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The Journal of Immunology

Cutting Edge: Nucleotide-Binding Oligomerization Domain 1-Dependent Responses Account for Murine Resistance against *Trypanosoma cruzi* Infection

This information is current as of August 4, 2022.

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J Immunol 2010; 184:1148-1152; Prepublished online 30 December 2009;
doi: 10.4049/jimmunol.0902254
http://www.jimmunol.org/content/184/3/1148

Supplementary Material **http://www.jimmunol.org/content/suppl/2009/12/30/jimmunol.0902254.DC1**

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Cutting Edge

THE JOURNAL OF IMMUNOLOGY

Cutting Edge: Nucleotide-Binding Oligomerization Domain 1-Dependent Responses Account for Murine Resistance against *Trypanosoma cruzi* Infection

Grace K. Silva,* Fredy R. S. Gutierrez,† Paulo M. M. Guedes,† Catarina V. Horta,* Larissa D. Cunha,* Tiago W. P. Mineo,† Juliana Santiago-Silva,† Koichi S. Kobayashi,‡,§ Richard A. Flavell,¶ João S. Silva,† and Dario S. Zamboni*

An effective innate immune recognition of the intracellular protozoan parasite *Trypanosoma cruzi* is critical for host resistance against Chagas disease, a severe and chronic illness that affects millions of people in Latin America. In this study, we evaluated the participation of nucleotide-binding oligomerization domain (Nod)-like receptor proteins in host response to *T. cruzi* infection and found that Nod1-dependent, but not Nod2-dependent, responses are required for host resistance against infection. Bone marrow-derived macrophages from Nod1$^{-/-}$ mice showed an impaired induction of NF-κB–dependent products in response to infection and failed to restrict *T. cruzi* infection in presence of IFN-γ. Despite normal cytokine production in the sera, Nod1$^{-/-}$ mice were highly susceptible to *T. cruzi* infection, in a similar manner to MyD88$^{-/-}$ and NO synthase 2$^{-/-}$ mice. These studies indicate that Nod1-dependent responses account for host resistance against *T. cruzi* infection by mechanisms independent of cytokine production. ***The Journal of Immunology*, 2010, 184: 1148–1152.**

Cells from the innate immune system express numerous pattern recognition receptors (PRRs) that are activated upon recognition of pathogen-associated molecular patterns (1). The best characterized PRRs are the TLRs, which are transmembrane proteins localized either at the cell surface or within the endosome membranes (1). Upon activation, most of these receptors initiate signaling pathways dependent on adaptor proteins such as MyD88 that culminate in activation of MAPKs and NF-κB. Despite TLRs, new families of intracellular PRRs have emerged as important components of the innate immune system that account for detection of intracellular microbial infection. Intracellular PRRs include viral sensors and the nucleotide-binding oligomerization domain (Nod)-like receptor protein (NLR) family, which are bona fide sensors of bacterial infection (reviewed by Ref. 2).

Nod1 and Nod2 were the first NLRs to be identified and are possibly the best characterized members of the NLRs (3–6). Whereas Nod1 is ubiquitously expressed, Nod2 is expressed in hematopoietic cells, and both cooperate to signal via the adaptor molecule Rip2, a protein kinase required for activation of NF-κB and MAPK cascades that culminate with production of a series of cytokines and chemokines (3, 5, 6).

Nod1 and Nod2 recognize muropeptides derived from cell walls of Gram-positive and Gram-negative bacteria (4, 7). These PRRs are localized in the cytoplasm and recognize bacterial pathogens that lyse the parasitophorous vacuole membrane and gain access to the host cell cytoplasm, such as *Listeria monocytogenes* and *Shigella flexineri* (8). Despite the bona fide role of Nod1 and Nod2 for recognition of intracellular bacterial infection, deletion of these receptors has little impact on the infection outcome, possibly because of the functional redundancy with TLRs (9). Although Nod1 and Nod2 were extensively characterized as PRRs for bacterial infection, little is known regarding the role of NLRs for recognition of intracellular parasites.

Trypanosoma cruzi is an intracellular protozoan parasite, which is the etiological agent of Chagas disease, a severe and chronic infectious illness that affects millions of people in Latin America. The development and severity of the disease depends on humoral and cell-mediated adaptive responses; thus, an effective innate immune recognition of the parasites by PRRs is key for host resistance (10–13). Previous studies have demonstrated that initial parasite recognition occurs mainly by TLR2, -4, and -9 (14–16). Engagement of these

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Received for publication July 27, 2009. Accepted for publication December 2, 2009.

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant 06/52867-4 to D.S.Z.), World Health Organization/TDR, and Instituto Nacional de Ciência e Tecnologia de Vacinas/ Conselho Nacional de Desenvolvimento Científico e Tecnológico. R.A.F. is an Investigator of the Howard Hughes Medical Institute, and D.S.Z. and J.S.S. are research fellows from Conselho Nacional de Desenvolvimento Científico e Tecnológico.

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The online version of this paper contains supplemental material.

Abbreviations used in this paper: BMM, bone marrow-derived macrophage; NLR, nucleotide-binding oligomerization domain-like receptor protein; Nod, nucleotide-binding oligomerization domain; NOS, NO synthase 2; PRR, pattern recognition receptor; WT, wild-type.

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receptors triggers activation of a MyD88-dependent pathway that culminates on activation of NF-κB and MAPKs (10). However, MyD88$^{-/-}$ mice still produce cytokines in response to *T. cruzi* infection (17). Therefore, we tested if Nod1 and Nod2, which are known to trigger NF-kB and MAPKs, participate in the innate immune recognition and host resistance against of *T. cruzi* infection. Strikingly, we found that despite normal cytokine production in the sera, Nod1$^{-/-}$, but not Nod2$^{-/-}$, mice were highly susceptible and succumbed to infection similar to MyD88$^{-/-}$ mice. Taken together, our data indicate that regardless of TLRs activation and MyD88 signaling, Nod1-dependent responses play a critical role in host resistance against *T. cruzi* infection.

Materials and Methods

Mice, parasites, and infection

Eight-week-old male mice were used for infection experiments. Nod1$^{-/-}$, Nod2$^{-/-}$, MyD88$^{-/-}$, and NO synthase 2 (NOS2)$^{-/-}$ mice were backcrossed to C57BL/6 (wild-type [WT]) for seven or more generations. Mice were infected i.p. with 10^3 blood-derived trypomastigotes from the Y strain of *T. cruzi.* Bone marrow-derived macrophages (BMMs) were generated from bone marrow cells as described (18) and treated or not treated with 10 or 100 U/ml IFN-γ (R&D Systems, Minneapolis, MN). The release of parasites from the infected cells was determined by counting the trypomastigotes in hemocitometer counting chambers. The determinations of intracellular parasites were performed by microscopic counting of Giemsa-stained parasites.

Real-time PCR, cytokines, and NO

Real-time PCR was performed as described previously (19). Primers used were β-actin-fwd 5′-AGCTGCGTTTTACACCCTTT-3′; β-actin-rev 5′-AAGCCATGCCAATGTTGTCT-3′; TNF-fwd 5′-GATCTCAAAGACAACCAACATGTG-3′; TNF-rev 5′-CTCCAGCTGGAAGACTCCTCCCAG-3′; IL-12p40-fwd 5′-AGACCAGAGACATGGAGTCATA-3′; IL-12p40-rev 5′-TGCTCCACACTTCAGGAAAG-3′; IFN-fwd 5′-GCATCTTGGCTTTGCAGCT-3′; IFN-rev 5′-CCTTTTTCGCCTTGCTGTTG-3′; NOS2-fwd 5′-CGAAACGCTYCACTTCCAA-3′; and NOS2-rev 5′-TGAGCCTATATTGCTGTGGCT-3′. The cytokine concentrations were assessed by OpTEIA ELISA sets (BD Biosciences, San Diego, CA) according to manufacturer instructions. Nitrite concentration was determined with Griess reagent as described previously (18).

Parasitemia and mortality

Parasitemia was monitored by microscopic analysis of 5 μl blood samples drawn from the tail vein during the first 3 wk of infection. Survival rates were determined by daily examination of the infected animals.

Statistical analysis

Data are expressed as mean ± SEM. In time course studies, one-way ANOVA was used followed by Tukey-Kramer post hoc analysis. Parasitemia data were analyzed by two-way ANOVA followed by Bonferroni posttest analysis. The Kaplan-Meier method was used to compare survival curves. All analyses were performed using the Prism 5.0 software (GraphPad, San Diego, CA).

Results and Discussion

NLR signaling accounts for cytokine and NO production by infected BMMs

To evaluate the possible participation of intracellular NLRs in the recognition of *T. cruzi*, we infected BMMs obtained from WT, Nod1$^{-/-}$, or Nod2$^{-/-}$ mice and measured expression of genes induced in response to NF-κB activation. We measured the levels of transcripts for cytokines and microbicidal components using real-time PCR and found that WT BMMs strongly induced NF-κB–dependent products in response to *T. cruzi* infection. In contrast, NLR-deficient BMMs showed an impaired production of transcripts for TNF-α, IL-12p40, IFN-γ, and NOS2 in response to *T. cruzi* infection (Fig. 1*A*). Next, we evaluated the production of IL-12p40 and TNF-α by

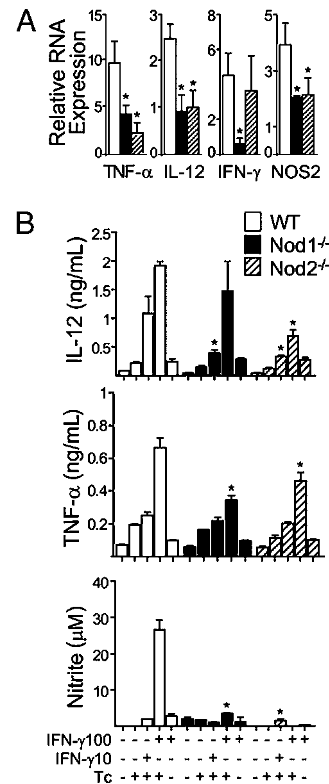

FIGURE 1. Nod1 and Nod2 account for induction of NF-kB–dependent products in response to *T. cruzi* infection. BMM obtained from Nod1$^{-/-}$ (black bars), Nod2$^{-/-}$ (hatched bars), or WT (white bars) mice were infected with a multiplicity of infection of 5. *A*, RNA was extracted from 1×10^6 cells 6 h poststimulation, and the levels of transcripts were estimated by real-time PCR. Data shown are the relative RNA expression of the messenger RNAs (TNF-α, IL-12p40, IFN-γ, and NOS2) normalized to β-actin. *B*, Cytokine concentrations (IL-12p40 and TNF-α) were estimated by ELISA from 5×10^5 cells after 24 h, and nitrite was estimated from cells infected for 48 h. Cells treated with 10 or 100 U/ml of IFN-γ and/or infected with *T. cruzi* (Tc). $^*p < 0.005$ as compared with similarly treated WT BMMs.

ELISA and the nitrite production by Griess. We found that whereas *T. cruzi* infection alone was not sufficient to trigger a robust production of IL-12, TNF-α, and NO, the infection of IFN-γ–stimulated macrophages trigger a robust production of these molecules (Fig. 1*B*). According to the data obtained by real-time PCR, we found that macrophages from mice deficient for Nod1 and Nod2 failed to produce NO and were slightly impaired in the production of IL-12 and TNF-α (Fig. 1*B*). Together, these results indicate that Nod1 and Nod2 participate in the BMM response against *T. cruzi* infection.

Nod1$^{-/-}$ BMMs fail to eliminate intracellular parasites in response to IFN-γ

Because NO is essential for restriction of *T. cruzi* infection by BMMs, we investigated if the impaired NO production observed in NLR-deficient BMMs influenced their ability to control intracellular parasite infection. In untreated cultures, we found similarly high numbers of intracellular amastigotes in WT, Nod1$^{-/-}$, and Nod2$^{-/-}$ BMMs (Fig. 2). However, in the presence of IFN-γ, we found that Nod1$^{-/-}$, but not WT and Nod2$^{-/-}$, BMMs contain a higher proportion of infected cells (Fig. 2*A*) and higher loads of intracellular amastigotes per infected cell (Fig. 2*B* and Supplemental Fig. 1). These results indicate that Nod1$^{-/-}$ BMMs failed to eliminate the intracellular parasites in response to IFN-γ.

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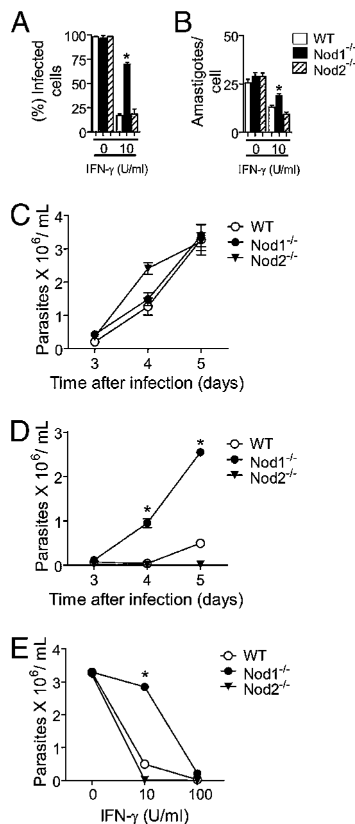

FIGURE 2. Nod1-dependent responses are required for elimination of intracellular parasites by IFN-γ–treated macrophages. BMM from $Nod1^{-/-}$, $Nod2^{-/-}$, or WT mice were cultivated at the concentration of 1×10^5 in the presence or absence of 10 or 100 U/ml of IFN-γ. Cells were infected with a multiplicity of infection of 5. Cells were stained with Giemsa after 48 h infection for determination of percentage of infected cells (*A*) and average amastigote per cell (*B*). Parasite release 3, 4, and 5 d postinfection in untreated (*C*) cultures or cultures treated with 10 U/ml of IFN-γ (*D*). *E*, Number of parasites liberated from cultures infected for 5 d in absence or presence of 10 or 100 U/ml of IFN-γ. *$p < 0.005$ as compared with similarly treated WT BMMs.

Next, we investigated the viability of the amastigotes found in IFN-γ–treated BMMs. Thus, we carried out the cultures for up to 5 d postinfection and measured release of the highly motile trypomastigotes into the tissue culture supernatants. We found that untreated BMMs gradually released trypomastigotes into the tissue culture supernatants (Fig. 2*C*). In contrast, in the presence of 10 U/ml IFN-γ, parasites were only detected in the supernatants of $Nod1^{-/-}$ BMMs (Fig. 2*D*). On day 5 postinfection, we detected high numbers of fully motile trypomastigotes in cultures of $Nod1^{-/-}$ treated with 10 U/ml; however, the treatments with 100 U/ml IFN-γ were sufficient to eliminate the parasite in all BMMs tested (Fig. 2*E*). Taken together, our data indicate that $Nod1^{-/-}$ BMMs failed to restrict *T. cruzi* infection in the presence of IFN-γ; these cultures supported the multiplication of intracellular amastigotes and the release of extracellular trypomastigotes. Therefore, Nod1-dependent responses are required for macrophage elimination of *T. cruzi* infection in response to IFN-γ.

$Nod1^{-/-}$ mice succumb to T. cruzi *infection and show higher loads of parasites in spleen and heart tissues*

To test if the impaired trypanocidal ability of $Nod1^{-/-}$ BMMs culminates with increased susceptibility in vivo, we compared infections of $Nod1^{-/-}$, $Nod2^{-/-}$, and WT mice. We found that $Nod1^{-/-}$ mice present high numbers of parasites in the blood, which peaked on day 9 postinfection. The numbers of parasites found in the blood of $Nod1^{-/-}$ mice were ~3 times higher than the similarly infected WT and $Nod2^{-/-}$ mice (Fig. 3*A* and Supplemental Fig. 2*A*). Strikingly, 100% of the $Nod1^{-/-}$ mice succumbed to infection 24 d postinfection in contrast to none of the WT or $Nod2^{-/-}$ mice (Fig. 3*B* and Supplemental Fig. 2*B*). The higher susceptibility of the $Nod1^{-/-}$ mice was accompanied by a lower inflammatory infiltrate in the cardiac tissues on day 14 postinfection (Supplemental Fig. 2*C*). However, no differences were detected in the hepatic inflammatory infiltrate in these mice (data not shown). Because cardiac muscle is one of the target sites for *T. cruzi* multiplication during chronic and acute infection, we aimed to estimate the amount of parasites in the cardiac tissues of these mice. By using immunohistochemistry, we found that $Nod1^{-/-}$ mice contained higher numbers of amastigote nests in the cardiac tissues when compared with WT and $Nod2^{-/-}$ animals (Supplemental Fig. 2*D*). Together, these results suggest that Nod1-dependent responses are important for induction of acute myocarditis observed upon *T. cruzi* infection. These responses may account for restriction of parasite multiplication in the cardiac tissues.

Our data show that $Nod1^{-/-}$ mice were highly susceptible to *T. cruzi* infection (Fig. 3); indeed, $Nod1^{-/-}$ BMMs failed to produce NO and restrict *T. cruzi* infection in response to IFN-γ (Figs. 1, 2). Therefore, we investigated if $Nod1^{-/-}$ mice were fully able to trigger the production NO and IFN-γ in response to *T. cruzi* infection in vivo. Initially, we

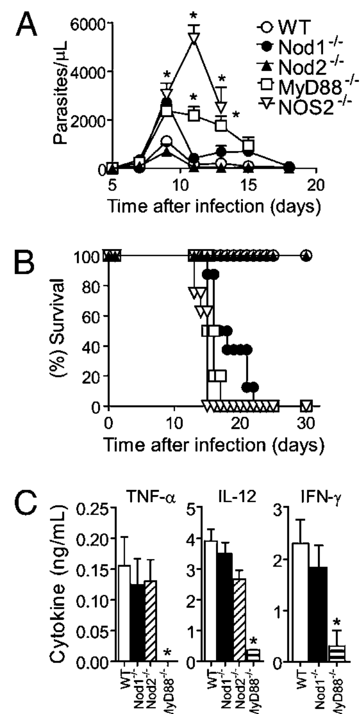

FIGURE 3. $Nod1^{-/-}$ mice are highly susceptible to *T. cruzi* infection in vivo. $Nod1^{-/-}$ (filled circle), $Nod2^{-/-}$ (filled triangle), $MyD88^{-/-}$ (open squares), $NOS2^{-/-}$ (open triangle), and WT (open circle) mice were infected i.p. with 1000 trypomastigotes. *A*, Parasitemia was quantified by counting the parasites in 5 μl of citrated blood obtained from the lateral tail veins at days 5, 7, 9, 11, 13, 15, and 17 postinfection. *B*, Mortality was evaluated by daily inspection of the cages. *C*, Concentration of TNF-α, IL-12p40, and IFN-γ in the sera of mice infected for 10 d. *$p < 0.005$ as compared with WT mice.

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estimated the expression of NOS2 in vivo by immunohistochemistry. We found that expression of NOS2 was reduced in the cardiac tissues of $Nod1^{-/-}$ and $Nod2^{-/-}$ mice (Supplemental Fig. 3*A*, 3*B*). Accordingly, $Nod1^{-/-}$ and $Nod2^{-/-}$ mice show an impaired production of NO as measured by nitrite production in the sera of animals infected for 14 d (Supplemental Fig. 3*C*). Collectively, these data indicate that both Nod1 and Nod2 are required for NO production in response to *T. cruzi* infection ex vivo and in vivo. Next, we measured the production of IFN-γ in different organs of mice on day 14 postinfection and found that $Nod1^{-/-}$, $Nod2^{-/-}$, and WT mice produced similar amounts of IFN-γ in serum, heart, spleen, and liver (Supplemental Fig. 4). To further evaluate the cytokine production in these mice, we stimulated spleen cells from naive mice with *T. cruzi* and measured cytokine production. We found that $Nod1^{-/-}$, $Nod2^{-/-}$, and WT spleen cells readily produced IFN-γ and IL-10 in response to *T. cruzi* (Supplemental Fig. 5*A*, 5*B*). In addition, spleen cells from previously infected WT, $Nod1^{-/-}$, and $Nod2^{-/-}$ mice were able to proliferate and to produce IFN-γ in response to Con A (Supplemental Fig. 5*C*, 5*D*). These results indicate that, regardless of Nod1 deficiency, T cells from these mice did not demonstrate apparent defects for proliferation and IFN-γ production. Moreover, ex vivo cultures of spleen cells obtained from $Nod1^{-/-}$ mice released high numbers of trypomastigotes into the tissue culture supernatants (Supplemental Fig. 6*A*). Therefore, the higher levels of IFN-γ detected in splenocyte cultures of $Nod1^{-/-}$ may be related to the increased parasitism in the organ. Interestingly, the higher parasite loads found in the spleen of $Nod1^{-/-}$ mice were accompanied by a slightly reduced splenomegaly of the $Nod1^{-/-}$ when compared with WT and $Nod2^{-/-}$ mice (Supplemental Fig. 6*B*, 6*C*). Together, these studies demonstrate that Nod1 deficiency does not impair the production of cytokine ex vivo and in vivo.

Next, we compared the infection of $Nod1^{-/-}$ with $NOS2^{-/-}$ and $MyD88^{-/-}$ mice, which have been previously characterized as highly susceptible to *T. cruzi* infection (10, 20). We found that $NOS2^{-/-}$ mice show an exacerbated parasitemia in the blood and succumbed to infection at very acute stages (Fig. 3*A*, 3*B*). $MyD88^{-/-}$ and $Nod1^{-/-}$ mice showed a similar high blood parasitemia on day 9 postinfection (Fig. 3*A*) and succumbed to the infection, respectively, between days 15–17 and 15–22 (Fig. 3*B*). Strikingly, $Nod1^{-/-}$ succumbed to infection soon after the $MyD88^{-/-}$ mice, which are known to be highly susceptible to *T. cruzi* infection (10). Therefore, these data support the unique role of Nod1 for host resistance against *T. cruzi* infection. $MyD88^{-/-}$ mice have been previously described as highly susceptible to *T. cruzi* infection because of the lack of cytokine production (10). Therefore, we measured TNF-α, IL-12, and IFN-γ in the sera of $MyD88^{-/-}$, $Nod1^{-/-}$, and WT mice on day 10 postinfection. We found that whereas $MyD88^{-/-}$ mice were severely impaired for cytokine production, $Nod1^{-/-}$ mice effectively produced TNF-α, IL-12p40, and IFN-γ in response to infection (Fig. 3*C*).

Collectively, our data indicate that Nod1 is required for murine resistance against infection with *T. cruzi*. Interestingly, the main signaling pathways downstream of Nod1 and Nod2 are MAPKs and NF-κB (3, 5). Thus, our data may explain previous reports, which demonstrated that IFN-primed macrophages trigger NO in response to *T. cruzi* infection via NF-κB and MAPK pathways (21, 22). Of note, the demonstration that Nod1-dependent pathways operate in response to *T. cruzi* provide mechanistic explanation for how innate immune cells are activated in response to intracellular parasites. In this context, it is possible that NLR cooperates with TLR signaling for innate immune detection of intracellular protozoan parasites. Interestingly, *T. cruzi* lack peptidoglycan or any known agonist for Nod1. In this context, it remains unknown if Nod1 directly senses a parasite pathogen-associated molecular pattern or if the Nod1 indirectly accounts for host resistance. The mechanisms underlying the Nod1-dependent resistance appear to be independent of cytokine production and possibly dependent on the impaired killing ability of the IFN-γ–activated BMMs. One can speculate that the IFN-γ–inducible p47GTPase participates in this process. It was previously demonstrated that the $LRG\text{-}47^{-/-}$ BMMs are defective in killing intracellular amastigotes despite normal expression of TNF-α and NOS2 (23). It is also possible that NO participates in the Nod1-dependent resistance. However, our data show that despite the reduced expression of NOS2 and production of NO, cells and animals deficient for Nod2 effectively restricted *T. cruzi* infection ex vivo and in vivo. Therefore, we cannot support a strong role for NO in the Nod1-dependent resistance against *T. cruzi* described in this study. Regardless of the participation of NOS2 and p47GTPase, other mechanisms may operate to trigger the Nod1-dependent resistance against *T. cruzi* infection. Identification of such mechanisms is under investigation in our laboratories. The future elucidation of the Nod1-dependent responses that operate in response to *T. cruzi* may account for our understanding of how NLR proteins operate to trigger host resistance against infectious diseases. Indeed, it may contribute to the rational development of drugs that can potentially help the treatment of Chagas disease.

Disclosures

The authors have no financial conflicts of interest.

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