INTRAPHAGOSOMAL PEROXYNITRITE AS A MACROPHAGE-DERIVED CYTOTOXIN AGAINST INTERNALIZED *TRYPANOSOMA CRUZI*

Consequences for oxidative killing and role of microbial peroxiredoxins in infectivity

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Macrophage-derived radicals generated by the NADPH oxidase complex and inducible nitric oxide synthase (iNOS) participate in cytotoxic mechanisms against microorganisms. Nitric oxide (•NO) plays a central role in the control of acute infection by *Trypanosoma cruzi*, the causative agent of Chagas disease, and we have proposed that much of its action relies on macrophage-derived peroxynitrite (ONOO− + ONOOH) formation, a strong oxidant arising from the reaction of •NO with superoxide radical (O2•−). Herein, we show that internalization of *T. cruzi* trypomastigotes by macrophages triggers the assembly of the NADPH oxidase complex to yield O2•− during a 60-90 min period. This does not interfere with IFN-γ-dependent iNOS induction and a sustained •NO production (ca. 24 h). The major mechanism for infection control via reactive species formation occurred when •NO and O2•− were simultaneously produced generating intraphagosomal peroxynitrite levels compatible with microbial killing. Moreover, biochemical and ultrastructural analysis confirmed cellular oxidative damage and morphological disruption in internalized parasites. Overexpression of tryparedoxin peroxidase (*TcCPX*) in *T. cruzi* neutralized macrophage-derived peroxynitrite-dependent cytotoxicity to parasites and favored the infection in an animal model. Collectively, the data provide for the first time direct support on the action of peroxynitrite as an intraphagosomal cytotoxin against pathogens, and that microbial peroxiredoxins facilitate infectivity via decomposition of macrophage-derived peroxynitrite.
species inside the phagosome (11,12,14-17). Due to its negative charge, the $O_2^\cdot$ radical ($pK_a = 4.8$) remains within this organelle while $^\cdot$NO, being a hydrophobic radical, diffuses from the cytosol or phagosomal membrane-associated iNOS (18) into the phagocytic vacuole. The reaction between $^\cdot$NO and $O_2^\cdot$ yields peroxynitrite (19,20), a strong one- and two-electron oxidant, and precursor of secondary species including hydroxyl ($^\cdot$OH), nitrogen dioxide ($^\cdot$NO$_2$) and carbonate ($CO_3^\cdot$) radicals (21). Peroxynitrite and peroxynitrite-derived radicals are capable of reacting with different biological targets including protein amino acid residues (e.g. thiol oxidation (22), tyrosine nitration (23)), transition metal-containing centers (24), lipids (25), and nucleic acids (26), reactions that can ultimately promote cell death by either apoptotic or necrotic pathways (27). The role of various oxidative mechanisms in the micropbicidal activity of macrophages depends on a number of factors including, in the case of the oxidative mechanisms, a dynamic balance between the activation state of macrophages and the sensitivity of targets cells to oxidants (12,28). However, the actual formation of peroxynitrite in the phagosome at levels that could be cytotoxic to invading microorganisms has remained elusive. While in simple biochemical systems the reaction of $^\cdot$NO with $O_2^\cdot$ is very efficient, approaching the diffusion-controlled limit (21), in biological systems, competition reactions (e.g. SOD-catalyzed $O_2^\cdot$ dismutation) (4), rapid diffusion of $^\cdot$NO across biomembranes (29) and temporal mismatch between $^\cdot$NO with $O_2^\cdot$ (30-32), among other factors, can preclude peroxynitrite formation. In addition, once formed, much of peroxynitrite may be decomposed by reacting with non-critical targets and antioxidant enzymes (i.e. peroxiredoxins) (reviewed in (21)), and therefore its potential cytotoxic effects are attenuated or even fully neutralized.

*Trypanosoma cruzi* is an obligate intracellular pathogen responsible for Chagas disease. It has been estimated that 8 million people are currently infected by the parasite, with 20% of Latin America’s population (109 million) at risk resulting in 41,000 new cases each year (33). Moreover, the disease is spreading worldwide as a result of migration (mammalian hosts and insect vectors), HIV-co-infection, blood transfusion and organ transplantation (www.cdc.gov/chagas). During its life cycle, *T. cruzi* undergoes extensive morphological and biochemical changes; non-infective epimastigotes proliferate in the gut of the insect vector where they differentiate into the infective metacyclic trypomastigotes. When initially infecting a host, the metacyclic form invades mainly host-macrophages, where they can be destroyed by host immune mechanisms, including phagocytosis, or transform into replicative intracellular amastigotes. After several cycles of binary division, host cell disruption occurs and infective forms access the bloodstream and are able to invade cells from any tissue, such as myocardium, smooth muscle and central nervous system.

Biologically relevant levels of peroxynitrite are cytotoxic to non-infective epimastigote parasites (14). *In vitro* experiments have revealed that peroxynitrite addition to *T. cruzi* epimastigotes results in severe alterations of energy charge, calcium homeostasis and depletion of trypanothione, the low-molecular-weight thiol found in trypanosomatids (34). We have shown that macrophage-derived peroxynitrite has the capacity to diffuse and exert cytotoxicity against epimastigotes in co-culture experiments (13). Additionally, parasites overexpressing peroxiredoxins, antioxidant enzymes known to readily detoxify peroxynitrite (35,36) and recently revealed as virulent factors in the human infection (37), are resistant to this reactive species (28). As early as 1974, the central role of macrophage phagocytosis in the control of *T. cruzi* infection was determined (38). Later, the importance of macrophages in the *in vivo* infection was evidenced by experiments where resistance to *T. cruzi* was associated with the production of IFN-$\gamma$ and IL-12 in animal models (39,40). Moreover, early treatment with IFN-$\gamma$ was able to reverse the course of infection (41-43), presumably by controlling the early replication of parasites in host macrophages, with participation of $^\cdot$NO (44,45).

Macrophage-derived $O_2^\cdot$ production triggered by *T. cruzi* is a matter of debate with discussion centered on the ability of trypomastigotes to activate NADPH oxidase (41,46,47). In regards to the modulation of $^\cdot$NO production, contradictory results have been reported: while it has been proposed a negative regulation of iNOS induction by epimastigotes (48,49), experiments performed with trypomastigotes have shown potentiation of cytokine-mediated iNOS induction (50). Thus, the occurrence of $O_2^\cdot$, $^\cdot$NO and their reaction...
product peroxynitrite, and how these reactive species independently or synergically participate in the ability of host macrophages to control *T. cruzi* infection, requires an unambiguous analysis. In this work, we first studied whether *T. cruzi* infection of activated macrophages resulted in the simultaneous production of *NO* and O$_2^-$ and lead to the intraphagosomal formation of peroxynitrite. We then evaluated the capacity of peroxynitrite versus that of *NO* and O$_2^-$ (and H$_2$O$_2$) alone to efficiently serve as cytotoxins to control the load of internalized parasites. Finally, we tested the role of the cytosolic parasite peroxiredoxin in neutralizing the effects of host cell-derived oxidants in vitro and in vivo with the possible consequent promotion of infectivity. The data presented herein assist in understanding the mechanisms of oxidant-dependent killing to internalized pathogens.

**Experimental Procedures**

**Reagents.** Murine recombinant IFN-$\gamma$, human MPO and the anti-human MPO antibody were purchased from Calbiochem. DMEM, lipopolysaccharide (LPS), luminol, N$^\delta$-monomethyl-L-Arginine (N$^\delta$-MMA), apocynin, [5,6-$^3$H] uridine (30 Ci mmol$^{-1}$), geneticin (G418), nitrobluetetrazolium (NBT), diphenyliodonium (DPI) and anti-N-terminal region of actin antibody were from Sigma Chemical Co. Lab-Tek tissue culture chamber/slides were from Nunc. 4’-6-Diamidino-2-phenylindole (DAPI), dihydroorhodamine (DHR), and Alexa-Fluor 488 and 594 conjugated anti-rabbit antibodies were from Invitrogen. 4,5-diaminofluorescein diacetate (DAF2-DA) from Alexis Biochemicals. All other reagents were of research grade quality.

**Parasite culture and differentiation.** *T. cruzi* epimastigotes (CL-Brener) were cultured at 28ºC in brain heart infusion medium (BHI) as previously described (51). Parasites overexpressing cytosolic tryparedoxin peroxidase (*Tc*CPX) were kindly provided by Dr. Shane Wilkinson from Queen Mary University of London, London, UK (52) and were cultured in BHI containing 250 $\mu$g ml$^{-1}$ of G418 (Sigma)(28). Parasites were differentiated into the infective metacyclic stage under chemical defined conditions as previously described (53). Briefly, epimastigotes were collected by centrifugation at 800 g for 10 min at 25ºC, washed 3 times in 10 ml of triatominic artificial urine media (TAU, 190 mM NaCl; 17 mM KCl; 2 mM MgCl$_2$; 2 mM CaCl$_2$; 8 mM phosphate pH 6; 0.035% sodium bicarbonate) and resuspended at a cell density of 3-5x10$^6$ cells ml$^{-1}$. After 2 h incubation at 28ºC, parasites were diluted in TAU-3AAG medium (TAU pH 6 supplemented with three amino acids: 10 mM L-proline; 50 mM sodium L-glutamate; 2 mM sodium L-aspartate and 10 mM glucose) at 3-5 x10$^6$ cells ml$^{-1}$. Cells were incubated for 96 h at 28ºC in 75 cm$^3$ culture flasks in a horizontal position to allow adhesion of epimastigotes to the flask surface, an important step in enabling parasites to transform into the infective metacyclic trypomastigote stage (54).

**Macrophages culture.** The murine macrophage cell line J774A.1 (American Type Culture Collection (ATCC- TIB-67)) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) supplemented with L-glutamine (2 mM), penicillin (100 U ml$^{-1}$), streptomycin (100 mg l$^{-1}$) and 10% heat-inactivated fetal bovine serum at 37ºC in a 5% CO$_2$ atmosphere.

**Macrophage O$_2^-$ production**

**Luminal Chemiluminescence.** Macrophages were cultured in 24 well plates and O$_2^-$ production assayed by chemiluminescence studies as previously (13). Briefly, macrophages were incubated in the different experimental conditions in Dulbecco’s PBS (dPBS) pH 7.3 containing 5 mM glucose, 1 mM L-arginine and 100 $\mu$M luminol; the time course of luminol chemiluminescence was followed for 100 min in a luminescence plate reader at 37ºC (Lumistar, BMG Labtechnologies).

**Intracellular detection of reduced NBT.** Macrophages were seeded in Lab-Tek tissue culture chamber/slides (Nunc) and infected with *T. cruzi* metacyclic trypomastigotes (5:1, parasite: macrophage ratio) in dPBS containing 1 mg/ml of NBT (55). Slides were incubated at 37ºC for 30 min, rinsed in warmed dPBS, fixed 10 min in 4 % v/v formaldehyde in PBS at room temperature and stained with DAPI (5 $\mu$g ml$^{-1}$). Cells that contained formazan-stained inclusions within phagocytic vacuoles were observed by DIC microscopy and internalized trypomastigotes evidenced by colocalization of the formazan deposits with DAPI-fluorescence.

**Oxygen consumption by macrophages.** Macrophage-mitochondrial respiration and
respiratory burst were evaluated using a high resolution electrode, Oxygraph 2K (Oroboros Instruments, Austria). Cells were cultured in 6 well plates and challenged with opsonised zymosan (56) or T. cruzi metacyclic trypomastigotes in DMEM. Interaction was synchronized by plate centrifugation (800 g, 5 min) and following 15 min incubation at 37°C non-internalized zymosan or parasites were removed. Macrophages were resuspended at 1 x 10^6 cells/ml in DMEM, and O2 consumption was recorded at 37°C. The rate of oxygen consumption was calculated using the equipment software (DataLab) and was expressed as nmol O2 min^-1/10^6 cells. Oxygen consumption inhibited by 1 mM potassium cyanide (KCN) corresponds to mitochondrial respiration. The cyanide-insensitive fraction was inhibited by 100 µM DPI and corresponds to NADPH oxidase activity (57).

**Macrophase •NO production.** The induction of the macrophage iNOS was triggered by incubating the cells with 200-300 U ml^-1 IFN-γ (Calbiochem) plus 3 µg ml^-1 LPS (Sigma) for 5 h (3). •NO production by non- or IFN-γ/LPS-activated macrophages was evaluated in tissue culture. Cells were seeded at 1 x 10^6 cells/well in the presence or absence of T. cruzi epimastigotes or chemically differentiated metacyclic trypomastigotes (see above). Following incubation (24 h), supernatants were collected and the concentration of nitrite (NO_2^-) determined spectrophotometrically at 540 nm using the Griess method with NaN_2O_3 as standard (3,58). Macrophage •NO production was also evaluated using the cell permeable fluorescence probe 4,5-diaminofluorescein diacetate (DAF-2DA) (59). After 5 h incubation with the iNOS inducers, the media was replaced with dPBS with 5 mM glucose and 1 mM L-arginine containing 5 µM DAF-2DA. Probe oxidation was followed in a fluorescence plate reader at 37°C (Fluostar, BMG Labtechnologies) with filters at \( \lambda_{exc} = 485 \) nm and \( \lambda_{em} = 520 \) nm. Under our experimental conditions, the peroxynitrite-dependent reactions (60) did not interfere with the fluorophore.

**Measurements of iNOS messenger ARN levels.** Macrophages were seeded in 6 well plates (5 x 10^6 cells/well) and infected with a 10:1 parasite: macrophage ratio. After 3 h of infection total RNA was extracted using the PureLink™ Micro-to-Midi™ Total RNA purification system (Invitrogen) and cDNA copy obtained using SuperScript™ III First-Strand System for RT-PCR (Invitrogen). iNOS mRNA in each condition was amplified using the following primers 5'CTAAGAGTCACCAAAATGGCTCCC-3' and 5'-ACCAGAGGCACACATCAA AGC-3' that yields an 875 bp band of murine iNOS. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to verify equal cDNA loading in each condition with the following primers 5'-CTGAGAACGGGAAGCTTGTC-3' y 5'-CCTGCTTCACCACCTTCTTG-3' that yields a 600 bp band. The polymerase chain reaction was carried out using 5µg of cDNA with an annealing temperature of 58°C. PCR products were resolved by electrophoresis in 2 % agarose gel, fragments visualized with ethidium bromide and gels registered with a digital Polaroid camera.

**Immunochemical evaluation of myeloperoxidase.** Mice neutrophils, used as positive control for MPO detection, were purified from peripheral blood as described (61). For protein extraction, neutrophils and J774A-1 macrophages were lysed in RIPA buffer (150 mM NaCl, 10 mM Na_2HPO_4 pH 7.2, 0.1 % SDS, 1 % Triton-X100 and 1% sodium deoxycholate) supplemented with a cocktail of protease inhibitors (Sigma). For immunoblotting, samples were separated by SDS-PAGE (10 %) and transferred onto PVDF membrane (Millipore). The membrane incubated with a rabbit polyclonal anti-human MPO antibody (Calbiochem), and horseradish peroxidase-conjugated as secondary antibody. The blots were developed with ECL reagent (Amersham Biosciences). The nitrocellulose membrane was stripped and rebotted with anti-actin antibody (SIGMA).

**Intracellular dihydrorhodamine oxidation.** Metacyclic trypomastigotes were incubated for 20 minutes in dPBS pH 7.4 containing 50 µM dihydrorhodamine (DHR) for intracellular probe incorporation and washed twice in dPBS before the assay. Macrophages were infected with DHR-loaded metacyclic trypomastigotes for 30 min at 37°C and washed in dPBS to remove non-internalized parasites. Intracellular rhodamine 123 (RH 123, oxidation product of DHR) formation under the different macrophage stimulation conditions was measured in a
fluorescence plate reader at 37°C (Fluostar, BMG Labtechnologies) with filters at $\lambda_{\text{exc}}= 485$ nm and $\lambda_{\text{em}}= 520$ nm (3). In order to evaluate rhodamine localization, cells were examined by fluorescence microscopy and digital photographs of infected macrophages recorded.

**Immunocytochemistry studies.** Macrophages cultured in Lab-Tek glass chamber slides, were exposed to different conditions and then fixed in 4% formaldehyde at room temperature for 5 min. They were then rinsed in PBS and permeabilized with 0.1% Triton in PBS previous to primary antibody exposition. Immunodetection of iNOS expression was performed using a rabbit anti-iNOS (1:100) (Sigma). After copious washes, slides were then incubated with a secondary goat anti-rabbit conjugated to Alexa Fluor 594 (Invitrogen) for 1 h diluted 1:500 in 0.005% v/v Triton X-100 in PBS. Immunodetection of DMPO (5,5-dimethylpyrroline-N-oxide)-nitrone protein adducts on internalized parasites was performed using a rabbit anti- (DMPO-nitrone) serum which binds to the one-electron oxidation product of the initial DMPO nitroxyl protein spin adduct (62). Parasites were incubated with DMPO (100 mM) in dPBS for 30 min at 28°C. After incubation, parasites were collected by centrifugation at 800 g and washed three times in dPBS pH 7.4, to eliminate non incorporated [5,6-3H]-Uridine. Macrophages were infected for 30 min at 37°C with [5,6-3H]-Uridine-loaded metacyclic trypomastigotes (parasite: macrophage ratio of 5:1). Infection was synchronized by plate centrifugation (800 g, 5 min) and following 30 min incubation at 37°C, cell supernatant containing non-internalized parasites (S1) was removed and conserved for quantification of total counts present in each condition. Macrophages were then washed three times with dPBS pH 7.4 and after 2 h incubation in DMEM at 37°C, supernatants were collected (S2, killed parasites). Pelleted, infected macrophages, were lysed in 200 µl of TET buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8 with 0.5% Triton X-100). Cell pellets (P) and supernatants (S1 and S2) were each mixed with 3 ml of scintillation fluid and radioactivity measured in a liquid scintillation counter Trilux 1450 Microbeta (Wallac Instruments). Results are expressed as percentage of [5,6-3H]-Uridine released during the 2h infection period (S2) with respect to total [5,6-3H]Uridine incorporation (P + S1 + S2) for each condition and represent the mean of three independent experiments.

**Transmission Electron Microscopy.** Infected macrophages were also prepared for examination by transmission electron microscopy. After the indicated incubation periods, the cells were fixed with a solution containing 4% (w/v) paraformaldehyde/0.2 % (w/v) glutaraldehyde in PBS pH 7.4, rinsed in the same buffer and post-fixed in 1% osmium tetroxide, dehydrated through graded ethanol solutions and embedded in Araldite resin. Sections were made on a RMC MT-X ultramicrotome with a DIATOME diamond knife. Ultrathin sections (40-60 nm) were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-1010 electron microscope operating at 80-kV. The images were obtained with a Hamamatsu C-4742-95 digital camera and processed with Photoimpact program.

**T. cruzi cytotoxicity assay.** T. cruzi metacyclic trypomastigotes were incubated 24 h in 10 µCi ml$^{-1}$ [5,6-3H]-Uridine at 28°C. After incubation, labeled parasites were collected by centrifugation at 800 g and washed three times in dPBS pH 7.4, to eliminate non incorporated [5,6-3H]-Uridine. Macrophages were infected for 30 min at 37°C with [5,6-3H]-Uridine-loaded metacyclic trypomastigotes (parasite: macrophage ratio of 5:1). Infection was synchronized by plate centrifugation (800 g, 5 min) and following 30 min incubation at 37°C, cell supernatant containing non-internalized parasites (S1) was removed and conserved for quantification of total counts present in each condition. Macrophages were then washed three times with dPBS pH 7.4 and after 2 h incubation in DMEM at 37°C, supernatants were collected (S2, killed parasites). Pelleted, infected macrophages, were lysed in 200 µl of TET buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8 with 0.5% Triton X-100). Cell pellets (P) and supernatants (S1 and S2) were each mixed with 3 ml of scintillation fluid and radioactivity measured in a liquid scintillation counter Trilux 1450 Microbeta (Wallac Instruments). Results are expressed as percentage of [5,6-3H]-Uridine released during the 2h infection period (S2) with respect to total [5,6-3H]Uridine incorporation (P + S1 + S2) for each condition and represent the mean of three independent experiments.

**Determination of parasite load in macrophages.** Macrophages were seeded in Lab-Tek tissue culture chamber/slides and incubated for 5 h with or without iNOS inducers (IFN-γ plus LPS) before infection with metacyclic trypomastigotes...
(parasite:macrophage ratio of 5:1) for 1 h at 37°C. Non-engulfed parasites were removed by washing twice in dPBS and macrophages further incubated for 24 h in DMEM at 37°C. Infected macrophages were fixed in 4% v/v formaldehyde solution in PBS for 10 min at room temperature, washed with PBS containing 100 mM glycine and permeabilized for 5 min with 0.1% v/v Triton X-100 in PBS. The number of parasites per macrophage was determined by DAPI staining (5µg ml⁻¹). Preparations were analyzed using a microscope (Nikon Eclipse TE-200 Inverted Microscope) at 1000 X magnification, and digital photographs of infected macrophages recorded. At least 2500 cells from five independent experiments were counted. Results are expressed as number of amastigotes per 100 macrophages under each condition and represent the media of five independent experiments.

Parasitemia and histopathological analysis. Balb/c mice (n = 6) were infected with 1 x 10⁶ metacyclic trypomastigotes by intraperitoneal injection with wild type parasites or T. cruzi overexpressing TcCPX. Blood (10 µl) of anesthetized mice was used to determine the parasite load and the number of trypomastigotes per 100 fields of view every ten days. After 60 days, tissue samples (heart and quadriceps muscle) of infected mice were recovered, fixed in 10% (v/v) fresh formaldehyde. Paraffin embedded tissue sections (four nonconsecutive slides) from the heart and quadriceps muscle of each mouse were stained with hematoxylin-eosin and examined in a light microscope (64).

Microscopy analysis. Imaging was performed using a Nikon Eclipse TE-200 Inverted Microscope coupled to a camera (SPOT RT, Diagnostic Instrument Inc). Images were analyzed using ImageJ, a public domain Java image processing program.

Data Analysis. Experiments were performed at least three times on independent days. All data are given in mean ± standard deviation unless otherwise noted and a P < 0.05 was considered significant. Means were compared by the Student’s t test. For comparison of more than two groups, an analysis of variance and the least significant difference (LSD) post hoc test was performed.

RESULTS

T. cruzi trypomastigotes trigger O₂⁻ production by macrophage NADPH oxidase

We examined the capacity of T. cruzi trypomastigotes to trigger O₂⁻ production by activation of NADPH oxidase during phagocytosis. The respiratory burst at the phagosome level was evaluated using a qualitative assay in which NBT is converted into a dark formazan precipitate following its reduction by O₂⁻: this can be visualized by microscopy (Fig. 1A, NBT). Unstimulated macrophages were infected with trypomastigotes (parasite: macrophage ratio = 5:1) for 30 min and examined using differential interference contrast (DIC) microscopy. Figure 1 shows the precipitated dark formazan inside the phagosome (indicated by arrows), which co-localized with DAPI-stained trypomastigote nuclei inside the vacuole (Fig. 1A, DAPI). This indicates that O₂⁻ formation is occurring on the luminal side of the phagosome. The formation of formazan upon T. cruzi invasion was completely abolished by pretreatment of macrophages with the NADPH oxidase inhibitor apocynin (Fig. 1A), a compound that prevents p47 PHOX subunit translocation and therefore assembly of the enzyme complex. Moreover, NBT reduction was also prevented by the flavoprotein inhibitor DPI (50 µM) and by SOD (2 µM) (not shown), fully confirming that the parasite generated respiratory burst is dependent on NADPH oxidase-derived O₂⁻.

To further characterize O₂⁻ production by macrophages, we conducted chemiluminescence experiments using luminol, which can serve as a probe for O₂⁻ and/or peroxynitrite (20,65), and allows evaluation of the steady state levels reached by these oxidants. Time-course experiments in unstimulated macrophages showed a O₂⁻ production for ~ 60-90 min, which peaked after 10 min of the infection (Fig. 1B, inset). Superoxide production triggered by the infective trypomastigote was five-fold higher than that observed with the non-infective epimastigote and was dependent on the parasite number used to infect macrophages (Fig. 1B). Luminol chemiluminescence was almost fully abolished by apocynin (Fig. 1B) and SOD (not shown; see also (13)). Peroxynitrite did not contribute to chemiluminescence in unstimulated macrophages as light emission did not decrease by pre-treatment of the cells with the iNOS inhibitor N⁶-monomethyl-L- arginine (L-
NMMA) (not shown), consistent with their lacking of significant iNOS activity (see below, Fig. 2). Interestingly, higher chemiluminescence signal was observed upon *T. cruzi* invasion of macrophages that had been pre-stimulated for *"NO formation (Fig. 1, B inset); in this case, light emission was inhibited by L-NMMA (Fig. 1B), reaching levels comparable to those observed in unstimulated macrophages and fully supporting the formation of peroxynitrite, an efficient inducer of luminol chemiluminescence (66).

To quantitate *O2*•− production by trypomastigote-challenged macrophages, we determined the cyanide-insensitive and DPI-inhibitable oxygen consumption, which corresponds to the respiratory burst. Macrophages challenged with *T. cruzi* caused an increase in oxygen consumption, the extent of which was dependent on the parasite number (0.72 ± 0.08 and 1.19 ± 0.37 nmol/min/10⁶ macrophages for *T. cruzi/macrophage ratios of 2:1 and 5:1, respectively). These data on NADPH activity obtained in macrophages during *T. cruzi* invasion was within the order of magnitude of that observed during phagocytosis of opsonized-yzmosan particles (not shown and (67)), the standard method for respiratory burst evaluation.

**Differential modulation of macrophage *"NO production by epimastigotes and trypomastigotes**

Experiments were conducted to explore the effect of trypomastigotes on *"NO output and iNOS-mRNA level in macrophages. Exposure the mammalian cells to infective *T. cruzi* before activation by IFN-γ and LPS did not affect the accumulation of nitrite, measured as an index of *"NO formation, at 24 h (Fig. 2A). In contrast, non-infectious epimastigotes caused an inhibition on *"NO production by iNOS that was dependent on the parasite number interacting with macrophages (Fig. 2A). Rates of *"NO production were evaluated by the L-NMMA-inhibitable DAF-2DA oxidation, an intracellular fluorescence probe for *"NO (59). Again, while the presence of trypomastigotes did not affect macrophage iNOS activity, epimastigotes strongly inhibited *"NO formation (Fig. 2B).

Further, we also explored the effect of trypomastigotes on macrophage iNOS mRNA levels. As shown on Figure 2C, trypomastigotes did not affect mRNA levels after the induction of iNOS by IFN-γ and LPS (lane 3). Moreover, in the presence of IFN-γ alone, trypomastigotes caused some further increase of mRNA levels for iNOS (lane 5); the relevance of this observation was confirmed by an increase in DAF-2DA oxidation when using macrophages activated by IFN-γ in the presence of trypomastigotes versus IFN-γ - activated macrophages alone (data not shown). Finally, while iNOS induction by IFN-γ plus LPS was inhibited by epimastigotes, full protein expression was obtained in the presence of trypomastigotes (Fig. 2D), consistent with the data obtained for *"NO production and iNOS mRNA levels.

**Phagocytosed *T. cruzi* trypomastigotes are targets of intraphagosomal peroxynitrite**

The concomitant formation of *O2*•− and *"NO during the infection by trypomastigotes is expected to yield peroxynitrite since these two free radicals react with each other at diffusion control rates (k ~10¹⁰ M⁻¹ s⁻¹). Once formed, peroxynitrite could diffuse to and react with molecular targets in the parasite directly or through formation of secondary radicals such as "OH, NO₂•, and CO₃•⁻ (13). In order to evaluate if peroxynitrite reaches the cytosol of phagocytosed cells, trypomastigotes pre-loaded with the redox-sensitive probe DHR were used to infect macrophages. DHR can be oxidized to the fluorescent rhodamine 123 (RH 123) by a group of strong oxidants (68); thus, appropriate control experiments are required to assist in revealing the nature of the main proximal reactive species responsible of its oxidation. Indeed, peroxynitrite-derived radicals efficiently oxidize DHR, which can be monitored by fluorescence spectroscopy and microscopy (20).

In fact, during phagocytosis maximal DHR oxidation yields were obtained in macrophages previously activated with IFNγ/LPS, where the simultaneous formation of *"NO and O2*•− is present, indicating peroxynitrite formation during the infection process (Fig. 3A). Under the experimental conditions where macrophages were stimulated to produce exclusively *O2*•−/H₂O₂ (i.e. unstimulated macrophages) or *"NO (i.e. IFNγ/LPS-activation plus apocynin), no significant DHR oxidation was detected (Fig. 3A). DHR oxidation in IFNγ/LPS-stimulated cells was also largely inhibited by iNOS inhibitors (not shown) as reported previously (3). When infected IFNγ/LPS-activated macrophages were observed by fluorescence microscopy, fluorescent RH 123 was found at locations...
compatible with phagocytosed trypomastigotes (Fig. 3B). Finally, to further confirm that peroxynitrite was formed in the phagosome, we examined protein oxidative modifications such as formation of protein-derived radicals and nitration of tyrosine residues, that could be attributed to radicals derived from peroxynitrite. Oxidative modifications of proteins were evidenced by immunocytochemical analysis by a) immunospin trapping using an anti-DMPO-nitrotrone antibody (69) and b) with anti-3-nitrotyrosine antibodies (63). Infected IFN-γ/LPS-activated macrophages, had positive immunostaining for both DMPO-nitrotrone (Fig. 4A) and 3-nitrotyrosine (Fig. 4B) adducts. The punctuate pattern observed in the infected cells demonstrated that these protein modifications occurred in the internalized trypomastigotes as they are localized in close proximity to the T. cruzi nuclei and kinetoplast (Fig. 4), in full support of oxidative damage to parasite proteins inside the phagosome. Importantly, considering that O₂^•− and NO can evolve in cellular systems to H₂O₂ (via dismutation) and nitrite (NO₂^−; via oxidation), respectively, and that myeloperoxidase (MPO) can participate in oxidative damage to microorganisms including T. cruzi (70,71) we assessed whether MPO-dependent reactions in the presence of H₂O₂ and NO₂^− (23,31,72,73) could play a role in macrophage-dependent oxidation and cytotoxicity towards T. cruzi. First, MPO protein was not detectable in control or LPS plus INF-γ stimulated J774 macrophages (Supplemental Data). Second, incubation of unstimulated macrophages with relevant levels of NO₂^− to test the contribution of MPO or even other existing peroxidase activity in macrophages (74) in the generation of oxidizing and nitrating species (31,72,75), did not result in either probe oxidation (i.e. luminol, DHR) (not shown) or T. cruzi protein oxidation/nitration (Supplemental Data). Thus, MPO does not contribute to the formation of oxidant species in T. cruzi infected J774A-1 macrophages.

**Intraphagosomal peroxynitrite in T. cruzi infection: microbicidal activity and role of parasite cytosolic peroxiredoxin**

We examined the integrity of the internalized trypomastigotes in control and INOS-containing macrophages by transmission electron microscopy at one-hour post-infection (Fig. 5). In unstimulated macrophages, where O₂^•− and H₂O₂ are the main oxidant species released to the phagosome during T. cruzi internalization, well preserved parasites were observed in the host cell, residing inside phagosomes. Parasite structures, such as nucleus, mitochondrion with kinetoplast, reservosomes and flagellar pocket were present and no major morphological alterations were observed (Fig. 5, upper panel). On the other hand, internalization in activated macrophages caused marked changes on the parasite ultrastructure, including compact condensation of nuclear and mitochondrial kinetoplast DNA and an intense cytoplasmic vacuolization (Fig. 5, lower panel). Moreover, visible disruption of the parasite membrane integrity was observed under this condition (Fig. 5, arrows).

In order to confirm that the cytotoxicity and damage observed in parasites was due to peroxynitrite, macrophages were infected with T. cruzi overexpressing TcCPX, an antioxidant enzyme known to detoxify peroxynitrite (k= 2 x 10^5 M⁻¹s⁻¹) (35,76). We have recently shown that epimastigotes overexpressing TcCPX are highly resistant to peroxynitrite generated in vitro or released to the extracellular medium from activated macrophages (28). Firstly, phagocytosed TcCPX overexpressing trypomastigotes loaded with DHR fully inhibited INF-γ/LPS-activated macrophages and peroxynitrite-dependent probe oxidation in contrast to wild type parasites. This is, in agreement with the enhanced capacity of these genetically-modified cells to detoxify peroxynitrite (Fig. 6A). Moreover, cytotoxicity studies were carried out in wild type and TcCPX overexpressing trypomastigotes pre-loaded with [5,6-3H]-uridine which can be used as a sensitive probe of parasite membrane permeability integrity (77). Macrophages activated with INF-γ and LPS were toxic to wild type trypomastigotes as evaluated by the release of radioactivity to the culture media supernatant at two-hours of infection, indicative of membrane permeability loss, while TcCPX overexpressing cells showed resistance (Fig. 6B), indicating that the simultaneous generation of O₂^•− and NO is involved in the cytotoxic effect of macrophages on internalized parasites at this time point of the infection. TcCPX also reacts fast with O₂^•− derived H₂O₂ (k= 3 x 10^7 M⁻¹s⁻¹) (76) in vitro. However, no significant inhibitory effects on probe oxidation or parasite toxicity by TcCPX overexpressing cells were observed when H₂O₂ from unstimulated macrophages was formed.
(Figs. 5 and 6). These data fully support that H₂O₂ is not a major effector in macrophage-dependent oxidative reactions in T. cruzi, and that the observed antioxidant and cytoprotective effects of TcCPX are consistent with a prime role on peroxynitrite detoxification, on line with previous observations (28).

Progression of an infection in macrophages was evaluated after 24 hours by counting the amastigote number per 100 macrophages (Table I). Given that the infection process itself triggers O₂•⁻ production, we first performed experiments on unstimulated macrophages in the absence and presence of apocynin to evaluate O₂•⁻ or H₂O₂-mediated cytotoxicity. This inhibitor did not increase the parasite load in unstimulated macrophages, giving further support to our argument that alone neither O₂•⁻ nor H₂O₂ contribute significantly to macrophage cytoxicity. On the other hand, INF-γ and LPS-activated macrophages significantly decreased the parasite load of wild type parasites by ~60 (based on amastigote count, Fig. 6B)) while TcCPX overexpressing cells showed a remarkable tolerance. Apocynin did not affect •NO production from activated macrophages (Fig. 2A). However, NADPH oxidase inhibition resulted on a reversal of the trypanocidal activity on iNOS-expressing macrophages, consistent with the synergistic action of O₂•⁻ on •NO-mediated cytotoxicity. Indeed, to evaluate the independent contribution these two reactive species make in controlling parasite load, experiments were also performed with iNOS induction after infection; this circumvents the problem associated with the simultaneous formation of both radicals. Under these conditions, a modest effect on the inhibition of parasite load over the control condition was observed (~30% inhibition on amastigote count), in agreement with a cytostatic role for •NO as previously described (78,79). Together, the results indicate that the major cytotoxic effect depends on the concomitant production of •NO and O₂•⁻ leading to peroxynitrite formation.

**Peroxynitrite participates in the control of T. cruzi infection in vivo**

Balb/c mice were infected by intraperitoneal injection with wild type or TcCPX overexpressers metacyclic trypomastigotes. Parasitemia were evaluated every ten days over a 60 day period after which the animals were sacrificed and estimations made to tissue damage. Fig. 7A shows that the overexpression of TcCPX in trypomastigotes augments virulence as evidenced by a three-fold increase on parasitemia by day 50. The analysis of heart and skeletal muscle tissue sections showed more prominent inflammatory infiltrates in both tissues in animals infected with the TcCPX overexpressers compared to the wild type trypomastigote infected group (Fig. 7B).

**DISCUSSION**

During the course of the infection process, macrophages produce reactive oxygen and nitrogen species that are cytotoxic to several microbes including bacteria and parasites (17,80-82). The production of O₂•⁻ depends on the activation of the NADPH oxidase complex, whose assembly is triggered by receptor-mediated phagocytosis. The precursor of reactive nitrogen species, •NO, is produced after the cytokine-dependent induction of iNOS. This has led to hypothesize (12,13,15,83) that after microorganisms are ingested, O₂•⁻ formed inside the phagocytic vacuole will react with •NO diffusing from the cytosol to form microbicidal amounts of peroxynitrite. However, direct evidence for this phenomenon at the phagosomal level during microbial infection to macrophages has not been provided.

The importance of oxidant production as the first line of defense of macrophages is underscored by the fact that some pathogens (84), mostly unicellular parasites, have evolved to evade the activation of these set of enzymes in order to efficiently infect the host. For instance, Leishmania donovani promastigotes are internalized by macrophages without eliciting a respiratory burst thereby avoiding O₂•⁻ production (85) and can also down-regulate macrophage •NO production (86). Additionally, T. cruzi as well as other pathogenic trypanosomatids have an ample and well-distributed set of antioxidant enzymes to cope with reactive oxygen and nitrogen species (28,52,87,88).

Thus, in order to test our hypothesis, it was first essential to evaluate the influence of the infective forms of T. cruzi on the routes of O₂•⁻ and •NO formation and whether these radicals were formed simultaneously during an infection. In this context, the ability of T. cruzi trypomastigotes to trigger NADPH oxidase
activity and promote the formation of significant levels of $O_2^•−$ was confirmed on infection experiments by three independent methods, namely NBT reduction, luminol oxidation and cyanide-insensitive oxygen consumption (Fig. 1 and data in the text). Macrophage-dependent $O_2^•−$ production was responsive to the parasite load and sustained over a 60-90 min period (Fig. 1B and oxygen consumption data). Luminol, which equilibrates among intra- and extracellular compartments, and oxygen consumption data reflect overall cellular $O_2^•−$ production but do not discriminate the cellular sites (e.g. intraphagosomal vs. extracellular) of $O_2^•−$ release. Thus, to assist in the site-specific detection of $O_2^•−$ in the phagosome of infected macrophages, NBT was used as a qualitative probe; the insoluble product from the $O_2^•−$-mediated reduction of NBT, formazan, was formed, deposited and co-localized in the same cellular compartment where the internalized trypomastigotes were located (Fig. 1A). In regards to the $NO$ pathway, non-infective epimastigotes inhibited $NO$ production released by activated macrophages as confirmed both by evaluating total NO$_2^−$ accumulation and the time course of $NO$-dependent oxidation of DAF-2DA and is consistent with iNOS mRNA levels and protein expression (Fig. 2), in agreement with previous reports (48,49). Importantly, this inhibitory effect did not operate for trypomastigotes that did not alter $NO$ production from macrophages; interestingly, trypomastigotes caused some stimulation of the INF-$γ$-mediated iNOS induction (Fig. 2). The effect of the infective stage on iNOS expression could be attributed to TOLL-2 receptor (TLR2)- and CD-14-mediated recognition of trypomastigotes, that up-regulate iNOS expression (89-92).

Previous work by our group has provided evidence for the cytotoxic effect of macrophage-derived peroxynitrite to $T. cruzi$ in model studies with the non-infective epimastigote stage (13,14,34,93). In this study, we focused on the infective trypomastigote stage and the formation of peroxynitrite inside the phagosome of activated macrophages during parasite internalization. The capacity of peroxynitrite to cause oxidative damage to target cells is limited by its short half-life in biological milieu and depends on the mean diffusion distance to its molecular targets (< 10 ms, corresponding to less of 10 µm) (13). Electron microscope studies revealed that distance between the parasite and the phagosomal membrane is quite small and determined to be ≤ 10-20 nm (Fig. 5). Therefore, more than 90 % of the peroxynitrite formed in the phagosomal lumen will reach parasite targets even in the presence of CO$_2$ (13). The previously reported lethal peroxynitrite dose for epimastigotes (13) is probably an underestimation when considering trypomastigotes, due to the apparent increase in antioxidant enzymes observed during metacyclogenesis (28,37,94). The amount of intraphagosomal peroxynitrite formation was sufficient to kill trypomastigotes, as evidenced by protein nitrosative modifications (Fig. 4), ultrastructural alterations (Fig. 5) and parasite killing (Table I). Peroxynitrite stands as a significantly more potent cytotoxic agent against trypomastigotes than H$_2$O$_2$ as revealed by the lack of killing in unstimulated macrophages (Table I) and is in good agreement with previous determinations with reagent peroxynitrite (13) and H$_2$O$_2$ (47), respectively.

Protein oxidative damage in phagocytosed trypomastigotes was detected by immune-staining with anti-DMPO-nitrone and anti 3-nitrotyrosine antibodies: this revealed modified-protein in close proximity to $T. cruzi$ nuclei and kinetoplast, which further supported the phagosomal generation of peroxynitrite (Fig. 4). This result is in contrast to that reported during the infection of macrophages by Salmonella typhimurium. In this case, the bacteria did not co-localize with protein 3-nitrotyrosine as interfered with NADPH oxidase activation, thereby preventing $O_2^•−$ and peroxynitrite formation in the vesicle (16,95). Alternative systems to account for $T. cruzi$ protein tyrosine nitration such as myeloperoxidase (MPO)-H$_2$O$_2$-NO$_2^−$ (23,31,72,74,75) were not operative due to the lack of MPO in J774A-1 macrophages (Supplemental Data). Peroxidase activity in J774A-1 macrophages may arise from cyclooxygenase-2 induction, but this may only be relevant at 15-20 h post-activation (32), a time frame beyond the experiments reported herein and in full agreement with the lack of oxidative effects on $T. cruzi$ upon macrophage incubation with NO$_2^−$ (Supplemental Data).

To further evaluate the role of peroxynitrite toxicity and its relation with the control of macrophage infection, we used chemically-transformed trypomastigotes overexpressing $Tc$CPX as a molecular tool:
previous work has shown that this enzyme can efficiently detoxify peroxynitrite in *T. cruzi* (28). While some data on peroxiredoxin activity toward peroxynitrite in biochemical and cellular systems have been provided (28,35,96-98), direct and unambiguous confirmation of its capacity to prevent peroxynitrite-mediated effects during microbial infection to mammalian cells was lacking. We found that RH 123 formation on phagocytosed DHR-loaded wild type trypomastigotes depended on peroxynitrite formation and that the observed fluorescence was confined to the phagosome (Fig. 3). Parasites with elevated levels of *Tc*CPX showed less DHR oxidation, in agreement with the cells increased ability to decompose peroxynitrite (Fig. 6A). Importantly, activated macrophages capable of simultaneously producing O₂⁻ and *NO* during internalization of wild type trypomastigotes limited the progression of the infection, causing a decrease of 60 % in parasite load when compared to macrophages producing only O₂⁻ or *NO*, or even both but with a temporal mismatch (Table I). Ultrastructural analysis of phagocytosed parasites by peroxynitrite producing activated macrophages showed marked damage of cellular organelles and disruption of cell membrane (Fig. 5). *Tc*CPX overexpressing trypomastigotes resisted cytotoxicity as evaluated by [H]-uridine release assay (Fig. 6B), compatible with an efficient neutralization of peroxynitrite and resulted in higher infection yields 24 h post-infection (Table I). In line with this, experimental infections of mice with trypomastigotes having higher levels of *Tc*CPX resulted in higher parasitemias and tissue inflammation compared to the infection with wild type parasites (Fig. 7). These observations recapitulate well with previous findings of our group, showing a positive association between the levels of peroxiredoxins in several parasite strains and their degree of virulence (37) The role of *NO* in the control of *T. cruzi* infection *in vivo* (99), and the fact that parasite peroxiredoxins do not decompose *NO* but rather *NO*-derived peroxynitrite, fully support the notion that peroxynitrite acts as a cytotoxin *in vivo* and the existence of an interplay between peroxynitrite and peroxiredoxins influencing the outcome of infection (100).

In summary, the present work demonstrates that peroxynitrite is generated within the phagosome of activated macrophages from the reaction of O₂⁻, arising from the activation of NADPH oxidase secondary to internalization of the parasite, with *NO* diffusing from the cytosol. Peroxynitrite was formed for about 60-90 minutes and in sufficient amounts to diffuse into the parasite and to cause substantial cytotoxicity, to a much larger extent than that of its precursor radicals and H₂O₂. The effects of peroxynitrite were neutralized by the overexpression of *Tc*CPX in the parasites which facilitated the development of the infection. The dynamic balance between intraphagosomal oxidant production and the activity of key antioxidant enzymes in the parasite at the onset of the infection critically determines parasite infectivity, providing additional insights for the observed differences on natural *T. cruzi* strains of diverse virulence and pathogenicity in the human disease (37).
REFERENCES

FOOTNOTES

1 The term peroxynitrite refers to the sum of peroxynitrite anion (ONOO⁻) and peroxynitrous acid (ONOOH), pKₐ = 6.8 (20, 21)

2 Given its high concentration in biological fluids (1-2 mM), CO₂ is a key reactant for peroxynitrite (k = 4.6 x 10⁴ M⁻¹ s⁻¹, 37° C, pH 7.4) limiting its diffusion distance. The product of this reaction is the transient nitrosoperoxocarboxylate adduct that undergoes fast homolysis to CO₃⁻ and NO₂⁻ in ~35% yields (21).

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ABBREVIATIONS

The abbreviations used are: iNOS, inducible nitric oxide synthase; NO, nitric oxide; O₂⁻, superoxide radical; H₂O₂, hydrogen peroxide; OH, hydroxyl radical; NO₂, nitrogen dioxide radical; CO₃⁻, carbonate radical; DHR, dihydrorhodamine; nitrobluetetrazolium (NBT), diphenyliodonium (DPI); 4,5-diamino-fluorescein diacetate, DAF2-DA; L-NMMA, N⁰-monomethyl-L-arginine TcCPX, T. cruzi cytosolic tryparedoxin peroxidase; MPO, myeloperoxidase
FIGURE LEGENDS

Figure 1. Phagocytosis of T. cruzi trypomastigotes is accompanied by intraphagosomal- and NADPH oxidase-dependent $\text{O}_2^{-}$ formation

(A) Superoxide dependent intraphagosomal NBT reduction. Macrophages were incubated with T. cruzi trypomastigotes (5:1 ratio) in the presence of NBT (1mg/ml, 30 min, 37°C) and apocynin (50 μM) as indicated; stained with DAPI as described in methods, and observed by fluorescence/Nomarski DIC microscopy (magnification, × 1000). Arrows indicate internalized parasites visualized by DAPI-stained nuclei (upper and lower left) within phagosomes as evidenced by formazan deposits (upper right). Apocynin completely abolished $\text{O}_2^{-}$ dependent formazan formation (lower right). Data shown are representative of at least three independent experiments performed on separate days.

(B) Luminol chemiluminescence. Macrophages (1 x 10⁶) incubated in dPBS with 200 μM luminol were exposed to: T. cruzi epimastigotes (2 x 10⁷) and trypomastigotes (1, 2 and 5 x 10⁷) corresponding to a trypomastigotes: macrophage ratio of 10:1; 20:1 and 50:1. The effect on luminol chemiluminescence of apocynin (apo), iNOS induction (IFN-γ/LPS) and inhibition by L-NMMA (IFN-γ/LPS + L-NMMA) was explored at a 20:1 ratio. Chemiluminescence was continuously measured immediately after T. cruzi addition to macrophages monolayer in a luminescence plate reader at 37°C. Data are mean ± S.E. of total counts in 100 min. Inset shows representative records obtained by the addition of trypomastigotes (20:1) to unstimulated (T. cruzi), apocynin treated- (T. cruzi + Apo) or pre-activated macrophages (IFN-γ/LPS + T.cruzi). (Ctl) correspond to macrophages not challenged with T. cruzi.

Figure 2. Nitric oxide production and iNOS levels in T.cruzi infected macrophages

(A) Nitrite production. Macrophages were exposed to IFN-γ/LPS for 24 hours in the absence or in the presence of epimastigotes (5:1 and 20:1; epimastigotes: macrophages) or trypomastigotes (20:1). The effect of apocynin (apo) and L-NMMA (2 mM) was also evaluated. Nitrite accumulation was determined using the Griess reaction. Epis (epimastigotes); CTL (control); trypos (trypomastigotes)

(B) DAF-2DA oxidation. Macrophages were exposed to IFN-γ/LPS in the presence of epimastigotes (20:1), trypomastigotes (20:1) or L-NMMA (2 mM) in DMEM at 37°C. Control experiments were performed in the absence of cytokine stimulation (CTL). After 5 h, the media was replaced with dPBS containing 5 μM DAF-2DA and probe oxidation was followed in a fluorescence plate reader at 37°C.

(C) iNOS mRNA levels of macrophages exposed to IFN-γ/LPS, IFN-γ/LPS + trypomastigotes (10:1), IFN-γ and IFN-γ + trypomastigotes (10:1) were evaluated by RT-PCR. Total RNA from 5 x 10⁶ macrophages of all conditions was extracted after 3 h of infection. Host iNOS and GAPDH cDNAs were amplified using primers defined in experimental procedures. (D) iNOS expression was evaluated by immunocytochemistry. Macrophages plated in slides were stimulated for 5 h with IFN-γ/LPS in the presence or absence of T. cruzi epimastigotes or trypomastigotes and immunocytochemical analysis of
iNOS expression was performed using polyclonal anti-NOS antibody and visualized with Alexa-594-conjugated anti-rabbit antibody (magnification, \times 400). Data shown are representative of at least three independent experiments performed on separate days.

**Figure 3. DHR oxidation inside phagocytosed trypomastigotes**

*T. cruzi* trypomastigotes pre-loaded with DHR were exposed to macrophages and the cells washed 30 min after incubation. (A) RH accumulation after 2 h of infection in unstimulated (*T. cruzi*) or pre-activated macrophages (IFN-\(\gamma\)/LPS + *T. cruzi*) was determined in a fluorescence plate reader. The effect of apocynin was evaluated in both conditions (+ apocynin). Data are mean \(\pm\) S.E. of three independent experiments. (B) Activated macrophages plated in slides were infected with DHR-loaded trypomastigotes (5:1 parasite: macrophage ratio). Merged DIC and fluorescence images were obtained 2 h after infection; the green fluorescence corresponds to oxidized DHR (magnification, \times 400).

**Figure 4. Detection of peroxynitrite-dependent protein oxidation in phagocytosed trypomastigotes**

(A) DMPO-protein adducts. Unstimulated (CTL) or activated (IFN-\(\gamma\)/LPS) macrophages were infected with DMPO-loaded trypomastigotes (20:1). After 2 h, cells were fixed, permeabilized and incubated with anti-(DMPO-nitrone) polyclonal antibody and visualized with Alexa-594-conjugated anti-rabbit antibody. DMPO-protein adducts are shown in red and nuclei in blue (magnification, \times 400). (B) Protein 3-nitrotyrosine. Unstimulated (CTL) or activated (IFN-\(\gamma\)/LPS) macrophages were infected with *T. cruzi* trypomastigotes (20:1). Following phagocytosis (2 h), macrophages were incubated with anti-3-nitrotyrosine antibody and visualized with Alexa-488-conjugated anti-rabbit antibody. 3-nitrotyrosine staining is shown in green, and the nucleus is stained in blue (magnification, \times 400). (C) Magnified field (\times 1000) of anti-(DMPO-nitrone) (red) or anti-3-nitrotyrosine (green) in activated macrophages. Arrows indicate the distinctive parasite kinetoplast or nuclei revealed by the blue DAPI staining in close proximity to the oxidized proteins. Results are representative of at least three independent experiments performed on separate days.

**Figure 5. Electron microscopy studies of *T. cruzi* infection**

Micrographs showing unstimulated (CTL) and activated (IFN-\(\gamma\)/LPS) infected macrophages, at 1 h post-infection; the arrows in the lower panel indicate the disruptions of membrane integrity. Abbreviations: N, macrophage nucleus; Tc, *Trypanosoma cruzi*; n, *T. cruzi* nucleus; k, kinetoplast; fp, flagellar pocket; r, reservosomes. Representative electron micrographs of at least three independent experiments.
Figure 6. *T. cruzi* killing by intraphagosomal peroxynitrite and protective effect of parasite cytosolic peroxiredoxin

(A) Wild type (WT) or *Tc*CPX overexpressing trypomastigotes pre-loaded with DHR were exposed to unstimulated (*T.cruzi*) or activated macrophages (IFN-γ/LPS + *T.cruzi*). RH accumulation after 2 h of infection was determined in a fluorescence plate reader. (B) Macrophages were infected with wild type or *Tc*CPX overexpressing trypomastigotes pre-loaded with [5,6-³H]-uridine. After incubation (2 h) supernatants were collected and radioactivity measured in a liquid scintillation counter as described in methods. Data are mean ± S.E. of three independent experiments.

Figure 7. Parasitemia time course and tissue inflammation of mice infected with wild type trypomastigotes or *Tc*CPX overexpressers

Behavior of wild type (WT) and *Tc*CPX overexpressers trypomastigotes in experimental mice infections. Two month-old Balb/c mice were infected by i.p. inoculation with 1x10⁶ metacyclic trypomastigote forms. Parasitemia and histopathology were analyzed as described in Experimental procedures. (A) Parasitemia (tryptomastigotes/50 fields) levels elicited by the wild type and *Tc*CPX overexpresser strains. Values are given as means ± S.E. (B) Microphotographs of muscle tissue sections from mice inoculated with wild type (WT) and *Tc*CPX overexpressers (*Tc*CPX) (magnification, × 400). Arrows in the right panel (*Tc*CPX) indicate inflammatory infiltrates.
Table I. Intraphagosomal peroxynitrite controls wild type T. cruzi infection

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<th>Macrophages treatment</th>
<th>T. cruzi amastigotes/100 macrophages&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>CL- Brener</td>
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<td>Unstimulated</td>
<td>37 ± 13</td>
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<td>+ apocynin</td>
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<td>IFN-γ/LPS pre-stimulated&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14 ± 3&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>+ apocynin</td>
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<td>IFN-γ/LPS post-infection&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27 ± 8</td>
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<sup>a</sup> IFN-γ/LPS were added to the macrophage culture 5 h before infection (pre-activated) or 30 min after infection (post-infection). Apocynin (50 µM) was used when indicated to avoid NADPH oxidase assembly.

<sup>b</sup>The load of T. cruzi amastigotes in the macrophage culture was evaluated at 24 h after infection with metacyclic trypomastigotes (parasite: macrophage ratio, 5:1). Data are mean ± S.E. of three independent experiments.

<sup>c</sup>non determined

<sup>*</sup><i>P < 0.01</i>
Figure 1A. Alvarez et al.
Figure 1B. Alvarez et al.
Figure 2A. Alvarez et al.
Figure 2B. Alvarez et al.
Figure 2C. Alvarez et al
Figure 2 D. Alvarez et al
Figure 3A. Alvarez et al.
Figure 3B. Alvarez et al.
A. Anti-(DMPO-nitrone)

B. Anti-3-nitrotyrosine

Figure 4. A and B. Alvarez et al.
Anti-(DMPO-nitrone)

IFN-γ/LPS

Anti-3-nitrotyrosine

IFN-γ/LPS

Figure 4C. Alvarez et al.
Figure 5. Alvarez et al.
Figure 6A. Alvarez et al.
$^{3}$H Uridine released (fold increase over control)

**Figure 6B. Alvarez et al**
Figure 7A. Alvarez et al
Intraphagosomal peroxynitrite as a macrophage-derived cytotoxin against internalized Trypanosoma cruzi: Consequences for oxidative killing and role of microbial peroxiredoxins in infectivity

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