (R837) was purchased from Invivogen (San Diego, CA). Phosphorothioate-stabilized unmethylated CpG-containing oligonucleotide (ODN 1826, 5'-TCCATGACTCTGCCGACT-3') was purchased from IDT Technologies (Coralville, IA). The transfection reagent Gene Juice was obtained from Novagen (Madison, WI). TsKb20 peptide, a CD8+ T cell epitope derived from *T. cruzi* transialidase, as well as the tetramer used to identify TsKb20-specific CD8+ T cells (21), were synthesized by L. Luescher from the tetramer facility from the Ludwig Institute for Cancer Research (Lausanne, Switzerland). Alum (Imject, Pierce) was obtained from Thermo Scientific (Rockford, IL).

**Mice**

C57BL/6 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). The 3d mice (C57BL/6 mice bearing a nonfunctional mutant UNC93B1 molecule) were generated by B. Beutler ( Scripps Research Institute, La Jolla, CA) (17). Mice deficient of TLR7, TLR9, and MyD88 were provided by S. Akira (Department of Host Defense, Osaka University, Osaka, Japan). Mice deficient of TLR3 were generated by R. Flavell (Yale University, New Haven, CN). Mice with triple deficiency of TLR3, TLR7, and TLR9 (TLR3/7/9−/−) were generated through interbreeding of single-knockout animals. All mice used were backcrossed to C57BL/6 background at least for eight generations. Age (5–8 wk old) and sex-matched groups of wild-type (WT) and knockout mice were used in all experiments.

**T. cruzi parasites, experimental infections, and vaccinations in mice**

Experimental infections were performed with blood trypomastigotes of the virulent CL-Brener strain, which were obtained by continuous passage in RAG−/− mice by intraperitoneal (i.p.) injection. Blood trypomastigotes were recovered from infected RAG−/− mice at the peak of parasitemia and inoculated by i.p. route at a dose of 100 trypomastigotes per mouse. Mice were then monitored daily for survival and every other day for parasitemia, or sacrificed at 13 d postinfection to collect blood and spleens for ex vivo immunological assays. Vaccination experiments were performed with the attenuated CL-14 strain, which was maintained as epimastigotes in liver infusion- tripropose medium, at 28°C. Epimastigotes were harvested from log-phase cultures, washed once in PBS, and suspended at 10⁸ parasites/ml. For vaccination, two doses of 100 µl (10⁷ parasites) were inoculated i.p., 8 wk apart. Four weeks after the last dose, animals were challenged with an i.p. injection of the virulent CL-Brener, as described above, or sacrificed for serum and spleen collection. Some animals were sacrificed after the first dose of CL-14, and spleen as well as serum samples were harvested for analysis of cellular and humoral immune responses, respectively. OT-I mice were vaccinated with three s.c. doses, 1 wk apart, containing 25 µg OVA and 18 µg ODN 1826 prepared in alum/v.

**T. cruzi lysates and RNA and DNA extraction from CL-Brener parasites**

*T. cruzi* total lysate Ag (TLA) was obtained from epimastigotes grown in liver infusion- tripropose medium. Epimastigotes obtained from log-phase cultures were washed, suspended in PBS at 10⁸ parasites/ml, and disrupted by repetitive cycles of freezing and thawing. After centrifugation of cellular debris, aqueous phase was recovered and protein content was determined by Bradford method (DC protein assay; Bio-Rad, Hercules, CA). Genomic DNA was purified from freshly harvested epimastigotes (10⁸ parasites), using the GFXTM genomic DNA purification kit (GE Healthcare, Piscataway, NJ). Total RNA was purified from trypomastigote forms, which were obtained from infected L6 cells grown in RPMI 1640 medium supplemented with 10% PBS, at 37°C and 5% CO₂. Trypomastigotes were purified from cells, as previously described (22), washed in PBS, and pelleted. Extraction of total RNA from parasite pellets (5 × 10⁻¹²–10⁸ trypomastigotes) was performed using RNeasy kit (Qiagen, Valencia, CA). Purified RNA samples were treated with DNase I (Fermentas, Glen Burnie, MD) and cleaned up using the RNeasy kit (Qiagen). RNA samples were assayed in PCR with two sets of *T. cruzi*-specific primers: aromatic 1α-hydroxy acid dehydrogenase (AHADH2, 5'-GGATTCGTTTCCGCCACTCG-3' and 5'-CACCTCTGGCAAGGGAATCTC-3') and mucin-associated surface protein (MASP, 5'-CAGGAGGTGACGCTGATGATG-3' and 5'-GTGGTGTCCCTGCTGGGGTACGGGT-3'). Approximately 500 ng total RNA was used in PCR reactions, with 200 µM dNTPs, 2 µM primers, and 1.25 U Taq polymerase in 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1% Triton X-100, and 1.5 mM MgCl₂.

**FIGURE 1.** Enhanced susceptibility and impaired proinflammatory cytokine production in 3d mice infected with *T. cruzi*. A. WT (C57BL/6), MyD88−/−, 3d, and TLR9−/− mice (five animals per group) were infected by i.p. injection of 100 blood trypomastigote forms of *T. cruzi*. Parasitemia (left panel) was evaluated every other day, and cumulative mortality (right panel) followed daily. Statistical analysis conducted at day 17 post-infection shows significant difference of parasitemia and mortality (p < 0.001) between MyD88−/− and remaining mouse groups. Analysis at later points (23 and 25 d postinfection) shows significant differences in parasitemia and mortality (p < 0.05 and p < 0.001 at 23 and 25 d, respectively) when comparing 3d with the groups of TLR9−/− and WT mice. B. Serum samples were obtained from wild-type (WT) and 3d infected mice, six animals (TLR9−/− and MyD88−/− infected mice) sacrificed at 13 d postinfection, or four noninfected animals from different groups. Both IL-12 (left panel) and IFN-γ (right panel) levels were quantified. Astersisks indicate that cytokine levels in sera from WT mice are significantly higher than in sera from 3d, TLR9−/−, and MyD88−/− animals (p < 0.05). Results are representative of one of two experiments, performed independently, and yielding similar results.
Spleen cell culture

Spleens were disrupted in cell strainers to obtain single-cell suspensions. RBCs were lysed in Red Blood Cell Lysing Buffer Hybi-Max (Sigma-Aldrich), and splenocytes were washed twice and resuspended in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% FBS, 25 mM HEPES, 1 mM L-glutamine, 2 mM sodium piruvate, 50 μM 2-ME, and 100 U/ml penicillin-streptomycin). Cells were cultured at 5 × 10^6/well in 24-well tissue culture plates in absence or presence of exogenous stimuli, as indicated in figure legends, and cell-free supernatants were collected 24 h later for cytokine measurement. Alternatively, purified CD11c^+^, CD4^+^ T cells, and CD8^+^ T cells were obtained from mouse spleens using EasySep cell separation kits from StemCell Technologies (Vancouver, BC, Canada), according to manufacturer’s instructions. The CD11c^+^ cells were then incubated in presence or absence of exogenous stimuli (as indicated in figure legends) and used as APCs. The CD11c^+^ APCs (5 × 10^5^) were cocultured with purified CD4^+^ T cells or CD8^+^ T cells (2 × 10^5^ cells) in 96-well plates. Cell-free culture supernatants were collected for IFN-γ measurement.

Flow cytometry

Total spleen cells (2 × 10^6^) were stained with fluorochrome-conjugated Abs (eBioscience, San Diego, CA) specific for T cell surface markers (CD3, CD4, and CD8), during 30 min on ice. Following two washes with PBS supplemented with 1% BSA (FACS buffer), spleen cells were stained for 45 min on ice with a PE-conjugated H-2K^b^ tetramer containing the TsKb20 epitope, for identification of anti-transialidase–specific T CD8^+^ cells. Cells were then washed in FACS buffer and fixed in 2% paraformaldehyde for 15 min on ice. After two washes in FACS buffer, cells were analyzed by flow cytometry in an LSRII cytometer (BD Biosciences, San Jose, CA). Data were acquired with DIVA software (BD Biosciences) and analyzed with FlowJo (Tree Star, Ashland, OR).

Cell culture

Immortalized bone marrow macrophages from C57BL/6, 3d, and TLR7-deficient mice were maintained in complete DMEM supplemented with 10% FBS, 20 mM HEPES, and 100 U/ml penicillin-streptomycin. Cells were seeded in 96-well plates at a density of 10^3^ cells/well and treated with different stimuli, as indicated in figure legends. Supernatants were collected 24 h later for cytokine measurement.

Cytokine measurement

Supernatants of splenocyte or macrophage cultures were assayed for proinflammatory cytokines with DuoSet ELISA kits from R&D Systems (Minneapolis, MN). Cytokine levels in mouse serum were assayed using the BD Cytometric Bead Array Mouse Inflammation kit (BD Biosciences).

Statistics

Comparisons of cumulative survival curves were performed by log-rank (Mantel Cox) test. Analysis of parasitemia points postinfection, as well as data obtained in the in vitro experiments, was performed by one- or two-way ANOVA test, with Bonferroni posttest. All statistic tests were performed using GraphPad Prism 5.0 Software (GraphPad Softwares, La Jolla, CA).

Results

Enhanced susceptibility of 3d mice to T. cruzi infection

A body of evidence indicates that innate response to acute T. cruzi infection is mediated by a combined action of different TLRs (2, 3). To assess the effect of the combined deficiency of intracellular TLRs in protection against trypanosomiasis, we used the 3d mice that express a nonfunctional form of UNC93B1 and are nonresponsive to agonists of nucleic acid-sensing TLRs (18, 19). We

![FIGURE 2. Impaired IFN-γ responses by T cells from 3d mice infected with T. cruzi. A, Spleen cells obtained at day 13 postinfection (+T. cruzi infection) or noninfected (−T. cruzi infection) WT, 3d, TLR9−/−, and MyD88−/− mice (three animals per group) were cultured in absence or presence of trypomastigote lysate Ag (TLA, 10 μg/ml), a CD8+ T cell epitope derived from T. cruzi transialidase (TsKb20, 10 μM), a CD8+ T control epitope derived from Leishmania A2 Ag (Ctrl pep, 10 μM), or the mitogen Con A (10 μg/ml). Supernatants from spleen cultures were harvested 24 h after Ag stimulation for measurements of IFN-γ levels. Asterisks indicate that IFN-γ levels produced by splenocytes from WT mice were significantly higher (p < 0.001) than from 3d, TLR9−/−, and MyD88−/− mice. B, Spleen cells obtained at 13 d postinfection and noninfected mice (three animals per group) were stained for CD8^+^ T cells positively stained with the tetramer was quantified by flow cytometry. Results are representative of one of two experiments that were performed independently and yielded similar results.](http://www.jimmunol.org/)

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infected 3d mice in parallel with TLR9 and MyD88 deficient (indicated by *+/−*) with the CL-Brener clone of *T. cruzi*. Although not as susceptible as the MyD88−/− mice, TLR9−/− mice showed enhanced parasitemia and accelerated mortality upon *T. cruzi* infection (12, 13). These results are presented in Fig. 1. Importantly, our experiments also show that 3d mice are highly susceptible to infection with *T. cruzi*, displaying an intermediary phenotype between MyD88−/− and TLR9−/− mice. We anticipated that in 3d mice, the lack of endosomal TLR function would also result in impaired cytokine production during *T. cruzi* infection. In fact, 3d mice infected with *T. cruzi* presented impaired production of proinflammatory cytokines, that is, IL-12p40 as well as IFN-γ (Fig. 1B), which are critical mediators of host resistance to *T. cruzi*.

**Impaired T cell responses in 3d mice infected with *T. cruzi***

T cells are an important source of IFN-γ and essential for protection against infection with *T. cruzi*. Thus, we investigated whether anti-*T. cruzi*-specific T lymphocytes were being properly activated in 3d mice. Our results show that IFN-γ produced by T lymphocytes was significantly impaired in 3d mice infected with *T. cruzi*. WT, 3d, TLR9−/−, and MyD88−/− mice were infected with *T. cruzi* and had their Ag-driven IFN-γ responses as well as the frequency of parasite-specific CD8+ T cells evaluated (Fig. 2). At 13 d postinfection, we observed an impaired IFN-γ production in response to TLA, in all mutant or TLR-deficient mouse strains. In addition, in the same experiment, we observed a decreased IFN-γ response to the immunodominant CD8+ T cell epitope TsKb20 of *T. cruzi* (21) by spleen cells from MyD88−/− and 3d, but not by cells from TLR9−/− mice (Fig. 2A). In contrast, the frequency of TsKb20-specific CD8+ T cells was not affected in MyD88−/−, 3d, or TLR9−/− mice infected with *T. cruzi* (Fig. 2B).

We also evaluated in vivo the essential requirement of UNC93B1 on development of acquired immunity and immunological protection against *T. cruzi* infection. WT and 3d mice were vaccinated with the highly attenuated CL-14 strain of *T. cruzi* (23, 24). The humoral response to *T. cruzi* Ags was identical when comparing 3d and WT after priming (Fig. 3A) and boost (data not shown) vaccination doses. In contrast, after both prime and boost vaccination doses, the cellular responses, that is, IFN-γ responses to TLA and to TsKb20, were significantly reduced in the 3d mice (Fig. 3A). Nevertheless, the humoral and cellular immune responses obtained with two doses of CL-14 were sufficient to protect 3d mice against a challenge with the virulent CL-Brener strain of *T. cruzi* (Fig. 3B).

**Enhanced susceptibility of TLR7−/− mice to *T. cruzi* infection**

In addition to TLR9, UNC93B1 mediates TLR3 and TLR7 translocation and function (18, 19). Altogether, our results suggest that the combined action of TLR9 with TLR3 and/or TLR7 would account for the UNC93B1 role on innate recognition and host resistance to infection with *T. cruzi*. To test this hypothesis, we evaluated the ability of TLR3−/− or TLR7−/− mice to control the infection with *T. cruzi*. The results shown in Fig. 4A demonstrate that TLR7−/− mice are more susceptible to *T. cruzi* infection, yielding a phenotype similar to that of TLR9−/− mice. In contrast, TLR3−/− mice were able to control parasitemia and survive infection. Importantly, the serum levels IL-12p40 and IFN-γ were greatly diminished in 3d and TLR7−/−, but not in the TLR3−/− mice (Fig. 4B, left and middle panels). Furthermore, the levels of IL-12p40 produced by spleen cells from 3d, TLR7−/−, or TLR9−/− (but not TLR3−/−) mice were severely impaired (Fig. 4B, right panel). Consistently, the IFN-γ response by T cells postinfection with *T. cruzi* was similarly affected in 3d and TLR7−/−, but not in TLR3−/− mice (Fig. 4C).

**FIGURE 3.** Impaired resistance of 3d mice is overcome by vaccination with an attenuated strain of *T. cruzi*. WT and 3d mice were vaccinated with one (prime) or two (boost) doses of the CL-14-attenuated strain of *T. cruzi*. As controls, animals were left without any immunizations (none). A, Serum samples were obtained 30 d after prime dose with CL-14 and quantification of anti-*T. cruzi* IgG performed by ELISA employing TLA, to confirm that all animals were successfully immunized (left panel). Spleen cells obtained 30 d after prime or boost dose of vaccine were restimulated in vitro with *T. cruzi* Ags (TLA at 10 μg/ml or TsKb20 peptide at 10 μM) or a control peptide (A2, 10 μM) (right panel). IFN-γ levels were evaluated in supernatants of spleen cultures by ELISA at 24 h after Ag stimulation. Asterisks indicate significant difference between WT and 3d mice. B, Prime-boost–vaccinated animals were challenged with an i.p. dose of 100 trypomastigotes of virulent CL-Brener strain, 30 d after the boost dose with CL-14 clone. Parasitemia (left panel) and mortality (right panel) were followed for 60 d after challenge. Nonvaccinated 3d mice showed high susceptibility, with significantly higher parasitemia (p < 0.001) starting at 21 d postinfection and 100% mortality by 25 d postinfection. In contrast, vaccinated 3d were as resistant to infection as the vaccinated WT animals. The representative results are from one of two experiments, which yielded similar results.

We also tested the ability of total RNA purified from *T. cruzi* to stimulate immortalized macrophages derived from WT, 3d, and TLR7−/− mice. parasite RNA induced a low TNF-α response, which was enhanced when RNA was delivered with a transfection reagent, in cells from WT but not from 3d or TLR7−/− mice. As expected, Imiquimod (R837) and an unmethylated CpG-containing oligonucleotide (Oligo 1826) did not activate macrophages from 3d mice, and R837 did not induce TNF-α production by TLR7−/− macrophages (Fig. 5A). To avoid RNA contamination with DNA, all RNA preparations were treated with DNase I. Absence of DNA was confirmed by PCR employing two different sets of *T. cruzi*-specific primers for AHADH2 and MASP. No amplification of parasite-specific genes was observed when RNA was used as template, contrasting with the results obtained, when we used parasite DNA as template (Fig. 5B). Nucleic acid-sensing TLRs account for the immunological defect found in 3d mice infected with *T. cruzi*. The possibility has also been raised that in addition to translocation of TLRs, UNC93B1 may also mediate other key functions in the immune system (17, 19), such as Ag presentation. With this in mind, we generated the TLR3/7/9−/− mice and compared the results with 3d mice, when both were challenged with *T. cruzi* in parallel infections. Importantly, the combination of TLR3, TLR7, and TLR9 deficiencies yielded a susceptibility phenotype identical
to that observed in the 3d mice, as measured by mortality and parasitemia (Fig. 6A). Because TLR3−/− mice did not display an altered phenotype (Fig. 4A), we believe that the combination of TLR7 and TLR9 deficiency accounts for the enhanced susceptibility observed both in the TLR3/7/9−/− and 3d infected with T. cruzi. We also evaluated the IFN-γ production by spleen cells from WT, 3d, and TLR3/7/9−/− mice infected with T. cruzi. Again, our results indicate a similar and significant impairment of IFN-γ production, when spleen cells from 3d or TLR3/7/9−/− mice were stimulated with either TLA or CD8 T cell epitope (Fig. 6B).

The defect of IFN-γ production by CD4 T cells from 3d was directly demonstrated by employing CD4 T lymphocytes obtained from infected WT, 3d, and TLR3/7/9−/− mice. Highly purified CD4 T lymphocytes were stimulated with APCs from WT mice pulsed with TLA (Fig. 6C). CD4 T lymphocytes derived from both 3d and TLR3/7/9−/− mice secreted significantly lower levels of IFN-γ, when compared with the CD4 T cells derived from WT mice (Fig. 6C). These results indicate that in vivo priming of the CD4 T cells and possibly differentiation of Th1 cells are compromised in the 3d and TLR3/7/9−/− mice.

Finally, our results indicated that the frequency of T. cruzi-specific CD8 T lymphocytes was similar between the WT and different mouse strains infected with T. cruzi. Nevertheless, we consistently observed reduction in IFN-γ secretion by CD8 T cells from 3d and TLR3/7/9−/− mice in response to the MHC I-binding epitope TsKb20 (Fig. 6B). Thus, we investigated whether an eventual defect in Ag cross-presentation contributed to the decreased IFN-γ production by CD8 T cells from 3d and TLR3/7/9−/− mice. For that, we isolated CD11c+ APCs from spleens of WT, 3d, and TLR3/7/9−/− mice. These cells were then pulsed with TLA, as an exogenous Ag, and then incubated with highly purified CD8 T cells obtained from syngeneic C57BL/6 mice infected with T. cruzi (Fig. 6D). Parallel experiments were performed with highly purified CD8 T cells obtained from OVA-vaccinated OT-I mice and CD11c+ APCs pulsed with exogenous particulate OVA. We observed that APCs from either WT, 3d, or TLR3/7/9−/− mice were equally efficient in presenting either TLA or OVA, as measured by IFN-γ production by CD8 T cells from WT mice (Fig. 6D). Thus, in our model, we were not able to observe differences in Ag cross-presentation, when comparing WT, 3d, and TLR3/7/9−/− mice.

**Discussion**

TLRs are important elements for host defense against every known category of human microbial pathogens (4), including protozoan parasites (3). The most convincing data indicating the importance of the Toll/IL-1R domain (TIR) in host resistance to protozoan infections are those obtained in MyD88−/− mice. Increased susceptibility associated with impaired production of the Th1-

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**FIGURE 6.** Enhanced susceptibility and impaired cytokine production by TLR7−/− mice infected with T. cruzi. A, WT, TLR3−/−, TLR7−/−, and TLR9−/− mice were infected by i.p. injection of 100 blood trypomastigote forms of T. cruzi. Parasitemia (left panel) and cumulative mortality (right panel) were evaluated every other day and daily, respectively. Statistical analysis conducted at days 20, 23, 25, and 27 postinfection denotes that the differences in parasitemia and survival curves are significant when comparing TLR7−/− and TLR9−/− to WT or TLR3−/− mice (p < 0.05 on day 20 and p < 0.001 on 23 through 27). B, Serum samples were obtained from WT, 3d, TLR3−/−, and TLR7−/− noninfected mice as well as at 13 d postinfection with 100 blood trypomastigote forms. Both IL-12 (left panel) and IFN-γ (middle panel) levels were measured. B, Right panel, Total spleen cells obtained from WT, 3d, TLR3−/−, TLR7−/−, and TLR9−/− at 13 d postinfection were cultured for 24 h, without any addition of exogenous Ag, for measurement of IL-12 levels in culture supernatants. C, Spleen cells from infected and noninfected 3d, TLR3−/−, and TLR7−/− were submitted to stimulation with T. cruzi Ags (TLA, 10 μg/ml or TsKb20, 10 μM), control peptide (Leishmania A2, 10 μM), or Con A (10 μg/ml). The levels of IFN-γ in culture supernatants were measured by ELISA 24 h later. Asterisks indicate significant differences (p < 0.05) when comparing cytokine levels in sera/supernatants derived from WT and TLR3−/− to 3d and TLR7−/− mice. Results are representative of one experiment. Each experiment was performed two independent times with similar results.
associated cytokines (e.g., IFN-γ and IL-12) (3) is observed in MyD88−/− mice infected with different protozoan parasites (3, 5–7), including T. cruzi (8). However, it has been difficult to assign specific TLRs that account for pronounced susceptibility to protozoan parasites observed in the MyD88−/− mice (8, 10, 12, 25). In this study, we demonstrated that 3d mice, which lack functional UNC93B1 that is responsible for translocation of nucleic acid-sensing TLRs (18, 19), are highly susceptible to experimental infection with T. cruzi. Furthermore, we demonstrated that TLR7−/− mice are more susceptible to T. cruzi. Thus, we propose that the enhanced susceptibility of 3d mice is a result of combined deficiency of TLR7 and TLR9, the results obtained with the 3d and TLR3/7/9−/− mice indicate that their combined action accounts for the role of UNC93B1 on IL-12 production by myeloid cells (e.g., DCs or macrophages) and, consequently, the optimal IFN-γ production by T lymphocytes and host resistance to T. cruzi infection.

Our previous studies demonstrated that upon infection with T. cruzi, TLR9−/− mice present impaired production of IL-12 by DCs, of IFN-γ by T lymphocytes, increased parasitemia, and accelerated mortality. We also demonstrated that TLR9 is recruited to the endolysosome compartment, where it probably interacts with DNA released from parasites that have been destroyed during the initial uptake by DCs. Furthermore, we found that immunostimulatory CpG motifs are abundant and preferentially distributed in regions of the genome that contains T. cruzi-specific genes, like multigene families encoding the mucin-like proteins, and the retroelement VIPER (26).

Importantly, upon infection with T. cruzi, the phenotype of TLR9−/− mice is less pronounced than that observed in MyD88−/− mice (12). Thus, we propose that host defense to primary T. cruzi infection is simultaneously orchestrated by different TLRs. This hypothesis is supported by the current study, which demonstrates that 3d animals that have combined deficiency of TLR3, TLR7, and TLR9 functions are highly susceptible to T. cruzi infection, with a phenotype that is intermediate between TLR9−/− and MyD88−/− mice. However, UNC93B1 was also demonstrated to be involved in activation of CD8+ T cells (17, 27), possibly by controlling translocation of elements of the cross-presentation machinery, similar to the way it mediates the movement of TLRs to endosomes (19). Thus, the enhanced susceptibility of 3d mice to T. cruzi infection could be explained by an impaired cross-Ag presentation in DCs. To address this question, we generated the triple TLR3/7/9-deficient mice and performed parallel experiments with the 3d mice. Our results demonstrate identical phenotypes in terms of parasitemia, survival curve, decreased IL-12 production by DCs, and induction of IFN-γ by CD4+ T as well as CD8+ T lymphocytes (31). We also proved in sequential experiments that TLR7 is a critical receptor in mediating activation of innate immune cells and host resistance to primary infection with T. cruzi. Because the TLR3 single-knockout mice did not display altered immune responses and resistance to T. cruzi infection, we assume that the combined lack of TLR7 and TLR9 was the main defect underlying the 3d susceptibility to the parasite. Thus, despite the redundant role of TLR7 and TLR9, the results obtained with the 3d and TLR3/7/9−/− mice indicate that their combined action accounts for the role of UNC93B1 on IL-12 production by myeloid cells (e.g., DCs or macrophages) and, consequently, the optimal IFN-γ production by T lymphocytes and host resistance to T. cruzi infection.

Altogether, our results demonstrate that UNC93B1 is critical for host resistance to T. cruzi. These data are highly reminiscent of a recent report showing that UNC93B1 is an essential element of host resistance to another intracellular protozoan, namely Toxoplasma gondii (32). However, in this particular case, a single deficiency on either TLR7 or TLR9 did not affect host resistance to infection. Thus, either the combined TLR7/TLR9 deficiency or a TLR-independent mechanism is required to explain the enhanced susceptibility of 3d mice to T. gondii. In contrast, in the case of T. cruzi infection, the enhanced susceptibility of 3d mice can be explained solely on the basis of predicted role of UNC93B1, which is the translocation of nucleic acid-sensing TLRs (18, 19).

Although TLR7 has been implicated in the recognition and host resistance to viral and bacterial infection (16, 33, 34) we now demonstrate that TLR7 is a critical innate immune receptor involved in recognition and host resistance to a protozoan infection. To date, TLR7 has been exclusively recognized as a receptor for ssRNA. By performing an in silico analysis, we found several potential TLR7 agonists, for example, guanosine- or uridine-rich single strand in the predicted parasite transcriptome. To this end, 27,752 potential transcribed elements of the genome were searched.
for GU motifs with 20 or more G and/or U residues allowing only one mismatch. The data set includes 23,218 protein coding regions, 2,424 retroelements, and 2,110 structural RNAs. A total of 1,338 GU motifs was found in 833 potential transcribed elements. Of these, 344 GU motifs were found in genes encoding surface proteins, such as gp85/trans-sialidase, MASP, mucin TcMUC, DGF-1, GP63, and serine-alanine proline-rich protein, all known to be highly expressed in the mammalian stages of the parasite (22, 35–38). However, this number of putative GU motifs is likely to be underestimated because a small proportion of T. cruzi untranslated regions was already mapped (39).

Importantly, we found a critical role for TLR7 in induction of IL-12p40 and consequent induction of IFN-γ by T cells during T. cruzi infection. Thus, we assume that the main source of IL-12 during T. cruzi are DCs and/or macrophages, which express TLR7. We believe that parasites that have been internalized by these professional phagocytic cells are destroyed, releasing RNA, which activates TLR7 in the phagolysosomes. As previously demonstrated in an experimental model of bacterial infection (34), TLR7 may also be involved in the induction of type I IFN, which is prominent during early stages of T. cruzi infection (40, 41).

It is noteworthy that 3d mice are consistently less susceptible than MyD88−/− mice, indicating that an additional TLR(s) or cytokine receptors are necessary to explain the hypersusceptibility of MyD88−/− mice to T. cruzi infection. Indeed, early studies indicate that GPI anchors and GIPL-ceramide from T. cruzi

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**FIGURE 6.** Combined TLR7 and TLR9 deficiency contributes to enhanced susceptibility and impaired cytokine production of 3d mice infected with T. cruzi. A, WT, 3d, TLR7−/−, TLR9−/−, and TLR3/7/9−/− mice were infected by i.p. injection of 100 blood trypomastigote forms of T. cruzi. Parasitemia (left panel) and cumulative mortality (right panel) were evaluated every other day and daily, respectively. Statistical analysis denotes that the differences in parasitemia and survival curves are significant when comparing WT, TLR7−/−, and TLR9−/− to TLR3/7/9−/− and 3d mice (p < 0.05 on day 20 and p < 0.001 on day 23), and when comparing WT to TLR7−/− and TLR9−/− mice (p < 0.001 on days 28–32). No statistical differences were observed when comparing 3d and TLR3/7/9−/− mice. B, Total spleen cells obtained from noninfected or WT, 3d, and TLR3/7/9−/− mice at 13 d postinfection were submitted to stimulation with T. cruzi Ags (TLA, 10 μg/ml or TsKb20, 10 μM), control peptide (Leishmania A2, 10 μM), or Con A (10 μg/ml). The levels of IFN-γ in tissue culture supernatants were measured by ELISA. Asterisks indicate significant differences (p < 0.05) when comparing IFN-γ levels in supernatants derived from WT to 3d and TLR3/7/9−/− mice. These cells were cocultured for 48 h with purified WT spleen CD11c+ APCs previously pulsed with TLA (10 μg/ml). Nontreated CD11c+ APCs (Medium) were used as control. IFN-γ was measured in cell-free culture supernatants by ELISA. The asterisk indicates significant difference (p < 0.001) when comparing IFN-γ production of WT CD4+ T cells to 3d and TLR3/7/9−/− CD4+ T cells. C, CD4+ T cells were purified from spleens of naive or T. cruzi-infected WT, 3d, and TLR3/7/9−/− mice. CD8+ T cells were purified from spleens of naive or OVA-vaccinated OT-1 mice. APCs and CD8+ T cells were cocultured for 96 h for induction of IFN-γ that was measured in cell-free culture supernatants by ELISA. All results are representative of one experiment. Each experiment was performed two independent times with similar results.
parasites activate primarily TLR2 and TLR4, respectively (9, 10). Furthermore, double TLR2/TLR4−/− are slightly more susceptible than TLR9−/− to T. cruzi infection (12). Thus, although deficiency of either TLR2 or TLR4 in C57BL/6 does not enhance susceptibility, it is possible that the lack of TLR2 (and/or TLR4) functions associated with combined TLR7/TLR9 deficiencies may account for the complete role of MYD88 on host resistance to T. cruzi infection. Alternatively, IL-1R, IL-33R, and/or IL-18R, whose functions are also dependent on MYD88 (30, 42, 43), may contribute to enhanced susceptibility to T. cruzi infection in MYD88−/−, although mice deficient in either IL-1R (data not shown) or IL-18R (30) are not more susceptible to experimental infection with T. cruzi.

In conclusion, our results indicate that the critical antiparasitic role for UNC93B1 is the control of IL-12 production of APCs and early IFN-γ responses by both CD4+ T as well as CD8+ T lymphocytes. The combined action of TLR7/TLR9 appears to mediate the UNC93B1 role during T. cruzi infection. Thus, our results indicate that UNC93B1 as well as TLR7/TLR9 nucleic acid-sensing TLRs play a fundamental role in activation of innate immunity, initiation of T cell responses, and host resistance to T. cruzi infection.

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Disclosures
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References


