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

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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 the 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 vacuolar membrane and gain access to the host cell cytoplasm, such as *Listeria monocytogenes* and *Shigella flexneri* (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...
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-κB and MAPKs, participate in the innate immune recognition and host resistance against 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 back-crossed to C57BL/6 (wild-type [WT]) for seven or more generations. Mice were infected i.p. with 10^6 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 hemocytometer counting chambers. The determinations of intracellular parasites were performed by microscopic counting of Giemsa-stained parasites.

Real-time PCR, cytokins, and NO

Real-time PCR was performed as described previously (19). Primers used were β-actin-fwd 5′-AGCTGCGTTGATACCTTT-3′; β-actin-rev 5′-AAACCTGCACTTTGTTGCT-3′; TNF-fwd 5′-GATCTCGCAAGACATGGAGTCAT-3′; TNF-rev 5′-CTCAGCTGAAAGCCTCCCTCCA-3′; IL-12p40-fwd 5′-AGACCCAGAGCTGGAGTCAT-3′; IL-12p40-rev 5′-TGCTCCACAGTTCGAAAG-3′; IFN-fwd 5′-GCATCTTTGCGTTTCCAGCT-3′; IFN-rev 5′-CCTTTTTGGCTTTGCTGTGCT-3′; NOS2-fwd 5′-CGAAGGCTCCTTTCAGAAG-3′; and NOS2-rev 5′-TGA- GCTATATTGCTGTGCTGCT-3′. The cytokine concentrations were assessed by OpTEIA ELISA sets (BD Biosciences, San Diego, CA) according to manufacture 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. 1A). Next, we evaluated the production of IL-12p40 and TNF-α by 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. 1B). 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. 1B). 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. 2A) and higher loads of intracellular amastigotes per infected cell (Fig. 2B and Supplemental Fig. 1). These results indicate that Nod1−/− BMMs failed to eliminate the intracellular parasites in response to IFN-γ.
that Nod1<sup>−/−</sup> 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<sup>−/−</sup> mice were ~3 times higher than the similarly infected WT and Nod2<sup>−/−</sup> mice (Fig. 3A and Supplemental Fig. 2A). Strikingly, 100% of the Nod1<sup>−/−</sup> mice succumbed to infection 24 d postinfection in contrast to none of the WT or Nod2<sup>−/−</sup> mice (Fig. 3B and Supplemental Fig. 2B). The higher susceptibility of the Nod1<sup>−/−</sup> mice was accompanied by a lower inflammatory infiltrate in the cardiac tissues on day 14 postinfection (Supplemental Fig. 2C). 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 <i>T. cruzi</i> 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<sup>−/−</sup> mice contained higher numbers of amastigote nests in the cardiac tissues when compared with WT and Nod2<sup>−/−</sup> animals (Supplemental Fig. 2D). Together, these results suggest that Nod1-dependent responses are important for induction of acute myocarditis observed upon <i>T. cruzi</i> infection. These responses may account for restriction of parasite multiplication in the cardiac tissues.

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

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. 2C). In contrast, in the presence of 10 U/ml IFN-γ, parasites were not detected in the supernatants of Nod1<sup>−/−</sup> BMMs (Fig. 2D). On day 5 postinfection, we detected high numbers of fully motile trypomastigotes in cultures of Nod1<sup>−/−</sup> 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. 2E). Taken together, our data indicate that Nod1<sup>−/−</sup> BMMs failed to restrict <i>T. cruzi</i> 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 <i>T. cruzi</i> infection in response to IFN-γ.

<i>Nod1<sup>−/−</sup></i> mice succumb to <i>T. cruzi</i> infection and show higher loads of parasites in spleen and heart tissues

To test if the impaired trypanocidal ability of Nod1<sup>−/−</sup> BMMs culminates with increased susceptibility in vivo, we compared infections of Nod1<sup>−/−</sup>, Nod2<sup>−/−</sup>, and WT mice. We found FIGURE 2. Nod1-dependent responses are required for elimination of intracellular parasites by IFN-γ-treated macrophages. BMM from Nod1<sup>−/−</sup>, Nod2<sup>−/−</sup>, or WT mice were cultured at the concentration of 1 × 10<sup>5</sup> 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). 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. *<i>p</i> < 0.005 as compared with similarly treated WT BMMs.

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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. 3A, 3B). 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. 3C). 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. 5A, 5B). 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. 5C, 5D). 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 tryptomastigotes into the tissue culture supernatants (Supplemental Fig. 6A). 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. 6B, 6C). 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. 3A, 3B). MyD88−/− and Nod1−/− mice showed a similar high blood parasitemia on day 9 postinfection (Fig. 3A) and succumbed to the infection, respectively, between days 15–17 and 15–22 (Fig. 3B). 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. 3C).

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-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 insective 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.

References


