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A Novel IFN Regulatory Factor 3-Dependent Pathway Activated by Trypanosomes Triggers IFN-β in Macrophages and Fibroblasts[1]

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Innate immune recognition of intracellular pathogens involves both extracellular and cytosolic surveillance mechanisms. The intracellular protozoan parasite *Trypanosoma cruzi* triggers a robust type I IFN response in both immune and nonimmune cell types. In this study, we report that signaling through TBK1 and IFN regulatory factor 3 is required for *T. cruzi*-mediated expression of IFN-β. The TLR adaptors MyD88 and TRIF, as well as TLR4 and TLR3, were found to be dispensable, demonstrating that *T. cruzi* induces IFN-β expression in a TLR-independent manner. The potential role for cytosolic dsRNA sensing pathways acting through RIG-I and MDA5 was ruled out because *T. cruzi* was shown to trigger robust expression of IFN-β in macrophages lacking the MAVS/IPS1/VISA/CARDif adaptor protein. The failure of *T. cruzi* to activate HEK293-IFN-β-*luciferase* cells, which are highly sensitive to cytosolic triggers of IFN-β expression including *Listeria*, Sendai virus, and transfected dsRNA and dsDNA, further indicates that the parasite does not engage currently recognized cytosolic surveillance pathways. Together, these findings identify the existence of a novel TLR-independent pathogen-sensing mechanism in immune and nonimmune cells that converges on TBK1 and IFN regulatory factor 3 for activation of IFN-β gene expression.   *The Journal of Immunology,* 2008, 181: 7917–7924.

Type I IFNs, traditionally regarded as mediators of antiviral defense, have recently emerged as important immunomodulatory cytokines that influence disease progression in a variety of nonviral pathogen infections (1). Pathogen triggers of type I IFN expression include those that engage cytosolic surveillance molecules (2) and those that activate TLRs, well-described pattern recognition receptors capable of recognizing a diverse range of pathogen products and nucleic acids (3, 4). TLRs that signal type I IFN expression include TLRs 3, 4, 7, and 9 and utilize the adaptor proteins TRIF and/or MyD88 (reviewed in Ref. 4). Nucleic acids in the host cytoplasm can also trigger type I IFN expression in a TLR-independent manner. Triphosphate RNA and dsRNA are recognized by the RIG-like receptors, a family of RNA helicases that include retinoic acid-inducible gene (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (5, 6). RIG-I and MDA5 both signal through the adaptor molecule MAVS (also called IPS1/VISA/CARDif) (7–10). Cytosolic DNA can also induce type I IFNs in a TLR-independent manner (11–13). A candidate sensor DNA-dependent activator of IFN regulatory factors (IRFs)[3] (DAI) has been identified; however, gene targeting of DAI

suggests it may not function as a cytosolic DNA receptor upstream of type I IFN expression (14–16). Cytosolic bacteria such as *Listeria monocytogenes* stimulate type I IFNs independently of TLRs and RIG-like receptors (17, 18) by a mechanism that requires the pore-forming molecule listeriolysin O to escape from the vacuole and enter the host cell cytosol, but the receptor for this pathway has not yet been identified (2, 13).

Members of the IRF family of transcription factors are central regulators of the type I IFN response (19). IRF3, which is sufficient to drive IFN-β expression, is phosphorylated on C-terminal serine and threonine residues by the noncanonical IκB kinases TANK-binding kinase 1 (TBK1) or inducible IKKε, which leads to the formation of IRF3 dimers, nuclear translocation, and binding to the IFN-β promoter (20–22). Secreted IFN-β binds to the type I IFN receptor (IFNAR), signaling an autocrine or paracrine response, resulting in the expression of a multitude of IFN-stimulated genes (ISGs) as well as additional IFN-β (23).

The obligate intracellular protozoan pathogen *Trypanosoma cruzi* causes human Chagas' disease, a leading cause of heart failure in Latin America. To initiate infection, *T. cruzi* trypomastigotes activate a number of host cell signaling pathways, resulting in plasma membrane invagination and entry into the endocytic pathway or targeted exocytosis of host cell lysosomes at the parasite attachment site (reviewed in Ref. 24). Consequently, invading parasites are rapidly trafficked to the host cell lysosomal compartment where they transiently reside before taking up residence in the host cell cytoplasm (25). Similar to *Listeria*, *T. cruzi* secretes a pore-forming molecule into the vacuole, which is thought to facilitate egress into the host cell cytosol (26). During the course of infection, *T. cruzi* triggers a strong type I IFN response in a variety of

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[3] Abbreviations used in this paper: IRF, IFN regulatory factor; DAI, DNA-dependent activator of IRF; TBK1, TANK-binding kinase 1; IFNAR, type I IFN receptor; MEF, mouse embryonic fibroblast; WT, wild type; Pen-Strep, penicillin-streptomycin; BMDM, bone marrow-derived macrophage; qRT-PCR, quantitative RT-PCR.

infected cell types (27–29) and in vivo at the site of intradermal infection of mice (A.-D. C. Chessler, M. Unnikrishnan, and B. A. Burleigh, manuscript in preparation), however, the mechanistic basis for *T. cruzi*-mediated induction of IFN-β is not well understood. In addition there is some indication that the type I IFN response influences the outcome of *T. cruzi* infection in vivo (28–30). In this study, we present evidence that *T. cruzi* induces expression of IFN-β in macrophages and fibroblasts independently of TLRs and known cytosolic sensors of nucleic acids with kinetics that suggest that IFN-β induction occurs before vacuole egress.

Materials and Methods

Cell culture and pathogens

IRF3$^{-/-}$, TRIF$^{-/-}$, MyD88$^{-/-}$, and TLR3$^{-/-}$ mouse embryonic fibroblasts (MEF) and matched WT controls, HEK293 IFN-β-*luc* reporter cells, and HEK293-*tlr3* cells were from K. Fitzgerald (University of Massachusetts, Worcester, MA). TBK1$^{-/-}$ and matched WT MEF were provided by W.-C. Yeh (University of Toronto, Toronto, Ontario, Canada) and MyD88$^{-/-}$, MyD88$^{-/-}$/TIRAP$^{-/-}$, and TLR2$^{-/-}$/TLR4$^{-/-}$ MEF and matched WT controls were from R. Medzhitov (Yale University, New Haven, CT). MEF, HEK293-tlr3, or HEK293-IFN-β-*luc* cells were maintained in DMEM containing 4.5 g/L glucose and 110 mg/L sodium pyruvate (Life Technologies 10313) supplemented with 10% FBS, 2 mM glutamine, and 100 μg/ml penicillin-streptomycin (Pen-Strep; DMEM-10).

Bone marrow-derived macrophages (BMDM) and MEFs were generated from WT, IRF3$^{-/-}$, TLR3$^{-/-}$, TLR4$^{-/-}$, TRIF$^{-/-}$, MyD88$^{-/-}$, MAVS$^{-/-}$, and C57BL/6 mice as previously described (31). IRF3$^{-/-}$ mice were a gift from T. Taniguchi (University of Tokyo, Tokyo, Japan). MyD88$^{-/-}$/TRIF$^{-/-}$ mice were bred from MyD88$^{-/-}$ (32) and TRIF$^{-/-}$ (33) mice provided by S. Akira (Osaka University, Osaka, Japan). TLR3$^{-/-}$ and TLR4$^{-/-}$ mice were from S. Akira (Osaka University, Osaka, Japan). MAVS$^{-/-}$ mice were a gift from Z. Chen (University of Texas Southwestern, Dallas, TX). Mice were bred and maintained at the University of Massachusetts Medical School and all experiments were conducted with Institutional Animal Care and Use Committee approval.

BMDM were flushed from femurs and cultured in 100-mm-diameter petri dishes for 6 days in BMDM medium (DMEM supplemented with 20% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/ml Pen-Strep, 55 μM 2-ME, and 30% L929 cell supernatant). MEF, HEK293-tlr3, or HEK293-IFN-β-*luc* cells were maintained in DMEM-10. Tissue culture-derived *T. cruzi* trypomastigotes (Y strain) were generated as previously described (34) and washed twice in DMEM containing 4.5 g/L glucose and 110 mg/L sodium pyruvate (Life Technologies 10313) supplemented with 2% FBS, 2 mM glutamine, and 100 μg/ml Pen-Strep (DMEN-2) before cell infection. Aldehyde-fixed parasites were fixed in 2% paraformaldehyde for more than 24 h and washed twice in DMEM-2 before use. Epimastigotes from the *T. cruzi* Y strain were maintained in liver infusion tryptose medium containing 10% FBS at 28°C (35) and washed twice in DMEM-2 before incubation with mammalian cells. Parasites were heat killed for 10 min at 56°C and washed twice in DMEM-2 before use. Overnight cultures in Luria-Bertani medium of *Listeria monocytogenes* strain 10403S were provided by D. Higgins (Harvard University, Boston, MA) and cells were infected with a multiplicity of infection of five bacteria per cell for 1 h in DMEM-10 without Pen-Strep, then medium was removed and fresh medium containing 50 μg/ml gentamicin was added.

Cell infection

Semiconfluent monolayers grown in 6-well plates were incubated with 5 × 10^7 parasites/well for 2 h at 37°C in 5% CO$_2$. Remaining extracellular parasites were removed by washing cells three times with PBS and DMEM-2 added in the presence or absence of bafilomycin A (50 nM; Sigma-Aldrich) as indicated and incubated for the indicated total infection times.

Western blot analysis

Infected or mock-treated control cells were washed three times in ice-cold PBS and then lysed in 100 μl of 95°C 2× Laemmli sample buffer. Lysates were separated on a 10% polyacrylamide gel and blotted onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked with TBS/5% milk and probed with rabbit anti-human phospho-IRF3 Ser^{396} (a gift from J. Hiscott, McGill University, Montreal, Quebec, Canada) or rabbit anti-human IRF3 (Santa Cruz Biotechnology) at 4°C overnight. After washing, bound Abs were detected with anti-rabbit IgG coupled to HRP (1/10,000) at room temperature for 1 h. The signal was visualized by using ECL (Amersham Pharmacia Biosciences). A representative blot of three independent experiments is shown.

Immunofluorescence

Glass coverslips (12 mm^2) with adherent cells were fixed in 2% paraformaldehyde/PBS, washed with PBS, and incubated for 10 min in 50 mM NH$_4$Cl/PBS. Ab dilutions, incubations, and washes were conducted in TBS/BSA (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% BSA) for staining of external Ags or permeabilized with TBS/BSA/0.1% saponin to detect intracellular Ags. *T. cruzi* immunostaining was performed with a rabbit anti-*T. cruzi* Ab (1/1500) (36) and Lamp-1 staining was performed with mouse anti-rat lamp-1 mAb LY1C6 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Secondary Ab conjugates used were Cy5-labeled goat anti-rabbit IgG (Amersham Pharmacia) and TR-conjugated anti-mouse (Molecular Probes). Ab incubations were conducted for 40 min with extensive washes for 25 min following primary and secondary incubation steps. Parasite and mammalian DNA was stained with 1.25 μg/ml 4′,6-diamidino-2-phenylindole (Pierce) for 3 min. Coverslips were washed with PBS and mounted in 10% Mowiol (Calbiochem) containing 2.5% 1,4-diazobicyclo-[2,2,2]-octane). Fluorescence images were obtained with a Nikon TE-300 inverted epifluorescence microscope equipped with an Orca-100 CCD camera (Hamamatsu) and analyzed using MetaMorph imaging software (Universal Imaging). For quantitative analysis of infection, internal parasites per cell were counted in a minimum of 100 cells/coverslip.

RT-PCR and real-time-PCR

Total RNA was harvested at the time points indicated with a Qiagen RNeasy Mini Kit. The Superscript II RT Kit (Invitrogen) was used for cDNA synthesis. cDNA templates were used for a quantitative real-time PCR using an Applied Biosystems 7000 or Applied Biosystems 7300 real-time PCR machine. The relative level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer, whereby data for each sample were normalized to *gapdh* and expressed as a fold change compared with untreated or uninfected controls. Primers and probes for human *ifnb* used were as follows: 5′-GACCAACAAGTGTCTCCTCCAAA-3′ (forward), 5′-AGCAAGTTGTAGCTCATGGAAAGAG-3′ (reverse), and 5′-6FAM-TCTCCTGTTGTGCTTCTCCACGA-3′ TAMRA (probe). The following TaqMan gene expression assays were used: mouse *ifnb* (Mm00439546_s1), human *gapdh* (4310884E), mouse *gapdh* (4308313) human *il6* (Hs00174131_m1), mouse *il6* (Mm00446190_m1), mouse *cxcl10* (Mm99999072_m1), mouse *viperin* (Mm00491265_m1) (Applied Biosystems). For quantification of mouse *lrg47* SYBR Green PCR Master Mix (Applied Biosystems) was used with the following primers: 5′-TGAGCTCAGCCTTCCCCTTT-3′ (forward) and 5′-TGGGACAATGTTGCCACAGT-3′ (reverse).

Quantification of luciferase expression

Quantification of luciferase expression was performed with the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocols using supplied reagents. Briefly, 24 h after treatment, cells were washed with PBS and lysed with 350 μl/well passive lysis buffer and 20 μl of sample was added to 100 μl of luciferase assay substrate in luciferase assay buffer II in a luminometer tube. Luciferase activity was measured using an AutoLumat LB953 luminometer (EG&G Berthold).

Statistics

Samples were compared using a two-tailed, unpaired *t* test with Welch's correction; $p < 0.05$ were considered to be significant.

Results

T. cruzi *induces a delayed IFN-β response in immune and nonimmune cells*

T. cruzi infection of isolated human foreskin fibroblasts is known to trigger robust up-regulation of a group of ISGs by 24 h after infection, correlating with synthesis and secretion of IFN-β from infected cells (27). In this study, we examine the kinetics of IFN-β mRNA expression in *T. cruzi*-infected MEF and BMDM by quantitative RT-PCR (qRT-PCR; Fig. 1). As compared with infection with Sendai virus, which strongly induces *ifnb* in both fibroblasts and macrophages with peak responses between 4 and 8 h after infection (Fig. 1, *A* and *B*, *second panels*), the peak response to *T. cruzi* was significantly delayed (Fig. 1, *A* and *B*, *first panels*). In *T.*

FIGURE 1. Kinetics of *ifnb* and ISG induction in *T. cruzi*-infected cells. Kinetics of IFN-β mRNA induction in WT or IFNAR$^{-/-}$ MEF (*a*) and BMDM (*b*) in response to *T. cruzi* or Sendai virus infection as determined by qRT-PCR. Induction of the ISGs *cxcl10*, *viperin*, and *lrg47* in response to 16 h of *T. cruzi* infection was confirmed in BMDM and MEF by qRT-PCR (*c*). Values reported represent the mean fold induction relative to mock-infected controls.
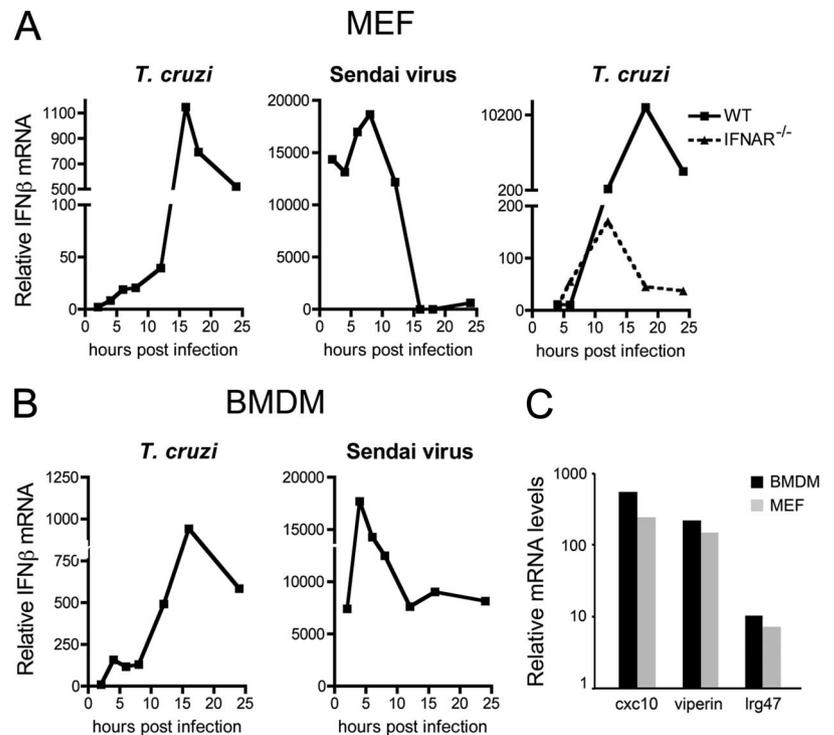
cruzi-infected fibroblasts, *ifnb* transcript levels gradually increased over the first 12 h of infection, followed by a dramatic rise in *ifnb* expression, peaking at 16 h after infection (Fig. 1A). A similar trend was observed in BMDM, although parasites triggered an initial peak of *ifnb* expression at 4 h after infection, followed by a large peak at 16 h (Fig. 1B).

T. cruzi infection of IFNAR$^{-/-}$ MEF revealed the absence of the 16-h peak of *ifnb* expression observed in WT MEF that corresponds to the IFN-dependent autocrine activation loop (Fig. 1A, *third panel*). In the absence of this loop, it is clear that the parasite-driven *ifnb* response increases steadily and peaks at ∼12 h after infection (Fig. 1B, *third panel*). Overall, these findings demonstrate that primary signals elicited by *T. cruzi* in fibroblasts and professional phagocytes trigger induction of *ifnb* expression with strikingly different kinetics as compared with virus-infected cells. In addition, IFNAR-dependent amplification of *ifnb* expression, as well as the induction of the ISGs *cxcl10*, *viperin*, and *lrg47* (Fig. 1C) is consistent with the production and secretion of active IFN-β from *T. cruzi*-infected cells, as previously reported (27).

Following *T. cruzi* entry into cells, parasites reside in a lysosomal vacuole (36, 37) for several hours before taking up residence in the host cell cytoplasm (25). We demonstrate that cytoplasmic localization of *T. cruzi* is a gradual process where loss of Lamp-1 staining of parasite vacuoles begins at ∼8 h and is complete by 16 h after entry (Fig. 2, *A* and *B*). Electron microscopic examination of intracellular *T. cruzi* over this time course demonstrates that loss of Lamp-1 correlates with loss of vacuole membrane (data not shown). Given that the initial induction of IFN-β mRNA in *T. cruzi*-infected cells precedes onset of egress from the parasitophorous vacuole, these data suggest that the trigger for *ifnb* expression is not linked to cytosolic localization of the parasite. Further support for this was obtained using bafilomycin A to block *T. cruzi* exit from the vacuole (25). Although bafilomycin A treatment exerted a strong inhibitory effect on *T. cruzi* vacuole egress (Fig. 2B), drug treatment did not block the initial rise in *ifnb* expression observed over the first 8 h of infection. Instead, bafilomycin A treatment attenuated *ifnb* expression at time points >10 h after infec-

tion, suggesting an impact on the autocrine activation loop, perhaps due to inhibition of IFNAR signaling from an endosomal compartment. Together, these data demonstrate that induction of *ifnb* by *T. cruzi* occurs before cytosolic localization and is likely to be initiated via extracellular receptors or from within the endosomal compartment in a manner that is not pH dependent.

To determine whether the ability of *T. cruzi* to trigger *ifnb* expression is strictly associated with intracellular infection, MEF were exposed to live or heat-killed trypomastigotes, the invasive life cycle stage, or epimastigotes, the insect stage of the parasite that does not infect mammalian cells. Heat-killed trypomastigotes were found to elicit strong induction of *ifnb* with more rapid kinetics than the response to live parasites (Fig. 2C). In contrast, a minimal IFN response to noninfective live or heat-killed epimastigotes was observed at both 6 and 16 h after treatment (Fig. 2C). These findings indicate that the ability of *T. cruzi* to trigger *ifnb* expression in mammalian cells is associated with the infective forms of the parasite, but that active invasion by live trypomastigotes is not necessary to trigger the response. Interestingly, neither aldehyde-fixed trypomastigotes or secreted/released trypomastigote-associated material present in "parasite-conditioned medium" triggers *ifnb* expression in MEF. Presently, it is not known whether live and heat-killed trypomastigotes trigger *ifnb* expression by engaging the same "receptors"/signaling pathways or whether the heat-killing process exposes a different set of ligands that causes this response.

TBK1 and IRF3 are required for T. cruzi-*dependent induction of IFN-β*

Transcriptional activation of the IFN-β promoter by IRF3 is central to IFN-β expression triggered by a diverse set of signals. Induction of *ifnb* in response to *T. cruzi* was completely abolished in IRF3$^{-/-}$ and TBK1$^{-/-}$ fibroblasts (Fig. 3A) as well as in IRF3$^{-/-}$ macrophages (Fig. 3B), confirming the critical role of this signaling axis in *T. cruzi*-dependent IFN-β expression. Comparable infection levels were observed in WT cells and IRF3- or TBK1-deficient MEF and no differences were observed in the kinetics of

FIGURE 2. Intracellular localization is not required for *ifnb* induction in *T. cruzi*-infected cells. Kinetics of egress from the parasitophorous vacuole was examined by immunofluorescence staining (*A*). Extracellular parasites were stained with *T. cruzi*-specific Abs (green), vacuole membrane with Lamp-1 (red), host and parasite DNA (blue) in *T. cruzi*-infected MEF at 4, 8, 16, and 24 h after infection. A subset of parasites in Lamp-1-positive vacuoles is highlighted by arrowheads and arrows are used to indicate cytosolic parasites. Fibroblasts treated with (gray triangles) or without (black squares) 50 nM bafilomycin A (BafA) were infected with *T. cruzi* and the percentage of Lamp-1-positive parasites (*B, first panel*) or IFN-β mRNA (*B, second panel*) was quantitated. MEF were exposed to infective *T. cruzi* trypomastigotes (live trypos), heat-killed trypomastigotes (hk), aldehyde-fixed trypomastigotes (fixed), parasite-conditioned medium (pcm), live noninfective *T. cruzi* epimastigotes (epi), and heat-killed epimastigotes (hk epi) to induce *ifnb*. Transcription was determined by qRT-PCR at 6 and 16 h after infection (*C*). Values reported represent the mean fold induction relative to mock-infected controls ± SD.
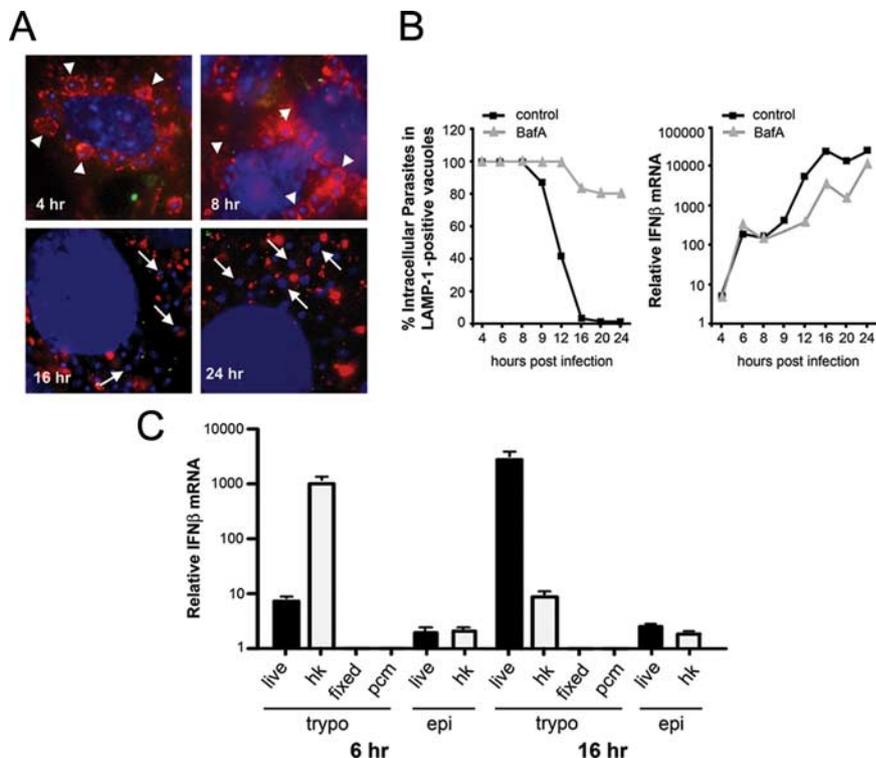

vacuole egress by *T. cruzi* in these cells (data not shown). Activation of IRF3 involves phosphorylation of multiple serine and threonine residues located in the C terminus, including Ser[396] which can be phosphorylated by TBK1 (38, 39). Using an Ab specific for phospho-Ser[396], we demonstrate elevated levels of phospho-IRF3 in *T. cruzi*-infected fibroblasts at 2 h after infection (Fig. 3*C*). The observation that IRF3 is phosphorylated at this early time point is consistent with our findings that *T. cruzi* triggers for IFN-β expression by engaging a signaling pathway that requires both TBK1 and IRF3 before cytosolic localization of the parasite.

T. cruzi induction of IFN-β is independent of TLR signaling

A variety of pathogen-associated molecular patterns can activate cells, triggering expression of type I IFNs through TLR-dependent and -independent pathways that converge on TBK1 and IRF3. In this study, we tested the ability of *T. cruzi* to induce *ifnb* expression in macrophages and fibroblasts that lack expression of the TLR adaptors MyD88 and TRIF. We find that *T. cruzi*-induced *ifnb* expression is not abrogated in MEF lacking MyD88 (Fig. 4*A*) or in MyD88$^{-/-}$ and TRIF$^{-/-}$ BMDM (Fig. 4*B*). As a control,
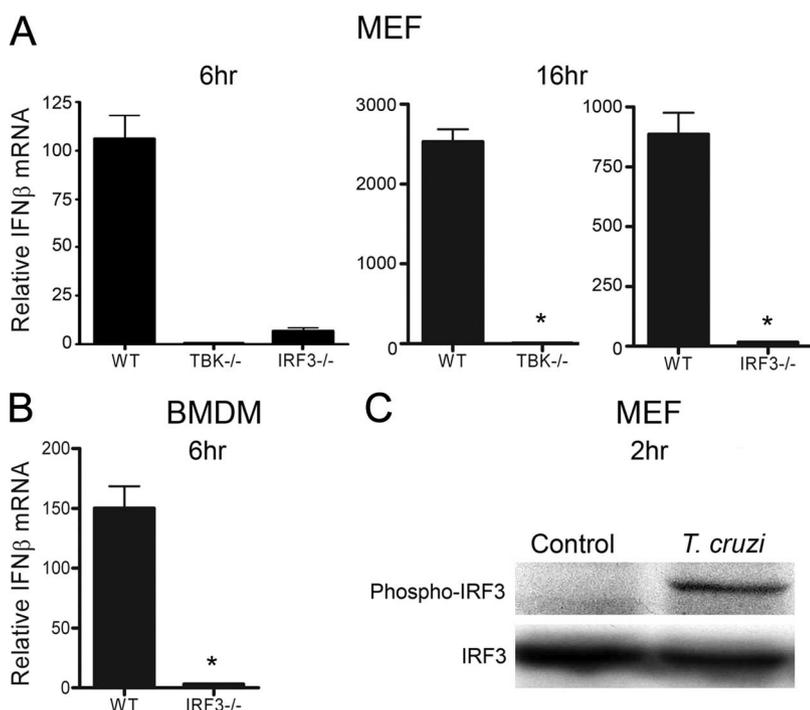
FIGURE 3. TBK1 and IRF3 are required for *T. cruzi*-induced IFN-β expression. *ifnb* transcript levels were measured by qRT-PCR in WT and IRF-3$^{-/-}$ or TBK1$^{-/-}$ MEF (*A*) and in IRF3$^{-/-}$ BMDM (*B*) following infection with *T. cruzi* for 6 and 16 h (MEF) or 6 h (BMDM). Values reported represent the mean fold induction relative to mock-infected controls ± SD. *C*, IRF3 is phosphorylated (Ser[396]) following a 2-h exposure of MEF to live *T. cruzi* trypomastigotes.
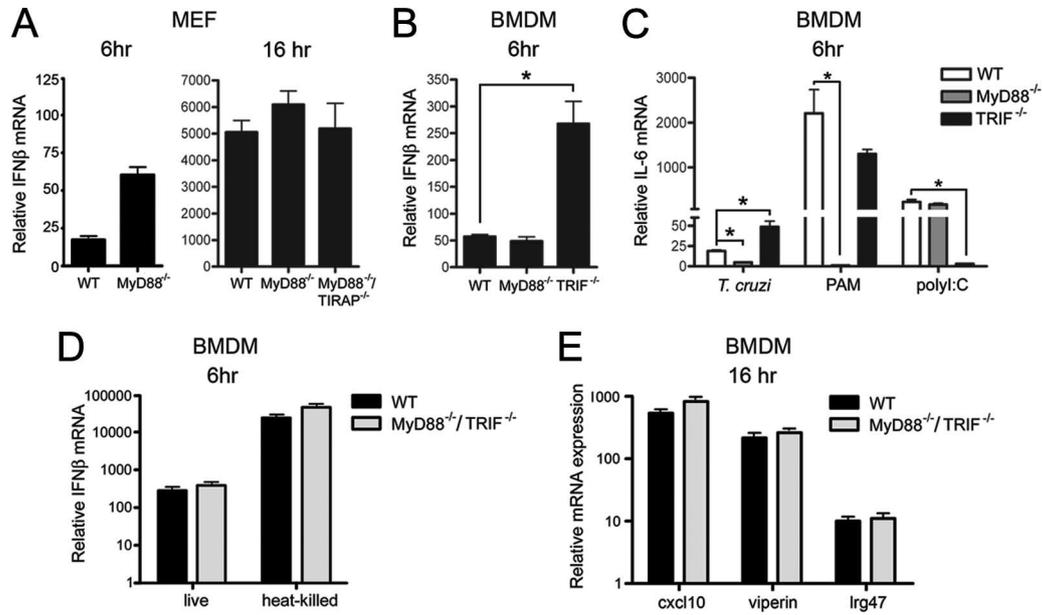
FIGURE 4. MyD88 and TRIF are not required for *T. cruzi* induction of IFN-β. Relative IFN-β mRNA levels were measured by qRT-PCR in *T. cruzi*-infected WT, MyD88$^{-/-}$, and MyD88$^{-/-}$/TIRAP$^{-/-}$ MEF 6 and/or 16 h after infection (*A*) or WT, MyD88$^{-/-}$, and TRIF$^{-/-}$ BMDM 6 h after infection (*B*). Relative IL-6 mRNA levels were measured by qRT-PCR in WT, MyD88$^{-/-}$, and TRIF$^{-/-}$ BMDM 6 h after infection with *T. cruzi* or treatment with Pam$_2$Csk$_4$ (PAM) or poly(I:C) (*C*). Relative induction of *ifnb* by live parasites or heat-killed *T. cruzi* trypomastigotes (*D*) and of the ISGs genes *cxcl10*, *viperin*, and *lrg47* by live *T. cruzi* trypomastigotes (*E*) was determined by qRT-PCR in WT or MyD88$^{-/-}$/TRIF$^{-/-}$ BMDM 16 h after exposure. Values reported represent the mean fold induction relative to mock-infected controls ± SD.

IL-6 mRNA levels were measured in WT, MyD88$^{-/-}$, and TRIF$^{-/-}$ BMDM following infection with *T. cruzi*, which triggers proinflammatory cytokine expression in a MyD88-dependent manner (Fig. 4*C*) (40). As expected, the IL-6 response triggered by *T. cruzi* or by stimulation with the TLR2 ligand, Pam$_2$Csk$_4$, is attenuated in MyD88$^{-/-}$ macrophages (Fig. 4*C*) and TRIF$^{-/-}$ BMDM fail to respond to exogenous poly(I:C) (Fig. 4*C*). Robust induction of *il6* (Fig. 4*C*) and *ifnb* (Fig. 4*B*) was observed in response to *T. cruzi* in TRIF-deficient cells. In fact, parasite-induced expression of *ifnb* and *il6* was ~3- to 5-fold higher in TRIF$^{-/-}$ BMDM when compared with WT cells (Fig. 4, *B* and *C*). Although the significance of the increased induction of *ifnb* in TRIF$^{-/-}$ BMDM is unknown, these data clearly indicate that the TLR adaptors TRIF and MyD88 are not required for *ifnb* induction in response to *T. cruzi*. This assertion is further confirmed by demonstrating that *T. cruzi*-dependent induction of downstream ISGs such as *cxcl10*, *viperin*, and *lrg47* occurs in MyD88/TRIF double knockout BMDM (Fig. 4*D*). Finally, we demonstrated that both live and heat-killed *T. cruzi* trypomastigotes trigger expression of *ifnb* in a TLR-independent manner (Fig. 4*E*), suggesting that a similar signaling pathway is engaged by live and heat-killed parasites.

Given that these data contradict a recent report indicating that *T. cruzi* activates expression of *ifnb* in BMDM in a TRIF-dependent manner (28), we investigated the role of TLR3 and TLR4, which are the only TLRs known to signal through TRIF to activate IFN-β gene expression (41). Consistent with data presented in Fig. 4, the absence of TLR3 or TLR4 does not inhibit *T. cruzi*-triggered *ifnb* expression in BMDM (Fig. 5*A*) or MEF (Fig. 5, *B* and *C*). Because a decrease in *ifnb* expression was observed in *T. cruzi*-infected TLR3-deficient MEF that approaches significance ($p > 0.09$; Fig. 5*C*), we sought to further test the potential role of TLR3 in *T. cruzi*-mediated *ifnb* expression. HEK293 stably expressing TLR3 become responsive to exogenous poly(I:C); however, *T. cruzi* failed to elicit *ifnb* expression in these cells (Fig. 5*D*). Together, these data exclude known MyD88- and TRIF-dependent TLR sig-

naling pathways and directly rule out a role for TLR3 and TLR4 as mediators of *ifnb* expression in response to *T. cruzi*. The data further suggest that *T. cruzi* may interact with an uncharacterized extracellular receptor or one of a growing number of putative cytosolic surveillance molecules to activate IFN-β.
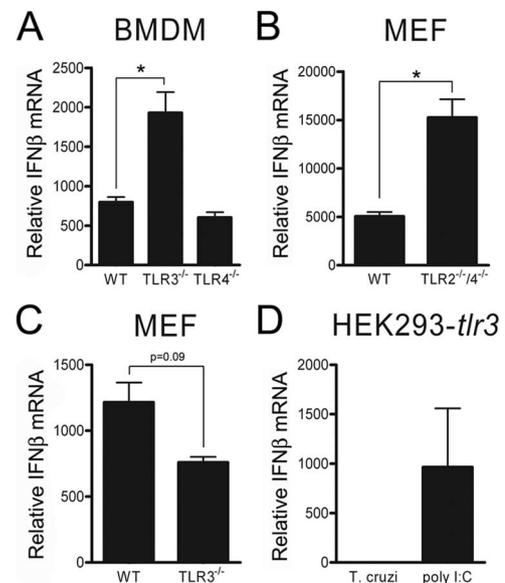
FIGURE 5. TLR3 and TLR4 are not required for *T. cruzi* induction of *ifnb*. Relative *ifnb* transcript abundance was measured by qRT-PCR in WT, TLR3$^{-/-}$, and TLR4$^{-/-}$ BMDM (*A*), WT and TLR2$^{-/-}$/TLR4$^{-/-}$ MEF (*B*), or WT and TLR3$^{-/-}$ MEF (*C*) following infection with *T. cruzi* for 16 h (MEF) or 6 h (BMDM). *D*, HEK293 cells stably expressing TLR3 HEK293-*tlr3* were infected with *T. cruzi* or activated with the TLR3 ligand poly(I:C) for 16 h and relative IFN-β mRNA levels were measured by qRT-PCR. Values reported represent the mean fold induction relative to mock-infected controls ± SD.
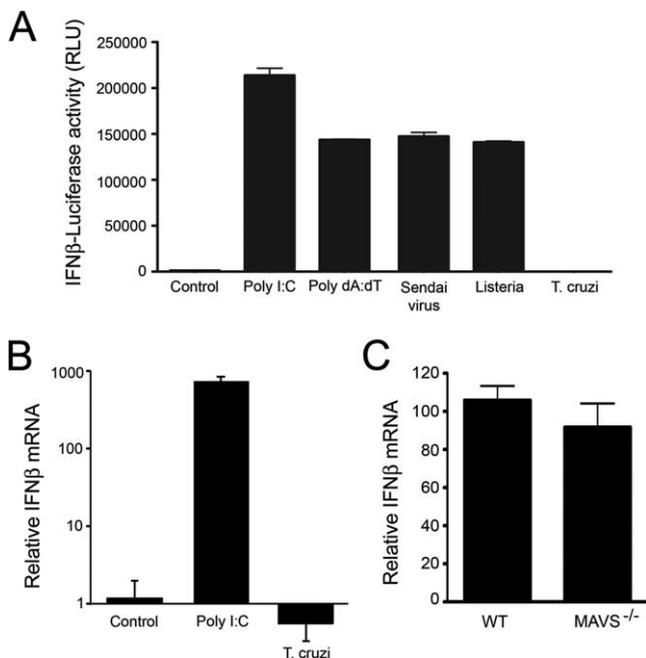
FIGURE 6. *T. cruzi* triggers IFN-β expression independently of characterized cytosolic pathways. HEK293-IFN-β-*luciferase* reporter cells were transfected with dsRNA (poly(I:C)) or dsDNA (poly(dA:dT)) or infected with Sendai virus, *L. monocytogenes*, or *T. cruzi*. Luciferase activity (*A*) or endogenous IFN-β mRNA (*B*) was measured 24 h after treatment. Relative *ifnb* transcript abundance was measured by qRT-PCR in WT and MAVS$^{-/-}$ BMDM after 6 h of infection (*C*). Values reported represent the mean fold induction relative to mock-infected controls $\pm$ SD.

T. cruzi *fails to induce IFN-β expression in HEK293 cells that respond to other cytosolic pathogen triggers*

HEK293 cells exhibit negligible TLR expression but remain responsive to a variety of cytosolic bacterial, viral, and nucleic acid triggers of IFN-β. Using HEK293-IFN-β-*luc* reporter cells, we were able to show that cytosolic delivery of dsRNA (poly(I:C)), or dsDNA (poly(dA:dT)) by lipofection lead to robust luciferase reporter activation (Fig. 6*A*). Similarly, infection of HEK293-IFN-β-*luc* cells with Sendai virus or *L. monocytogenes* caused reporter activation within 6 h (data not shown), with high levels of luciferase expression observed at 24 h (Fig. 6*A*). In stark contrast, *T. cruzi* failed to activate reporter expression at 6 h (data not shown) or 24 h after infection (Fig. 6*A*). To exclude the possibility that parasite infection interferes with the ability to assay luciferase activity, we also examined endogenous IFN-β mRNA by qRT-PCR and found that *T. cruzi* failed to induce *ifnb* gene expression in this cell type (Fig. 6*B*) despite achieving a high level of intracellular infection (data not shown). These data suggest that induction of *ifnb* expression by *T. cruzi* does not occur through the characterized dsRNA-sensing pathways involving RIG-I/MDA5 or through the less characterized dsDNA-sensing pathway. To directly test the role of dsRNA- sensing RIG-like receptors in the IFN response to *T. cruzi*, MAVS-deficient BMDM were infected and shown to mount a response that was similar to WT macrophages (Fig. 6*C*). Given that HEK293 cells responded to both cytosolic dA:dT and *Listeria* infection, which may be triggered by bacterial DNA in the host cell cytoplasm (13), our data strongly suggest that the previously described cytosolic DNA-sensing mechanisms do not play a role in eliciting a type I IFN response to *T. cruzi*. However, it is possible that *T. cruzi* DNA is recognized by a DNA recognition molecule/pathway that is not expressed in HEK cells. Coupled with data showing that known TLR-dependent pathways are not

required for *T. cruzi*-induced *ifnb* expression, our results provide evidence that the intracellular protozoan pathogen *T. cruzi* engages a unique pathway to trigger expression of IFN-β in fibroblasts and macrophages.

Discussion

Pathogen-dependent activation of the type I IFN response in cells is accomplished by engaging one of two general pathways involving recognition by plasma membrane and endosomal TLRs or by cytosolic RNA and DNA-sensing molecules (reviewed in Refs. 4 and 42). In this study, we examined the mechanistic basis for induction of IFN-β in immune and nonimmune cells by the intracellular protozoan parasite *T, cruzi*. In this study, we show that *T. cruzi* triggers IFN-β expression in host cells by engaging a novel TLR-independent pathway that requires both TBK1 and IRF3, but does not involve known cytosolic nucleic acid sensors. We demonstrate that the induction of *ifnb* is stage specific, as the epimastigote stage of the parasite does not induce this response, suggesting that the ligand that triggers the IFN response is differentially expressed within the parasite life cycle. Based on evidence that epimastigotes significantly differ from trypomastigotes with regard to the lipid (43), carbohydrate (44), and protein (45–47) composition of their surface coats, it is not surprising that the host response to these parasite life cycle stages would differ. Additionally, we demonstrate that *T. cruzi*-mediated induction of IFN-β mRNA results in a functional type I IFN response, leading to expression of the ISGs *cxcl10*, *viperin*, and *lrg47*, in fibroblasts and macrophages. Interestingly, IFN-γ- induced *lrg47* is known to play a vital role in control of *T. cruzi* infection in vivo (48). The demonstration here that *T. cruzi*-induced type I IFNs can also result in the expression of this critical player in the immune response to the parasite suggests a possible mechanism for the type I IFN response to play a protective role in *T. cruzi* infection.

Results from this study demonstrate that, comparable to many IFN-β triggering signals, TBK1/IRF3 signaling is required for *T. cruzi*-induced *ifnb* expression. We further demonstrate that the intracellular signaling pathways involved in *ifnb* induction by *T. cruzi* are TLR independent. Infection of cells deficient for expression of the TLR adaptor proteins TRIF and/or MyD88 showed no impairment of *ifnb* expression in response to *T. cruzi*. We also showed that TLR3 and TLR4, which signal through TRIF to activate *ifnb* expression (33), are dispensable for *T. cruzi*-elicited *ifnb* expression. These data provide strong evidence that *T. cruzi* triggers type I IFN expression in a TLR-independent manner. These findings contrast with results of a previous study that reported a role for TRIF in *T. cruzi*-triggered expression of *ifnb* in BMDM and dendritic cells (28). The reason for this difference is unclear but gross differences in experimental design do not appear to account for this. Both studies used BMDM isolated from mice on a C57BL/6 background; control treatments demonstrate that both TRIF$^{-/-}$ and MyD88$^{-/-}$ cells are no longer sensitive to poly(I:C) or the TLR2 agonist, Pam$_2$Csk$_4$, respectively, and both studies clearly show MyD88-dependent induction of proinflammatory cytokines in BMDM in response to *T. cruzi*. Because different parasite strains were used in the two studies, Y strain here and Tulahuen strain in the previous study (28), a plausible explanation may relate to differences in the *T. cruzi* strains used in the two studies. An enormous amount of genetic diversity exists between the >100 *T. cruzi* strains (49) and future studies should address the possibility that different strains of the parasite might differentially activate innate immune signaling pathways. If so, these differences may impact the nature of the host response and pathophysiology of

Chagas' disease. Irrespective of potential differences, our data provide a compelling argument that *T. cruzi* Y strain can induce IFN-β expression by engaging a TLR-independent pathway.

Similar to *Listeria*, *T. cruzi* invades cells and transiently resides within a vacuole before escaping into the host cell cytosol (25). Rupture of the *Listeria* vacuole is critically dependent upon expression of a pore-forming molecule, listeriolysin O (50). *T. cruzi* also secretes a pore-forming molecule into the vacuole, optimally active at low pH (TcTOX; Ref. 26) which is thought to be involved in vacuole membrane disruption facilitating parasite delivery to the cytoplasm. As shown in this study, *T. cruzi* resides within a Lamp-1-positive vacuole for ≥ 8 h after entry before the gradual process of egress begins. The signaling events initiated by *T. cruzi* to trigger IFN-β expression clearly precede cytosolic localization of the parasite because parasite-induced phosphorylation of IRF3 and *ifnb* expression are detectable by 2 h after infection. These observations suggest that signals leading to *ifnb* expression in response to *T. cruzi* are likely to be initiated from a plasma membrane receptor or from within the parasite vacuole. The rapid and robust response of cells to heat-killed trypomastigotes, which are not internalized, argues in favor of an extracellular signal. Alternatively, destabilization of the parasite membrane as a result of heat killing may also result in shedding of membrane vesicles that are internalized by the endocytic pathway more rapidly or in higher quantity than the route of ligand delivery that occurs during the invasion process. Although both live and heat-killed trypomastigotes trigger a robust IFN-β response in a TLR-independent manner, we cannot rule out the possibility that the initiating signals differ in these two conditions. If endocytic trafficking is involved in the mechanism of IFN-β induction by *T. cruzi*, the signaling events leading to *ifnb* expression are not dependent on maintenance of a pH gradient. Attempts to block the induction of *ifnb* by blocking the vacuolar H^+-ATPase with bafilomycin A failed to change the early kinetics of *ifnb* expression in *T. cruzi*-infected cells, while causing a marked delay in vacuole egress for the first 8 h. Similarly, bafilomycin A pretreatment of cells failed to block the IFN response to heat-killed trypomastigotes (data not shown). Thus, the events leading to *ifnb* expression following *T. cruzi* invasion appear to be pH independent and not triggered by vacuole egress as reported previously for *Listeria* (2). Furthermore, as evidenced from the differential ability to trigger *ifnb* expression in HEK293 cells, the pathways engaged by *T. cruzi* and *Listeria* to trigger IFN-β expression are clearly distinct.

Cytosolic surveillance mechanisms play an important role in TLR-independent pathogen recognition and induction of IFN-β (2, 51). Infection of cells with certain viruses can trigger activation of RIG-I-like receptors that recognize dsRNA in the cytoplasm (5). In addition, DNA-sensing pathways may play a role in generating a response to certain intracellular bacteria or viruses (13); however, definitive cytosolic DNA receptors have yet to be identified. A cytosolic DNA-sensing pathway involving Z DNA-binding protein 1 (ZBP-1), also known as DAI was initially reported to act as a cytosolic DNA receptor upstream of *ifnb* expression (15, 16); however, it was recently demonstrated that DAI is not responsible for cytosolic DNA-dependent *ifnb* signaling after examining the IFN response in DAI null cells (14).

Considering our finding that *T. cruzi* triggers IFN-β in a TLR-independent manner, we postulated that one of the known cytosolic recognition pathways is activated by internalized parasites. The cytosolic RNA signaling pathways acting through RIG-I and MDA5 were ruled out following infection of MAVS-deficient macrophages which mounted a normal IFN response to *T. cruzi*. Although DNA isolated from different organisms can trigger expression of type I IFNs when delivered to the cytoplasm of mam-

malian cells (11, 13), there is no clear evidence to support the idea that DNA is the principal ligand recognized by the host during the infection process. A recent report demonstrating that *T. cruzi* genomic DNA is able to induce a potent proinflammatory response when delivered to the endosomes of TLR9-expressing cells (52) suggests that parasite DNA in the vacuole could readily access the host cell cytoplasm and trigger IFN-β expression through a DNA-sensing mechanism. The ability to couple the proinflammatory cytokine response with IFN-β expression via TLR-dependent and -independent mechanisms in infected host cells is an attractive idea that warrants further investigation. Although we were unable to directly assess the role of dsDNA-sensing mechanisms in the parasite-mediated IFN response, HEK293 IFN-β-*luc* reporter cells, which lack TLRs but remain responsive to many cytosolic triggers of *ifnb* transcription, including dsDNA and dsRNA, were completely unresponsive to parasite infection. These findings strongly suggest that *T. cruzi* does not engage known cytosolic surveillance pathways. In the future, HEK293 cells could be used as a potential tool for genetic complementation approaches toward identification of the components of the signaling pathway that are engaged by *T. cruzi* to trigger type I IFN expression.

Findings reported here indicate the existence of a novel TLR-independent pathway that is distinct from the cytosolic dsRNA or DNA recognition pathways upstream of IFN-β expression that have been previously described in the literature. In addition to the novel pathway through which *T. cruzi* induces IFN-β described in this study, a recent report by Ishii et al. (14) also describes a novel TBK1-dependent pathway for type I IFN induction. Although these novel TBK1-dependent pathways have not been characterized at the molecular level, together these findings highlight the concept that the full range of pathogen and cytosolic recognition receptors has yet to be identified. A more complete understanding of the novel pathway through which *T. cruzi* triggers IFN-β in immune and nonimmune cells will provide valuable insight into the complexity of the signaling networks that trigger IFN-β expression and regulation of the innate immune response to intracellular pathogens.

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Disclosures

The authors have no financial conflict of interest.

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