Overexpression of *Trypanosoma rangeli* trypanothione reductase increases parasite survival under oxidative stress

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SUMMARY

The infectivity and virulence of pathogenic trypanosomatids are directly associated with the efficacy of their antioxidant system. Among the molecules involved in the trypanosomatid response to reactive oxygen or nitrogen species, trypanothione reductase (TRed) is a key enzyme. In this study, we performed a molecular and functional characterization of the TRed enzyme from *Trypanosoma rangeli* (*Tr*TRed), an avirulent trypanosome of mammals. The *Tr*TRed gene has an open reading frame (ORF) of 1473 bp (~490 aa, 53 kDa) and occurs as a single-copy gene in the haploid genome. The predicted protein contains two oxidoreductase domains, which are equally expressed in the cytosol of epimastigotes and trypomastigotes. Nicotinamide adenine dinucleotide phosphate (NADPH) generation is reduced and endogenous H_2O_2 production is elevated in *T. rangeli* Choachí strain compared with *T. cruzi* Y strain epimastigotes. Oxidative stress induced by H_2O_2 does not induce significant alterations in *Tr*TRed expression. Overexpression of *Tr*TRed did not influence *in vitro* growth or differentiation into trypomastigotes, but mutant parasites showed increased resistance to H_2O_2-induced stress. Our results indicate that *T. rangeli* constitutively expresses TRed during the entire life cycle, with reduced levels during infective and non-replicative trypomastigote stages.

Key words: *Trypanosoma rangeli*, Trypanothione reductase, antioxidant defence.

INTRODUCTION

During infection of the mammalian host, trypanosomatids are exposed to a variety of oxidizing environments. The reduced form of the thiol trypanothione, which is unique to trypanosomatids, is thought to play a central role in the redox defence systems of these parasites to such stresses (Flohé, 2012; Peloso *et al*. 2012; Paiva and Bozza, 2014). This reduced form is generated by the action of trypanothione reductase (TRed), an enzyme belonging to the flavoprotein disulphide oxidoreductase family. The substrate of this enzyme is trypanothione disulphide. TRed is a specific NADPH-dependant homodimeric protein that requires flavin-adenine dinucleotide (FAD) as a coenzyme and a redox-active cysteine disulphide in the active site (Fairlamb and Cerami, 1992). TRed and peroxidases play a role in protecting these parasites against oxidative stress that may arise internally as a result of their aerobic metabolism and externally by the action of the host immune response (Fairlamb *et al*. 1985; Shames *et al*. 1986).

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Among the trypanosomatids that are trypanothione-dependent organisms, *Trypanosoma rangeli* is a non-virulent protozoan parasite of mammals that is genomically related to *Trypanosoma cruzi*, the aetiologic agent of Chagas disease (Guhl and Vallejo, 2003; Stoco *et al*. 2014). Compared with *T. cruzi*, *T. rangeli* possesses striking new genomic features such as (1) considerably less amplification of the gene copy number within multigene virulence factor families, such as mucin-associated proteins (MASPs), trans-sialidases and mucins; (2) the presence of vestigial orthologues of the interference RNA (RNAi) machinery, which are insufficient to constitute a functional pathway; and (3) a reduced repertoire of genes encoding antioxidant defence enzymes (Stoco *et al*. 2014). These parasites also occur in sympatry in a wide overlapping area in South and Central America, infecting triatomine bugs, humans and a variety of sylvatic and domestic mammals (Coura *et al*. 1996; Grisard *et al*. 1999; Guhl and Vallejo, 2003). Moreover, *T. rangeli* and *T. cruzi* can share at least 60% of their soluble antigenic constitution, which can lead to a false-positive diagnosis of chagasic infection in people carrying *T. rangeli*, with consequent socio-economic effects (Azambuja *et al*. 2005; Ferreira *et al*. 2010).

Trypanosoma cruzi and *Trypanosoma brucei* express high levels of TRed (Piacenza *et al*. 2009*b*), supporting its essential role in parasite viability.

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Trypanosoma brucei parasites became more susceptible to H_2O_2-induced oxidative stress and were avirulent only when TRed levels were reduced to <10% (Krieger *et al*. 2000). Interestingly, TRed levels were not increased in *T. cruzi* during metacyclogenesis (Piacenza *et al*. 2009*a*, *b*).

Little is known regarding the behaviour of *T. rangeli* in response to oxidative stress or the relevance of TRed to survival and adaptation in response to the new environments encountered by the parasite during its life cycle. Thus, in the present study, we characterized the *T. rangeli* TRed gene and its expression levels during the parasite life cycle and under oxidative stress conditions. The relevance of this enzyme for the parasite response to reactive oxygen species (ROS) is highlighted.

MATERIALS AND METHODS

Parasites

Trypanosoma rangeli (Choachí strain) and *T. cruzi* (Y strain) epimastigotes were maintained at 27·5 °C in liver infusion tryptose medium (LIT) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units mL^{-1} penicillin and 100 $\mu g\ mL^{-1}$ streptomycin by weekly passages after cyclic mouse-triatomine-mouse passages. Polymerase chain reaction (PCR) showed that all samples tested negative for the presence of *Mycoplasma* sp. For DNA or protein extraction and for *in vitro* oxidative stress experiments, epimastigotes in the exponential growth phase were harvested during the late *log* phase and washed twice with sterile PBS (pH 7·2). *Trypanosoma rangeli* trypomastigotes were obtained *in vitro* using previously described conditions (Koerich *et al*. 2002). *Trypanosoma cruzi* culture-derived trypomastigotes (TCT) were obtained by infection of Vero cells as previously described (Eger-Mangrich *et al*. 2001).

Isolation of DNA and RNA

Total RNA was extracted from *T. rangeli* epimastigotes using the TRIzol method (Invitrogen) and then treated with DNase I, according to the manufacturer's instructions. *Trypanosoma rangeli* and *T. cruzi* genomic DNA (gDNA) was isolated using a standard phenol–chloroform method (Sambrook *et al*. 2001). The purity and quality of the obtained DNA and RNA were assessed spectrophotometrically and via agarose gel electrophoresis.

Protein extraction

Epimastigote or trypomastigote forms were washed once with PBS (pH 7·2), lysed by repeated pipetting in ice-cold lysis buffer (0·25 M sucrose, 0·25% Triton X-100, 10 mM EDTA) containing a protease inhibitor cocktail (2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0·3 μM aprotinin, 116 μM bestatin, 14 μM E-64, 1 μM leupeptin and 1 mM EDTA) (Sigma-Aldrich). Cellular debris were removed by centrifugation at 12 000 ***g*** for 20 min at 4 °C (Romero *et al*. 2014). The protein concentration was determined by the Bradford method (Bio-Rad) using BSA as a standard (Bradford, 1976). Parasite extracts were stored at −20 °C until use.

cDNA Synthesis and qPCR

For first-strand cDNA synthesis, 1 μg of total RNA was used in a 20-μL final reaction volume containing 200 U Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT), 1X First Strand Buffer, 200 μM dNTP, 10 pmoles oligodT-Anchor, 10 mM dithiothreitol (DTT), 40 U RNaseOUT™ (Invitrogen). The mixture was incubated at 37 °C for 50 min, heat-inactivated at 70 °C for 15 min and then diluted 5-fold in nuclease-free water. The diluted cDNA was used for qPCR amplification on ABI Prism® 7900HT *Sequence Detection System* (Applied Biosystems) equipment using specific primers (qTRed*Tr*-F: 5′-CGA AAG ACT GAT CAC ACC CG-3′ and qTRed*Tr*-R: 5′- CGA GTG CCG TCT TCT CTA TTC CTC-3′). GAPDH (GenBank AUPL01004827) (GAPDH-F: 5′-GCG ACA CCA GCA TCA AAG AG-3′/ GAPDH-R: 5′- CTG TGC TCA CAA GTT CCT CG-3′) and RNA60S (GenBank AUPL01005494) (RNA60S-F: 5′-CGA TGA AGC TCA AGT GGA CC-3′/RNA60S-R: 5′-CGG TTG TAC TTG ACG GGA AC-3′) genes were used as references, and a negative control containing no cDNA was included in each reaction set. Reactions consisted of 0·3 μM of each q*Tr*TRed primer, *Maxima® SYBR Green/ROX qPCR Master Mix* reagent (Thermo Scientific), 2 μL template cDNA and nuclease-free water up to 10 μL. The temperature profile for all reactions was 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C for 15 s) and annealing/extension (61 °C for 1 min). A final step was included to obtain the dissociation curve (95 °C, 60 °C and 95 °C for 15 s each). The reaction efficiency for each primer set was calculated after a 5-point serial dilution (1:2) of cDNA pools and gDNA. All qPCR assays were performed using biological and technical triplicates. Raw quantification cycle (C_q) results were obtained using SDS 2·4 data analysis software (Applied Biosystems) and normalized to the geometrical average value of two reference genes for each sample. The amplification efficiency was obtained with the $E = 10(-1\ \text{slope}^{-1})$ formula (Pfaffl, 2001). The relative quantification method (Livak and Schmittgen, 2001) was used to assess variations in transcript levels. Comparison of transcript levels between two samples was conducted using the

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unpaired Student's *t*-test with Prism 5·0 (GraphPad) and $P < 0{\cdot}05$.

Identification and Cloning of T. rangeli *TRed (TrTRed)*

Using the *T. cruzi* TRed gene as the query (GenBank Accession Number CAA78360), the complete sequence of the *Tr*TRed gene was retrieved via Blast from *T. rangeli* genomic and transcriptomic sequences available in GenBank and TriTrypDB (www.tritrypdb.org) (Aslett *et al*. 2010), as well as from sequences recently generated using the Illumina platform at the Science for Life Laboratory, Karolinska Institutet, Sweden. The entire *Tr*TRed open reading frame (ORF) was PCR-amplified using primers *Tr*TRed*Bgl*II (5′-AGA TCT ATG AAA GCC TTT GAT TTG GTT G-3′) and *Tr*TRed*Kpn*I (5′- GGT ACC AGC CTC TAG AGC CGT TTC CG-3′), containing the respective restriction enzyme sites to allow downstream cloning. PCR assays were performed on a Mastercycler® Gradient (Eppendorf) thermocycler using 35 cycles of the following thermal profile for denaturation (95 °C, 1 min), annealing (64 °C, 1 min) and extension (72 °C, 1 min), including a final extension step at 72 °C for 5 min. The PCR product was cloned into pGEM-T-Easy vector (Promega), and the resulting constructs were verified by sequencing on an Abi3500 automated DNA sequencer (Life Technologies) using the BigDYE 3·1 kit (Life Technologies) according to the manufacturer's instructions. Both DNA strands were sequenced for each obtained clone, and those presenting a high quality (Phred ≥ 20), as indicated by the Phred/Phrap/Consed package (Ewing *et al*. 1998), were clustered and compared with the public databases using the BLAST algorithm (Altschul *et al*. 1990).

Comparative analysis of TRed expression by T. rangeli *and* T. cruzi

Soluble protein extracts (50 μg) of different life cycle stages of *T. rangeli* and *T. cruzi* were resolved by sodium dodecyl sulphate- 12% polyacrylamide gel electrophoresis (SDS- 12% PAGE) and electroblotted onto nitrocellulose membranes (GE Healthcare) using an appropriate buffer (25 mM Tris; 192 mM glycine; 20% v/v methanol, pH 8·3). After blocking with 5% non-fat milk in blotting buffer (25 mM Tris–HCl pH 7·4, 150 mM NaCl, and 0·1% Tween-20) overnight at 4 °C (Gallagher *et al*. 2008), the membranes were incubated for 1 h with an anti-r*Tc*TRed mouse polyclonal antiserum (1:500) (kindly provided by Dr. Silvane Murta – CPqRR/Fiocruz) or anti α-tubulin monoclonal antibody (Cell Signaling Technology) (1:1000) as a loading control. Detection of expressed TRed was conducted using anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:10 000), followed by the enhanced chemiluminescence (ECL) kit (Pierce) according to the manufacturer's recommendations. The Western blots were digitally recorded and analysed using the ImageJ 1·463r software package. The relative protein expression was determined by the ratio of the TRed and α-tubulin band intensities.

Assessment of the enzymatic activity of T. rangeli *TRed*

The *Tr*TRed activity was assessed spectrophotometrically by measuring nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm as formerly described (Jockers-Scherübl *et al*. 1989). Kinetic assays were conducted in 96-well microplates at 27 °C in an incubation medium containing 20 mM HEPES pH 7·4, 30 mM NaCl, 0·1 mM EDTA, 150 μM NADPH, 50 μM $T(S)_2$ and 1·5 $\mu g\,\mu L^{-1}$ of soluble *T. rangeli* epimastigote protein extract in a final volume of 200 μL. The reaction was started by the addition of trypanothione disulphide ($T(S)_2$) and followed for 3 min. A negative (no substrate) and positive control (0·1 $\mu g\,\mu L^{-1}$ of r*Tc*Tred) were included in each enzymatic assay. One unit of TRed activity was defined as the amount of enzyme required to oxidize 1 μM of NADPH to $NADP^+$ per minute at 27 °C. The results were expressed as $\mu M\,min^{-1}$ mg of $protein^{-1}$. The reference values used were an NADPH molar extinction coefficient $\varepsilon M = 6220\ M^{-1}\,cm^{-1}$ and assuming a 1-cm path length.

Oxidative stress induced by H_2O_2 in T. rangeli

Exponential growth phase epimastigotes (5×10^7 cells) were harvested by centrifugation (3000 ***g*** for 10 min) and washed twice in sterile PBS pH 7·2. The parasites were then resuspended in LIT medium supplemented with 10% FBS and incubated in the presence of 67 μM H_2O_2 for 30, 60 or 90 min at 27 °C. The IC_{50} was determined as previously described (Romero *et al*. 2014). Considering that such a concentration of H_2O_2 in a single dose could induce bias in an *in vitro* system, the parasites were exposed to increasing concentrations of H_2O_2 every 20 min up to 1 h while another sample received no H_2O_2 (negative control). After incubation, the parasites were centrifuged (3000 ***g*** for 10 min) and washed twice with PBS (pH 7·4), and the pellet was stored at −20 °C for protein extraction. TRed expression and activity were measured as described above.

Detection of intracellular ROS in T. rangeli *epimastigotes*

The presence of intracellular ROS in *T. rangeli* and *T. cruzi* epimastigotes was assessed using the

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2′–7′-dichlorodihydrofluorescene diacetate (DCFH-DA) probe (Sigma-Aldrich) as previously described (Wang and Joseph, 1999). Briefly, 5×10^5 parasites well^{-1} (200 μL) were incubated in LIT medium supplemented with 10% heat-inactivated FBS in the presence of different H_2O_2 concentrations (0–1 mM) in black 96-well plates. After 10 min, 10 μM of DCFH-DA well^{-1} was added, and the fluorescence intensity was determined in a TECAN Infinite M200 spectrofluorometer (Switzerland) for 1 h using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Untreated parasites (no H_2O_2) and 10 μM of DCFH-DA probe in lysis buffer were used as negative controls. Two independent experiments were performed in quadruplicate. The results are expressed as relative fluorescence units that are directly proportional to the increase in the ROS concentration over the time course.

NADPH production through the measurement of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) activities

NADPH production was determined as previously described (Mielniczki-Pereira *et al*. 2007). Briefly, cells were collected by centrifugation and resuspended in PBS pH 7·2 in the presence of a protease inhibitor cocktail (Cocktail Set III, Calbiochem, Catalogue #: 539134). The combined activities of G6PD and 6PGD were measured by $NADP^+$ reduction at 340 nm after the addition of parasites (5×10^7 cells mL^{-1}) to the reaction mixture (50 mM Tris–HCl, pH 7·6, 50 mM KCl, 0·1% Triton X-100, 250 μM NADP+, 2 mM $MgCl_2$, 1 mM 6-phosphogluconate and 1 mM glucose-6-phosphate). 6PGD activity was assessed in the same medium but in the absence of glucose-6-phosphate.

Determination of hydrogen peroxide release

H_2O_2 release by *T. rangeli* epimastigotes was determined as previously described (Peloso *et al*. 2011). Briefly, 10^8 cells mL^{-1} were incubated in PBS pH 7·2 1 mM^{-1} $MgCl_2$ in the presence of 5 mM succinate, 60 μM digitonin, 1 U mL^{-1} of HRP and 25 μM Amplex Red (Molecular Probes). The fluorescence was monitored at excitation and emission wavelengths of 563 nM and 587 nM, respectively, using a Hitachi F2500 fluorescence spectrophotometer with continuous stirring. The quantitative correlation between the fluorescence and H_2O_2 release by the cells was determined as previously described (Barros *et al*. 2004).

Effect of antioxidants on the growth of T. rangeli *and* TRed *expression*

Epimastigotes (2×10^6 mL^{-1}) were grown in LIT medium (10% FBS) for 15 days at 27 °C in the presence of reduced glutathione (GSH) or N-acetylcysteine (NAC) (Sigma-Aldrich) at two different concentrations (1 and 2·5 mM). The experiments were performed in triplicate, and the parasites were counted every day in a Neubauer chamber. Samples were collected on days 1, 3, 5, 7, 9 and 11 to analyse TRed expression by Western blotting using anti-rTcTRed and anti α-tubulin antibodies.

Overexpression of TRed in T. rangeli

The *Tr*TRed gene was excised from the pGEM-T-Easy vector using *Bgl*II/*Kpn*I restriction enzymes and then subcloned into the pLEXSY-NEO2 vector (Jena). All constructs were checked for their restriction profiles and sequenced prior to transfection into *T. rangeli* epimastigotes by electroporation using e Nucleofector® device (Lonza). Briefly, 5×10^6 epimastigotes in the *log* growth phase were washed twice in PBS (pH 7·2), mixed with 100 μL of human T cell Nucleofector buffer (Lonza) and 10 μg of pLEXSY-NEO2 –*Tr*TRed construct DNA and transfected using program U-033. After transfection, the parasites were transferred to Neal, Novy, Nicolle-LIT medium and grown at 27·5 °C for 48 h. The transfectants were then selected via incremental exposure to geneticin (Gibco), from 25 μg mL^{-1} up to a final concentration of 300 μg mL^{-1}. Parasites transfected with empty vector were generated and compared with wild-type parasites in terms of growth and TRed expression levels. Overexpression of *Tr*TRed (*Tr*TRed+) by transfected parasites was assessed by Western blotting using an anti-His6-tagged antibody (1:200) (Santa Cruz Biotechnology). After confirmation, the *Tr*TRed+ parasites were harvested during the exponential and late *log* phases for protein extraction and for the *in vitro* oxidative susceptibility assay as described above, using non-transfected wild-type (WT) parasites as a control. Additionally, WT and *Tr*TRed+-overexpressing parasites were comparatively evaluated according to their *in vitro* growth curve and differentiation into trypomastigote forms.

*Cytolocalization of the r*Tr*TRed expression sites*

The expression sites of *Tr*TRed+ by *T. rangeli* were assessed by indirect immunofluorescence assays (IFA). Exponential growth phase WT and *Tr*TRed+ epimastigotes were harvested from LIT by centrifugation (3000 ***g***, 10 min), washed twice with PBS pH 7·2 and adhered onto 13-mm circular glass coverslips for 20 min. The parasites were fixed with 4% paraformaldehyde (w/v) in PBS and then permeabilized with 0·1% NP-40 or 0·1% Triton X-100. The cells were then blocked with 2% BSA diluted in PBS and probed with anti-His6-tagged antibody diluted 1:100 in blocking solution. After 90 min, the slides were washed

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extensively with PBS and then incubated for 1 h with Alexa Fluor 488-conjugated goat-anti-mouse IgG diluted 1:10 000 in blocking solution. After washing 3× with PBS, the parasites were stained for 5 min with 1 μg mL^{-1} of DAPI (4,6-diamidino-2-phenylindole), and the coverslips were mounted over microscopy slides using Hydromount (National Diagnostics). Images were obtained using an Olympus Bx40–FL fluorescence microscope (Olympus).

Susceptibility of TRed-overexpressing T. rangeli *epimastigotes to oxidative stress*

The susceptibility of *T. rangeli*-overexpressing *Tr*TRed to oxidative stress was compared with that of WT cells using Alamar Blue (AB) assays as previously described (Räz *et al*. 1997; Decuypere *et al*. 2012). Briefly, 5×10^5 *T. rangeli* and *T. cruzi* epimastigotes were exposed to hydrogen peroxide (Sigma-Aldrich) prepared at different dilutions (0–1 mM) and incubated for 48 h at 26 °C in quadruplicate in 96-well plates. After incubation for 24 h, 20 μL of AB reagent (Invitrogen) was added to each well to assess the parasite viability via fluorescence emission at 600 nM. Data from treated and non-treated cultures were used to calculate the IC_{50} by a sigmoidal regression analysis (with a variable slope) using GraphPad Prism v.5.0. Untreated control parasites and reagent blanks were included in each test plate.

In vitro interaction of THP-1 cells and T. rangeli *overexpressing* Tr*TRed*

Cells of the human acute monocytic leukaemia cell line THP-1 (ATCC#TIB-202) were cultured at 37 °C in a 5% CO_2 atmosphere using RPMI 1640 medium (HiMedia Laboratories) supplemented with 1% glutamine, 10% heat-inactivated FBS, 12·5 mM HEPES (Gibco), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (Gibco), 2 mM Glutamax® (Gibco) and 1 mM sodium pyruvate (Gibco). Monocytes were harvested during the logarithmic growth phase and transferred to medium containing 50 μg mL^{-1} phorbol myristate acetate (Sigma-Aldrich) to induce adherence and differentiation of the macrophages (Schwende *et al*. 1996). Prior to the *in vitro* infection assays with *T. rangeli*, a total of 1×10^5 macrophages were seeded onto 13-mm circular glass coverslips placed in 6-well plates and then incubated for 72 h at 37 °C in a 5% CO_2 atmosphere (Romero *et al*. 2005). After removal of non-adherent cells by washing the plate wells twice with sterile PBS (pH 7·2), THP-1-derived macrophages were incubated with *T. rangeli* trypomastigotes overexpressing *Tr*TRed for 1 h at 37 °C in a 5% CO_2 atmosphere using a parasite-to-cell ratio of 25:1. Non-adherent parasites were removed by washing with PBS, which was considered the initial time point of infection (T0), and the progression of the parasite–cell interaction was observed at 2 (T2), 4 (T4), 6 (T6) and 24 hours (T24) after infection. The cells were then stained with Giemsa, mounted on microscope slides with Entellan® (Merck) and observed by light microscopy. The number of infected cells was determined by counting 200 randomly chosen cells per coverslip for each of the time points, strains and species. The assays were performed using biological triplicate samples and non-infected cells were used as controls.

Statistical analyses

All experiments were performed in triplicate, and the results are presented as the mean and standard deviation (S.D.M.) or standard error of the mean (S.E.M.). Significant differences were assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test or Student's *t*-test using GraphPad Prism v.5.0 software as indicated in the figure legends.

RESULTS

In silico analysis revealed that the *T. rangeli* TRed gene (*Tr*TRed) has an ORF of 1473 nt encoding a predicted protein of 491 aa (~53·5 kDa) with a predicted isoelectric point of 6·33. *Tr*TRed is present as a single copy in the parasite genome and displays conserved catalytic domains, particularly the disulphide-binding site, FAD and NADH binding domains among different kinetoplastid species (Fig. 1).

Assessment of the intracellular localization of *Tr*TRed in *T. rangeli* epimastigotes overexpressing the enzyme by IFA revealed dispersed fluorescence throughout the parasite cytoplasm, suggesting a cytosolic localization (Fig. 2), which is consistent with the *in silico* prediction for the native protein.

The relative abundance of the *Tr*TRed protein assessed by Western blotting in *T. rangeli* epimastigotes and trypomastigotes revealed no significant differences (Fig. 3A). The absence of TRed stage-specific expression was also observed for the homologous protein in *T. cruzi* (*Tc*TRed).

Trypanosoma rangeli TRed was active in both epimastigotes and trypomastigotes (3·11 and 1·82 μM min^{-1} mg of protein^{-1}, respectively), suggesting that its activity is significantly increased in the replicative parasite forms (Fig. 3B). Interestingly, *T. cruzi* TRed was more active in trypomastigotes than in epimastigotes (2·74 and 1·71 μM min^{-1} mg of protein^{-1}, respectively), but no significant differences were observed between the infective and replicative forms of the parasite. Considering this difference, we also comparatively assessed the *Tr*TREd mRNA levels between replicating

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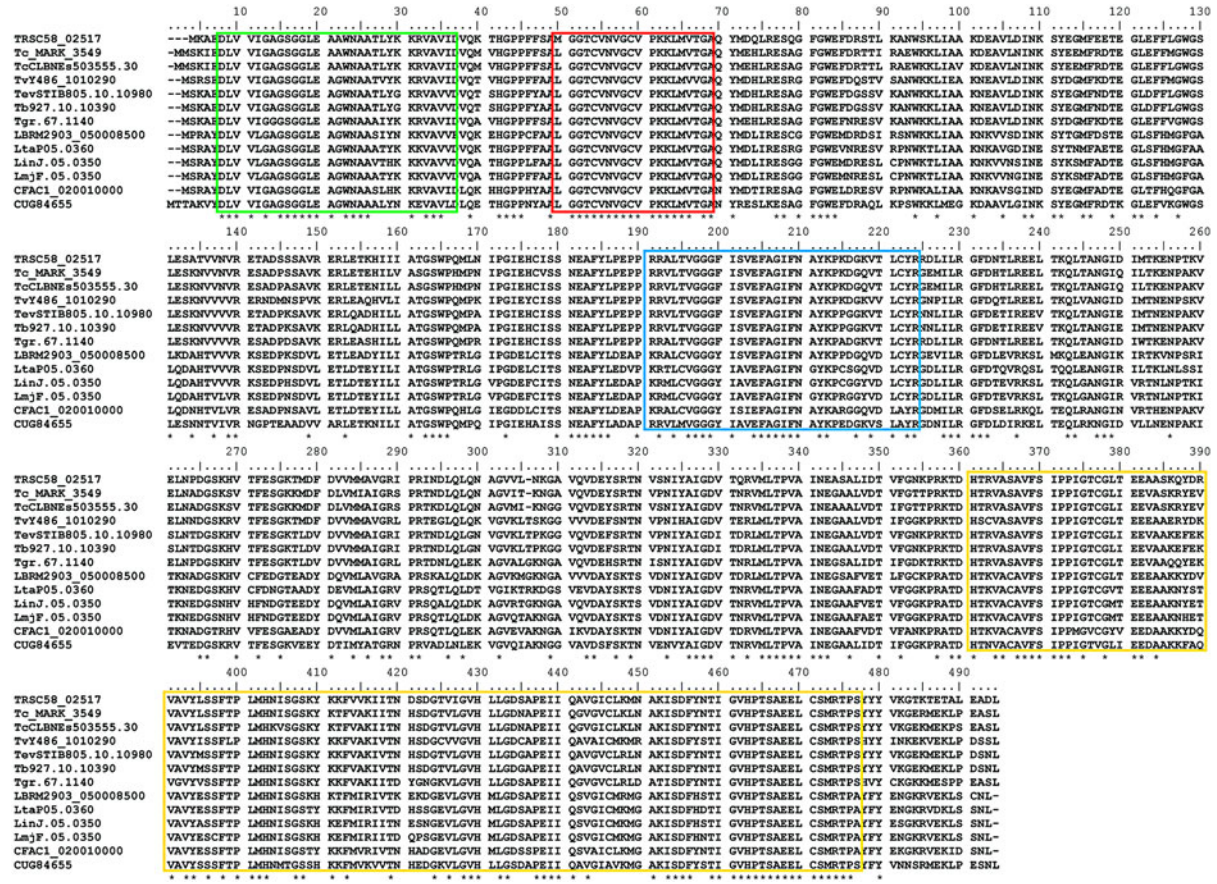

Fig. 1. *Trypanosoma rangeli* trypanothione reductase (TRed) has conserved catalytic domains. Alignment of the amino acid sequences of TRed from *T. rangeli* and other kinetoplastid species. Red rectangle- catalytic site; Green rectangle- FAD binding domain; Blue rectangle- NAD(P) binding domains; Yellow rectangle- dimerization domain. Accession numbers for TRed from *T. rangeli* – TRSC58_02517; *Trypanosoma cruzi marinkellei* – Tc_MARK_3549; *T. cruzi* CLBrener – TcCLBNEs503555·30; *Trypanosoma vivax*- TvY486_1010290; *Trypanosoma evansi* – TevSTIB805.10.10980; *Trypanosoma brucei* -Tb927.10.10390; *Trypanosoma grayi* – Tgr.67·1140; *Leishmania braziliensis* – LBRM2903_050008500; *Leishmania tarentolae* – LtaP05·0360; *Leishmania infantum* -LinJ.05·0350; *Leishmania major* – LmjF.05·0350; *Crithidia fasciculata* – CFAC1_020010000; *Bodo saltans* – CUG84655.

epimastigotes and infective trypomastigotes. The results are shown in Fig. 3C and indicate a significant decrease in the mRNA levels of *Tr*TRed in trypomastigotes.

Having determined the basal *Tr*TRed transcriptional and expression levels, we also evaluated whether *Tr*TRed expression by *T. rangeli* epimastigotes was influenced by H_2O_2-induced oxidative stress. The Western blotting shown in Fig. 4A indicates that there was no significant increase in *Tr*TRed expression up to 1 h after exposure to 67 μM H_2O_2. This concentration of H_2O_2 was based on the IC_{50} determined in previous studies (Stoco *et al*. 2014; Romero *et al*. 2014). For all times and conditions tested, no significant differences were observed in the densitometric analysis, which indicated that *Tr*TRed expression by parasites remained constant regardless of exposure to oxidative stress.

Modulation of enzyme expression was evaluated during oxidative stress induced by hydrogen peroxide in *T. rangeli*, also considering the endogenous production of H_2O_2 by the parasite. Using the DCFH-DA probe, we observed that oxidative stress induced by concentrations of H_2O_2 up to 125 μM did not induce changes in fluorescence emission among treated and non-treated parasites. However, at concentrations of 250 μM and higher, an effective and significant effect of the stressor was observed for both *T. rangeli* and *T. cruzi* in comparison with non-H_2O_2-treated parasites (Fig. 4B).

The amount of NADPH produced by *T. rangeli* epimastigotes, as determined by the combined measurement of G6PDH and 6PGD activities, was 1·1 ± 0·05 nM min^{-1} 10^7 $cells^{-1}$, whereas *T. cruzi* epimastigotes produced 2·13 ± 0·09 nM NADPH min^{-1} 10^7 $cells^{-1}$. This difference was significantly different ($P < 0·05$) and indicated that the pentose pathway is more effective in *T. cruzi* than in the other parasite. Based on this difference and considering the distinct virulence of these species to their mammalian hosts, we determined the amount of mitochondrial hydrogen peroxide released by these parasites. Using digitonin-permeabilized cells and succinate as a substrate for the mitochondrial

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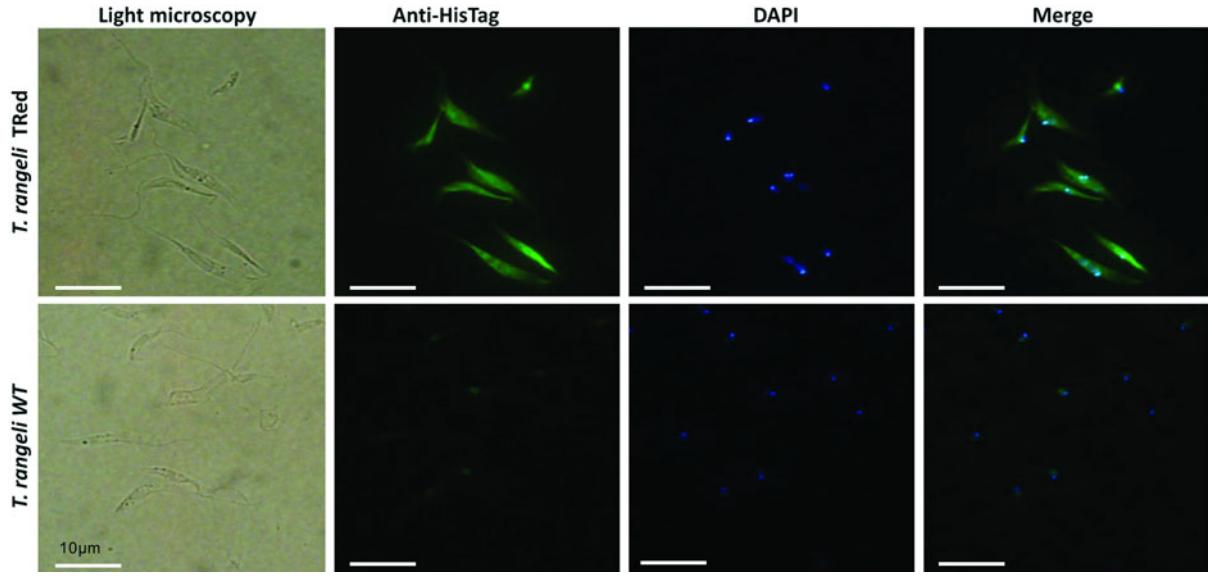

Fig. 2. Homologous trypanothione reductase overexpressed by *Trypanosoma rangeli* has a cytosolic localization. Immunolocalization of the expression sites of r*Tr*TRed in *T. rangeli* epimastigotes (*T. rangeli* TRed) by IFA assays using anti-His6-tag antibody. *T. rangeli* wild type (WT) parasites were used as controls. (1) Light microscopy, (2) Detection of r*Tr*TRed using anti-His6-tagged antibodies, (3) DAPI staining and (4) merged images. Bars indicate 10 μm.

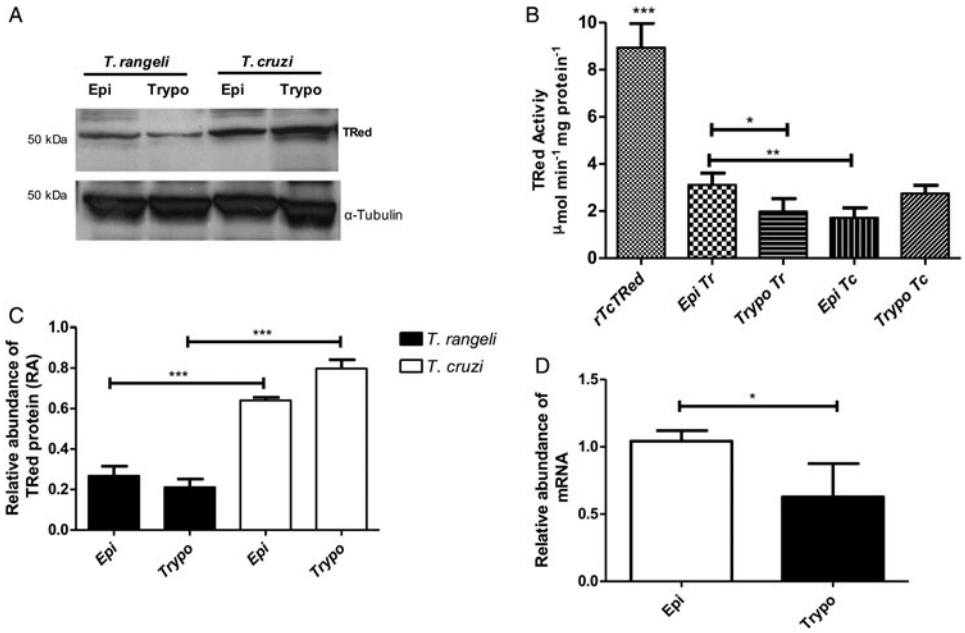

Fig. 3. TRed expression and activity are mainly observed in *Trypanosoma rangeli* epimastigotes. (A) Western blot analysis of TRed expression in soluble *T. rangeli* and *T. cruzi* protein extracts obtained from epimastigote (Epi) and trypomastigote (Trypo) forms and comparative densitometric analysis of the blotting results using ImageJ software. Significant differences were determined by one-way ANOVA followed by the Bonferroni test (***$P < 0{\cdot}001$). Detection of α-tubulin served as a loading control and for normalization of the densitometry analysis. (B) Enzymatic activity of TRed in soluble protein extracts of *T. rangeli* and *T. cruzi* epimastigotes and trypomastigotes. The recombinant *T. cruzi* TRed (r*Tc*TRed) was used as a positive control, and the results represent the mean ± S.D.M. (standard deviation of the mean) from 3 independent experiments performed in triplicate. Significant differences were assessed by one-way ANOVA followed by the Bonferroni test (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$). (C) Detection of *Tr*TRed mRNA levels in *T. rangeli* epimastigotes and trypomastigotes by qPCR using GAPDH and RNA60S for normalization. Data represent the mean ± S.D.M. from two independent experiments conducted with technical quadruplicates. Significant differences were assessed by Student's *t*-test (*$P < 0{\cdot}05$).

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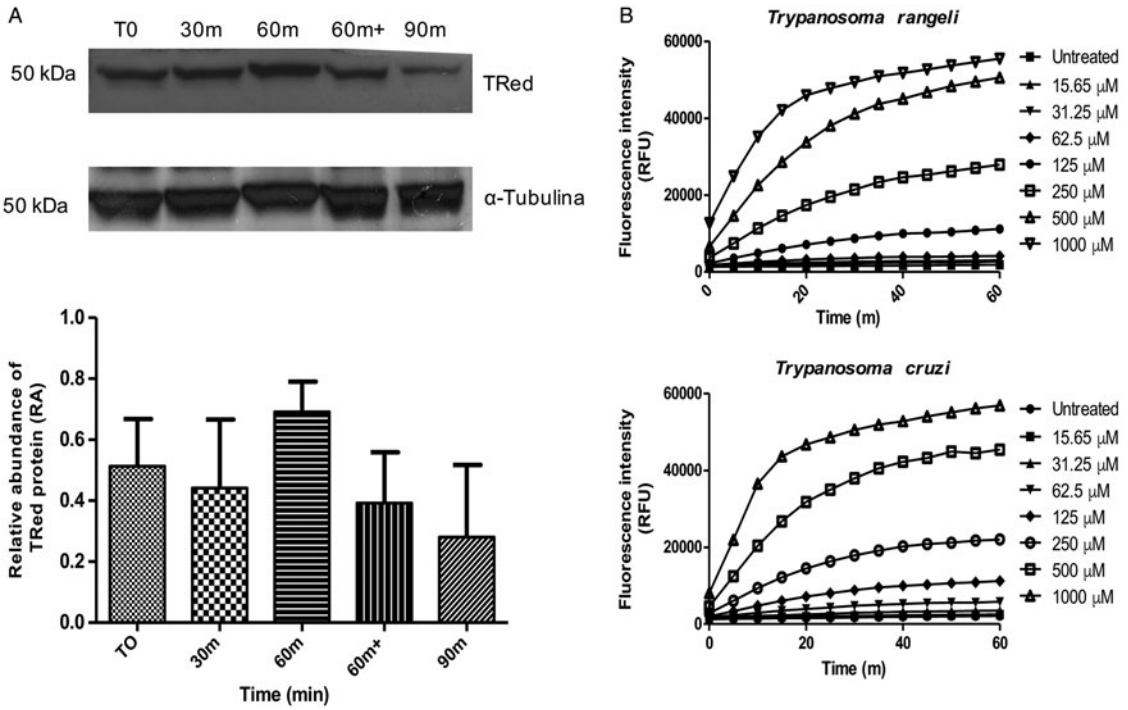

Fig. 4. *Trypanosoma rangeli* and *Trypanosoma cruzi* epimastigotes exhibit similar responses to *in vitro* oxidative stress induced by H_2O_2. (A) *Western blot* analysis of *Tr*TRed expression in soluble protein extracts obtained at different time points from *T. rangeli* epimastigotes under oxidative stress induced by 67 μM H_2O_2. Comparative densitometric analysis of the blotting results using ImageJ software. Data represent the mean ± S.D.M. from three independent experiments. Significant differences were assessed by one-way ANOVA followed by the Bonferroni *post-hoc* test (*$P < 0{\cdot}05$). Detection of α-tubulin served as loading control and for normalization in the densitometry analysis. (B) Detection of fluorescence emitted by the DCFH-DA probe in parasites exposed to increasing concentrations of hydrogen peroxide. The results are expressed as the mean ± S.D.M. from two independent experiments performed using technical quadruplicates. Significant differences were detected by one-way ANOVA following by the Bonferroni *post-hoc* test (***$P < 0{\cdot}001$).

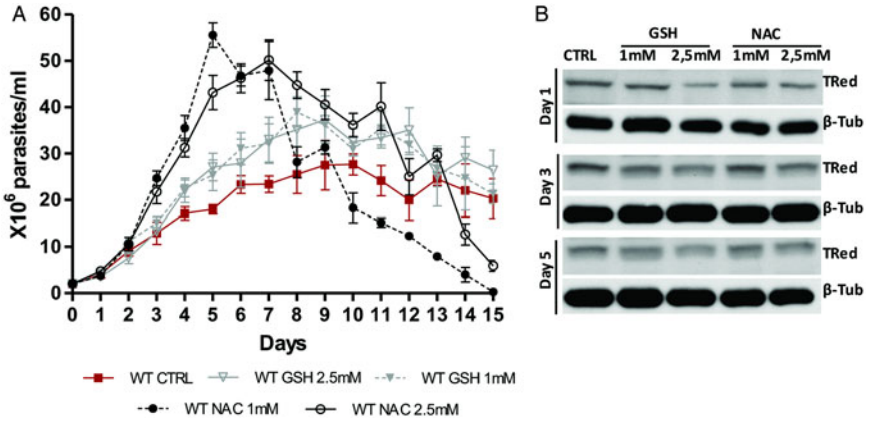

Fig. 5. N-acetylcysteine treatment improves the growth of *Trypanosoma rangeli* epimastigotes. (A) Comparative *in vitro* growth curve of *T. rangeli* submitted to treatments with GSH and NAC at 1 and 2·5 mM. The results are expressed as the mean ± S.D.M. of triplicates. Significant differences were detected using two-way ANOVA followed by the Bonferroni *post-hoc* test. (B) Representative *Western blot* analysis of *Tr*TRed expression in soluble protein extracts from *T. rangeli* with different treatments at days 1, 3 and 5 revealed by anti-*Tc*Red antibody. Detection of α-tubulin served as a loading control and for normalization in the densitometry analysis.

respiratory chain, we were able to determine that *T. rangeli* epimastigotes released 14·2 ± 1·6 pM of H_2O_2 min^{-1} 10^7 $cells^{-1}$ (data not shown), which is higher than the levels observed for *T. cruzi* under the same experimental conditions (Silva *et al*. 2011).

The presence of antioxidants improved the *in vitro* growth of *T. rangeli* epimastigotes (Fig. 5A). Both treatments with NAC (1 and 2·5 mM) induced dramatic growth of the parasites from day 3–7 ($P <$ 0·001). Parasites grown in the presence of 1 mM

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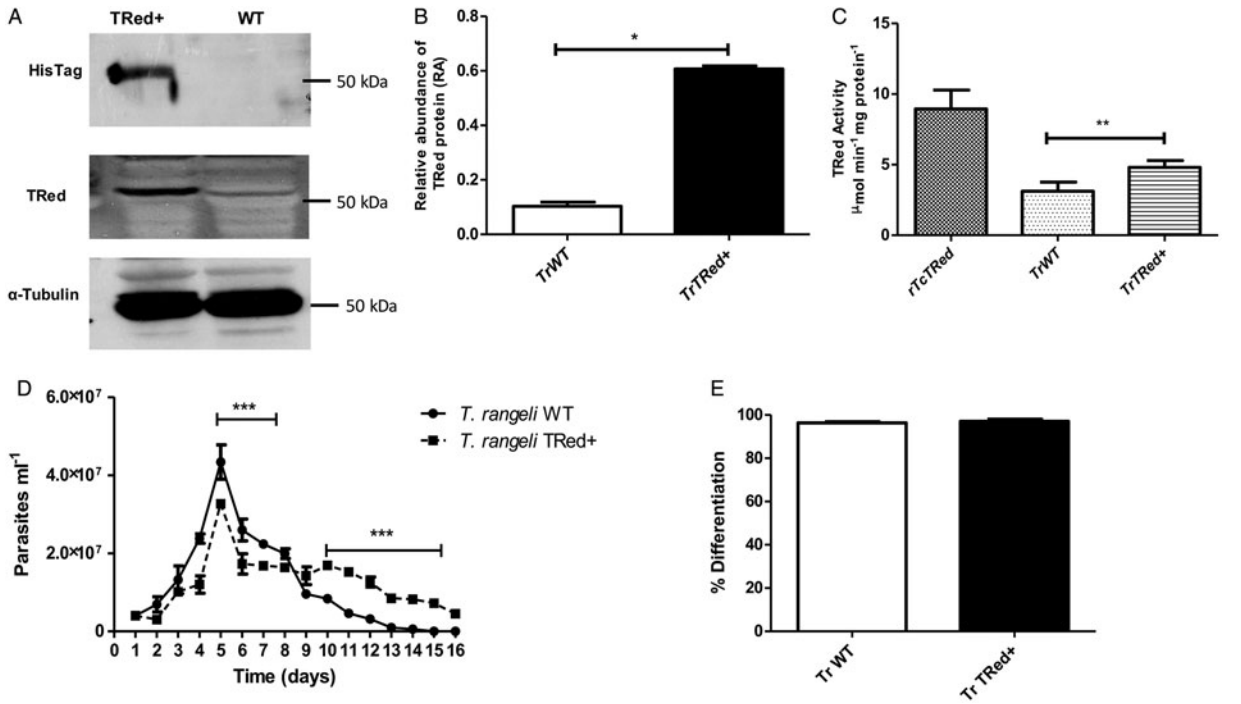

Fig. 6. Overexpression of TRed by *Trypanosoma rangeli* epimastigotes does not affect parasite biology *in vitro*. (A) *Western blot* analysis of *Tr*TRed expression in soluble protein extracts from *T. rangeli* overexpressing *Tr*TRed+ and WT revealed by anti-His6-tag and anti-*Tc*Red antibodies. Detection of α-tubulin served as a loading control and for normalization in the densitometry analysis. (B) Comparative densitometric analysis of the blotting results using ImageJ software. (C) TRed enzymatic activity in protein extracts from *T. rangeli* WT and *Tr*TRed+-overexpressing epimastigotes. r*Tc*TRed was used as a control. (D) Comparative *in vitro* growth curve of *Tr*TRed+ and WT *T. rangeli* strains starting from 4×10^6 parasites mL^{-1}. (E) Comparative percentages of the *in vitro* differentiation of *T. rangeli* WT and TRed+ mutants. The results shown in panels B and C represent the mean ± S.D.M. obtained from three independent experiments using biological triplicates. For panels B and C, significant differences were assessed using Student's *t*-test (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$). The results in panel D represent the mean ± S.D.M. obtained from three independent experiments performed in duplicate. Significant differences were assessed by two-way ANOVA (***$P < 0{\cdot}001$).

NAC reached the largest number of parasites ($5{\cdot}8 \times 10^7$ parasites mL^{-1}) on the 5th day of culture. Treatments with GSH (1 and 2·5 mM) also improved parasite growth from day 5–12 ($P < 0{\cdot}01$). Although important differences in parasite growth were observed in the presence of antioxidants, the reducing environment did not affect the expression of TRed (Fig. 5B). The protein levels remained constant in parasites grown under different conditions on the days evaluated.

Overexpression of homologous *Tr*TRed by *T. rangeli* epimastigotes revealed a 5-fold increase in the protein expression level compared with wild type parasites (Fig. 6A and B). As expected, the specific TRed activity in parasites overexpressing this gene was 1·3-fold higher than the TRed activity in control WT parasites (Fig. 6C). Overexpression of *Tr*TRed by *T. rangeli* revealed no major differences in *in vitro* growth when compared with WT parasites (Fig. 6D). The maximum number of parasites for both WT ($4{\cdot}3 \times 10^7$ parasites mL^{-1}) and overexpressing parasites ($3{\cdot}3 \times 10^7$ parasites mL^{-1}) was observed on day 5 of culture. While the growth curve of the wild type strain exhibited a typical decrease in the number of live parasites from day 6 forward, overexpressing parasites appeared to be sustained in culture up to day 14 and then started to decrease. Significant differences were observed in comparisons of the number of parasites between days 4–7 and 10–15. We may conclude that *Tr*TRed-overexpressing parasites have slightly higher growth and survival rates *in vitro* compared with WT parasites. It is noteworthy that overexpression of *Tr*TRed had no effect on the ability of parasites to undergo *in vitro* differentiation into infective trypomastigotes compared with the parental WT strain (Fig. 6E).

Parasites overexpressing *Tr*TRed protein were also more resistant (2·3 times) to H_2O_2 than wild-type parasites. The dose-response analysis revealed a marked difference in resistance to H_2O_2 between *T. rangeli* WT and mutant strains at the 125 μM H_2O_2 concentration, in which the viability of the mutant parasites was 13·4-fold higher compared with the WT parasites (Fig. 7A).

Due to this increased resistance to H_2O_2 stress, we also assessed whether the increased expression of *Tr*TRed by *T. rangeli* would modulate its ability to infect and survive within a phagocytic cell. Although *Tr*TRed-overexpressing parasites seemed

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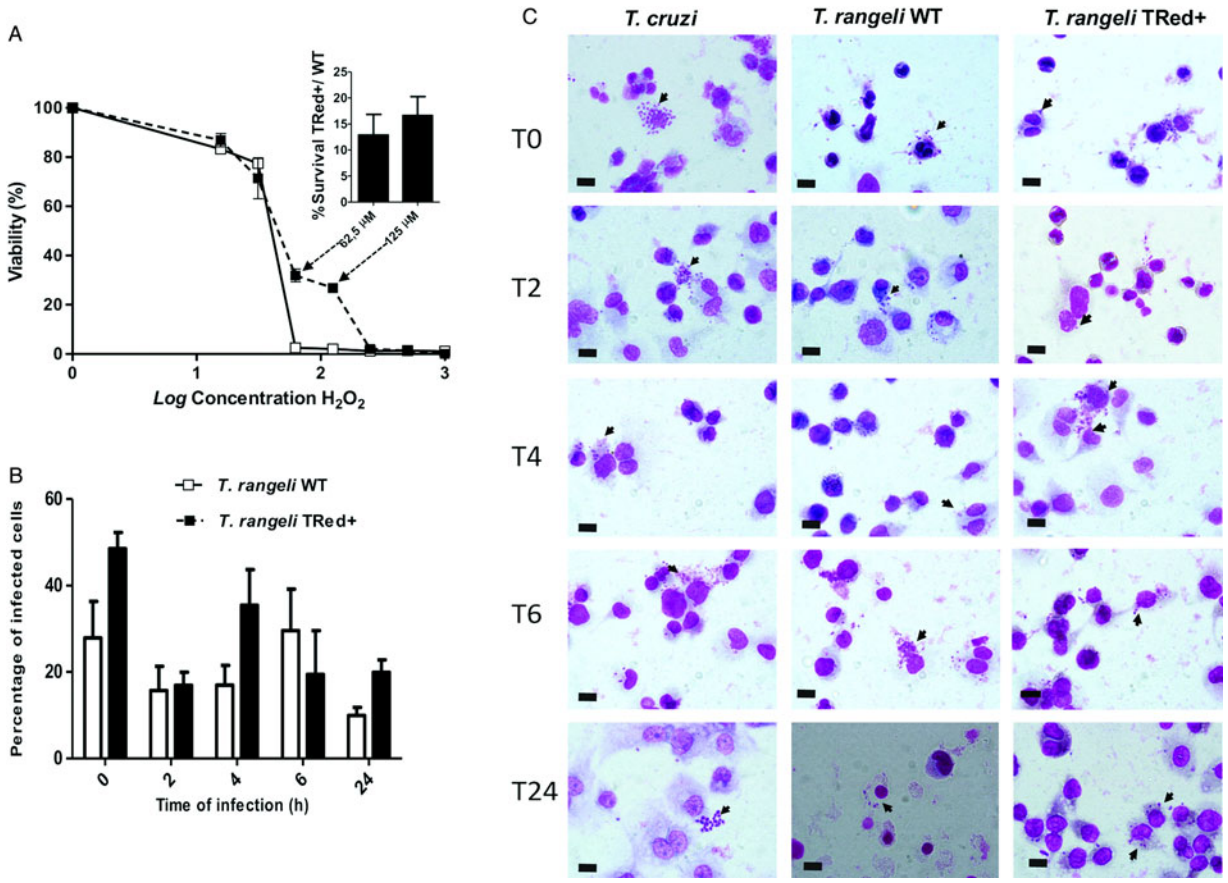

Fig. 7. Overexpression of homologous *Tr*TRed by *Trypanosoma rangeli* epimastigotes increases parasite survival under oxidative stress *in vitro*. (A) Viability of *T. rangeli* WT and *Tr*TRed+ epimastigotes in response to increasing concentrations of H_2O_2 assessed by Alamar Blue staining. The bars represent the survival ratio between *Tr*TRed+ and WT at 62,5 and 125 μM. (B) The percentages of THP-1 cells infected by *T. rangeli* WT or *Tr*TRed+ mutants during the time course of the *in vitro* cell-parasite interaction. (C) Light micrographs of distinct time points (T0-T24 hours) of THP-1-derived human macrophages infected by *T. rangeli* WT or *Tr*Tred+ mutants. *Trypanosoma cruzi*-infected THP-1 cells were used as a control. Cells were stained with Giemsa, black arrows indicate parasites and bars represent 10 μM. The results shown in panels A and B represent the mean ± S.D.M. from three independent experiments performed in triplicate.

to be more infective on T0, T4 and T24, the kinetic study of the interaction of WT and mutant parasites with THP1-derived macrophages *in vitro* revealed no significant differences between the parasite strains ($P < 0{\cdot}05$) in terms of the number of infected cells (Fig. 7B and C). No signs of *T. rangeli* multiplication were observed for WT or mutant parasites. In conclusion, increased expression of homologous TRed by *T. rangeli* enhanced its resistance to oxidative stress but did not induce detectable phenotypic changes in the *in vitro* parasite-THP-1 cell interactions.

DISCUSSION

Members of the Trypanosomatidae family are digenetic parasites with a life cycle that presents multiple differentiation forms alternating between invertebrate and vertebrate hosts (Teixeira and DaRocha, 2003). During each stage of differentiation, a stage-specific protein expression pattern participates in the invasion and survival processes of the parasite (Macleod *et al*. 2007; Díaz *et al*. 2011).

Redox homoeostasis appears to be efficiently regulated in kinetoplastids because the parasites can successfully survive the oxidative burst generated at the onset of infection and adapt to the different metabolic and environmental conditions (Peloso *et al*. 2012). At present, antioxidant defence is directly related to the success of the infection process because the proteins making up this system are among those that are most expressed during differentiation from replicative epimastigotes to infective trypomastigotes (Atwood *et al*. 2005; Piñeyro *et al*. 2008; Piacenza *et al*. 2009*a*, *b*; Gretes *et al*. 2012). In *T. cruzi*, increased expression of antioxidant defence-related proteins enables the parasite to survive in the complex and highly oxidative environment within macrophages (Atwood *et al*. 2005; Araujo and Teixeira, 2011). According to Irigóin *et al*. (2008), this increase is a preadaptation of the parasite prior to invasion of the vertebrate host in preparation for environments in which it may be exposed to ROS and RNS (reactive nitrogen species) such as those generated by immune cells. However, an evaluation

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of antioxidant enzymes in *T. cruzi* virulent strains compared with attenuated ones revealed that the protein contents observed in *Tc*TRed remained constant across the different parasites stages (Piacenza *et al*. 2009*a*, *b*).

The susceptibility of *T. rangeli* to oxidative stress was evaluated by subjecting epimastigotes to H_2O_2-induced stress conditions *in vitro* according to Stoco *et al*. (2014) and Romero *et al*. (2014). These authors demonstrated that *T. rangeli* was more sensitive than *T. cruzi* to oxidative stress, showing an IC_{50} of 53 μM, which is significantly less than the IC_{50} obtained for *T. cruzi* epimastigotes (188·3 μM). Previous studies using distinct *T. cruzi* strains have shown a wide range of IC_{50} for the H_2O_2 stress, varying from 98 to 190 μM (Finzi *et al*. 2004; Mielniczki-Pereira *et al*. 2007).

Taylor *et al*. (1994) described an absence of *Leishmania donovani* TRed expression modulation during the induction of oxidative stress by H_2O_2. These authors state that in contrast to peroxidases, which are upregulated due to direct involvement in the detoxification of H_2O_2-induced stress, TRed is not directly involved in such detoxification. For *T. rangeli*, TRed appears to be constitutively expressed during the parasite life cycle, as described in this study and in accordance with Taylor *et al*. (1994). It also appears to participate in the mechanisms that protect the parasite from ROS. Although the results obtained herein were obtained from only one strain of each species (*T. rangeli* Choachí and *T. cruzi* Y), they yielded some interesting insights regarding comparisons of the antioxidant defence of these parasites.

Although expressed during the entire life cycle of *T. rangeli*, elevated activity of *Tr*TRed was observed during the replicative epimastigote stage. Unlike *T. cruzi*, which faces oxidative stresses in the mammalian host and within the digestive tract of the triatomine vector, *T. rangeli* epimastigotes are found within the intestinal tract and haemolymph of triatomines, where the parasite is exposed to successive and distinct oxidative and/or nitrosative stresses. In this study, reduced *Tr*TRed expression was observed for the *in vitro*-derived trypomastigote forms. Because these parasites are biochemically distinct from the *T. rangeli* metacyclics observed within the triatomine salivary glands and bloodstream trypomastigotes found in infected mammals, the reduced *Tr*TRed expression observed *in vitro*-derived trypomastigotes remains to be examined in *in vivo* parasites.

The present observations can be related to a reduction in NADPH production in *T. rangeli* compared with *T. cruzi* epimastigotes. Unlike *T. cruzi, T. rangeli* is able to evade the intestinal tract of the triatomine by penetrating through the gut epithelium towards the haemocoel, where the parasites undergo successive division cycles prior to invading the salivary glands (Stoco *et al*. 2014). These highly oxidative environments (Garcia *et al*. 2009) demand increased synthesis of NADPH by the parasite as a primary source of reducing equivalents for biosynthetic reactions and the oxidation-reduction involved in protecting against the toxicity of ROS (Igoillo-Esteve *et al*. 2007; Leroux *et al*. 2010). In this sense, several enzymes are also co-responsible for the maintenance of this reducing environment, such as the NADP-linked glutamate dehydrogenase (Barderi *et al*. 1998), the NADP-linked malic enzyme (Cannata *et al*. 1979) and the two dehydrogenases in the pentose phosphate pathway (PPP) (Leroux *et al*. 2010), which is the major source of reduced TRed (Krauth-Siegel and Comini, 2008; Leroux *et al*. 2010; Peloso *et al*. 2011).

By analysing the PPP-related enzymes in different *T. cruzi* life stages, Maugeri and Cazzulo (2004) demonstrated increased expression in trypomastigotes that probably resulted from the need to respond to the oxidative stress created by the mammalian host.

Trypanosoma cruzi and *T. brucei* depend on their NADP-linked dehydrogenases, such as malic enzymes, for NADPH production in low or no glucose environments, in the gut of their insect vectors or within cells, because under such conditions, the PPP cannot act as a source of essential redox mechanisms (Hellemond *et al*. 2005; Leroux *et al*. 2010; Allman *et al*. 2013). The relative roles of the PPP and alternative reactions to provide NADPH have not yet been investigated in *T. rangeli*.

The major and continuous source of cellular ROS, including H_2O_2, is the mitochondrial electron transport chain. H_2O_2 can easily diffuse through the plasma membrane and interact with membrane-bound components (Cosentino-Gomes *et al*. 2009). As determined in this study, endogenous production of H_2O_2 was higher in *T. rangeli* than in *T. cruzi* (Peloso *et al*. 2011). This distinct behaviour could be due to differences in the mitochondrial antioxidant defences of the two species. In addition to the reduced TRed expression compared with *T. cruzi*, the reduced NAPDH production and higher susceptibility to H_2O_2-induced stress by *T. rangeli* allowed us to hypothesize that these two species have distinct superoxide dismutase expression profiles. Thus, it is noteworthy that overexpression of superoxide dismutase by *T. cruzi* induces a reduced sensitivity to trypanocidal agents (Temperton *et al*. 1998).

N-acetylcysteine (NAC) has been widely used as an antioxidant *in vivo* and *in vitro*. It is a powerful scavenger of hypochlorous acid (H--OCl) and reacts with hydroxyl radical and H_2O_2 (Aruoma *et al*. 1989; Rossato *et al*. 2014). GSH is the major intracellular antioxidant thiol that protects mammalian cells against oxidative stress induced by oxygen- and nitrogen-derived reactive species (Romão *et al*.

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1999). Our results demonstrated that the presence of these antioxidants (NAC and GSH) promoted improved growth of epimastigotes cultivated in LIT medium. Conversely, the presence of the same antioxidants dramatically impaired *T. cruzi* epimastigote proliferation (Nogueira *et al.* 2015), and the presence of the pro-antioxidant heme induced epimastigote proliferation. Thus, *T. rangeli* appeared to require a reduced environment for epimastigote proliferation, whereas *T. cruzi* required an oxidative environment. Both parasites develop inside triatomines; however, differences related to the biological cycle of these parasites in this host could explain the differences in proliferation. Epimastigotes from *T. cruzi* multiply and colonize the entire gut (oxidative environment), whereas *T. rangeli* epimastigotes rapidly cross the gut epithelium and invade the haemolymph. The haemolymph of *Rhodnius prolixus* has been described to have ten times more urate than human plasma (Souza *et al.* 1997; Graça-Souza *et al.* 2006). Urate is considered as one of the most important antioxidants in human plasma. Thus, the effect of the antioxidants NAC and GSH in *T. rangeli* cultures would improve their ability to grow by simulating the role of urate in the haemolymph. Despite the improved growth and reducing environment, we did not observe any modifications in the expression levels of *Tr*TRed.

The role of TRed in *T. rangeli*, like in *T. cruzi* and *L. donovani* (Kelly *et al.* 1993; Tovar *et al.* 1998), has been investigated by overexpressing the homologous enzyme. Kelly *et al.* (1993) transfected *L. donovani* and *T. cruzi* with an expression vector containing the *Ld*TRed gene and achieved 10–14-fold enzyme activity in transformed cells. *Trypanosoma cruzi* cells overexpressing the sense construct displayed a 4-fold increase in TRed protein levels and enzyme activity (Tovar and Fairlamb, 1996). The difference between the species is probably due to the distinct basal expression levels of TRed.

In addition to numerous tools used to unravel the mechanisms of *T. cruzi* infection, such as cellular tropism, infectivity and intracellular replication, *in vitro* infection of cell cultures has been utilized (Duran-Rehbein *et al.* 2014). Non-epithelial cells such as monocytes/macrophages are highly parasitized after very short incubation times, providing insight into the initial stages of *T. cruzi* infection and the subsequent immune response. Furthermore, survival within active macrophages seems to be highly dependent on the efficiency of ROS reduction (Leirião *et al.* 2004; Piacenza *et al.* 2008, 2013).

By overexpressing the homologous *Tr*TRed, we have demonstrated an apparent increase in the ability of *T. rangeli* to address the intracellular reducing environment within human macrophages, where there is a high demand for trypanothione (Dumas *et al.* 1997). This study also indicates that TRed does not appear to be a pivotal molecule involved in the response of *T. rangeli* to antioxidant environments in vertebrate host cells; however, it plays a role in other parts of the parasite life cycle, such as in the insect gut or haemocytes where ROS production limits the infection and colonization of *R. prolixus* by *T. rangeli* (Cosentino-Gomes *et al.* 2014).

ROS have been traditionally viewed as a 'necessary evil' in the battle against pathogens, and their production is coupled to antioxidant responses that are important for mitigating oxidative damage in cells and tissues (Andrews, 2012). However, Paiva *et al.* (2012) and Goes *et al.* (2016) have demonstrated that oxidative stress actually enhances infection by the protozoan *T. cruzi*. In others words, *T. cruzi* seems to thrive in an oxidative environment, and antioxidant responses suppress infections by this protozoan parasite. Likewise, *Leishmania donovani* promastigotes treated with sub-lethal doses of H_2O_2 have been found to be more virulent and more potent for infecting macrophages *ex vivo* via a metabolic reconfiguration designed to replenish the NADPH pool for protection against oxidative challenges.

Therefore, future investigations will be designed to elucidate the role of ROS during *T. rangeli* infection. In this work, the role of H_2O_2 appeared to be a determinant in the host protection against parasite infection, and the inability of the parasite to respond to oxidative stress is directly related to its virulence.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ETHICAL STANDARDS

All procedures involving animals were previously approved by the Ethical Committee on Animal Use of Federal University of Santa Catarina (Reference number 23080·025618/2009-81).

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REFERENCES

Allman, S., Morand, P., Ebikeme, C., Gales, L., Biran, M., Hubert, J., Brennand, A., Mazet, M., Franconi, J. M., Michels, P. A. M., Portais, J. C., Boshart, M. and Bringaud, F. (2013). Cytosolic NADPH homeostasis in glucose-starved procyclic *Trypanosoma brucei* relies on malic enzyme and the pentose phosphate pathway fed by gluconeogenic flux. *Journal of Biological Chemistry* **288**, 18494–18505.

Andrews, N. W. (2012). Oxidative stress and intracellular infections: more iron to the fire. *Journal of Clinical Investigation* **122**, 2352–2354.

Araujo, P. R. and Teixeira, S. M. (2011). Regulatory elements involved in the post-transcriptional control of stage-specific gene expression in *Trypanosoma cruzi*: a review. *Memórias do Instituto Oswaldo Cruz* **106**, 257–266.

Aruoma, O. I., Halliwell, B., Hoey, B. M. and Butler, J. (1989). The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radical Biology & Medicine* **6**, 593–597.

Aslett, M., Aurrecoechea, C., Berriman, M., Brestelli, J., Brunk, B. P., Carrington, M. and Wang, H. (2010). TriTrypDB: a functional genomic resource for the Trypanosomatidae. *Nucleic Acids Research* **38**, D457–D462.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.

Atwood, J. A., Weatherly, D. B., Minning, T. A., Bundy, B., Cavola, C., Opperdoes, F. R., Orlando, R. and Tarleton, R. L. (2005). The *Trypanosoma cruzi* proteome. *Science* **309**, 473–476.

Azambuja, P., Ratcliffe, N. A. and Garcia, E. S. (2005). Towards an understanding of the interactions of *Trypanosoma cruzi* and *Trypanosoma rangeli* within the reduviide insect host *Rhodnius prolixus*. *Anais da Academia Brasileira de Ciências* **77**, 397–404.

Barderi, P., Campetella, O., Frasch, A. C. C., Santomé, J. A., Hellman, U., Pettersson, U. and Cazzulo, J. J. (1998). The NADP-linked glutamate dehydrogenase from *Trypanosoma cruzi*: sequence, genomic organization and expression. *Biochemical Journal* **330**, 951–958.

Barros, M. H., Bandy, B., Tahara, E. B. and Kowaltowski, A. J. (2004). Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **279**, 49883–49888.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Cannata, J. J. B., Frasch, A. C. C., Cataldi De Flombaum, M. A., Segura, E. L. and Cazzulo, J. J. (1979). Two forms of malic enzyme with different regulatory properties in *Trypanosoma cruzi*. *Biochemical Journal* **184**, 409–419.

Coura, J. R., Fernandes, O., Arboleda, M., Barrett, T. V., Carrara, N., Degrave, W. and Campbell, D. A. (1996). Human infection by *Trypanosoma rangeli* in the Brazilian Amazon. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**, 278–279.

Cosentino-Gomes, D., Russo-Abrahão, T., Fonseca-de-Souza, A. L., Ferreira, C. R., Galina, A. and Meyer-Fernandes, J. R. (2009). Modulation of *Trypanosoma rangeli* ecto-phosphatase activity by hydrogen peroxide. *Free Radical Biology and Medicine* **47**, 152–158.

Cosentino-Gomes, D., Rocco-Machado, N. and Meyer-Fernandes, J. R. (2014). *Rhodnius prolixus*: modulation of antioxidant defenses by *Trypanosoma rangeli*. *Experimental Parasitology* **145**, 118–124.

Decuypere, S., Vanaerschot, M., Brunker, K., Imamura, H., Müller, S., Khanal, B., Rijal, S., Dujardin, J. C. and Coombs, G. H. (2012). Molecular mechanisms of drug resistance in natural *Leishmania* populations vary with genetic background. *PLOS Neglected Tropical Diseases* **6**, e1514.

Díaz, M. L., Solari, A. and González, C. I. (2011). Differential expression of *Trypanosoma cruzi* I associated with clinical forms of Chagas disease: overexpression of oxidative stress proteins in acute patient isolate. *Journal of Proteomics* **74**, 1673–1682.

Dumas, C., Ouellette, M., Tovar, J., Cunningham, M. L., Fairlamb, A. H., Tamar, S., Olivier, M. and Papadopoulou, B. (1997). Disruption of the trypanothione reductase gene of *Leishmania* decreases its ability to survive oxidative stress in macrophages. *EMBO Journal* **16**, 2590–2598.

Duran-Rehbein, G. A., Vargas-Zambrano, J. C., Cue´llar, A., Puerta, C. J. and Gonzalez, J. M. (2014). Mammalian cellular culture models of *Trypanosoma cruzi* infection: a review of the published literature. *Parasite* **21**, 1–9.

Eger-Mangrich, I., De Oliveira, M. A., Grisard, E. C., De Souza, W. and Steindel, M. (2001). Interaction of *Trypanosoma rangeli* Tejera, 1920 with different cell lines *in vitro*. *Parasitology Research* **87**, 505–509.

Ewing, B., Hillier, L., Wendl, M. C. and Green, P. (1998). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research* **8**, 175–185.

Fairlamb, A. H., Blackburn, P., Ulrich, P., Chait, B. T. and Cerami, A. (1985). Trypanothione: a novel bis (glutathionyl) spermidine cofactor for glutathione reductase in trypanosomatids. *Science* **227**, 1485–1487.

Fairlamb, A. H. and Cerami, A. (1992). Metabolism and functions of trypanothione in the Kinetoplastida. *Annual Review of Microbiology* **46**, 695–729.

Ferreira, L. L., Lorenzo, M. G., Elliot, S. L. and Guarneri, A. A. (2010). A standardizable protocol for infection of *Rhodnius prolixus* with *Trypanosoma rangeli*, which mimics natural infections and reveals physiological effects of infection upon the insect. *Journal of Invertebrate Pathology* **105**, 91–97.

Finzi, J. K., Chiavegatto, C. W., Corat, K. F., Lopez, J. A., Cabrera, O. G., Mielniczki-Pereira, A. A., Colli, W., Alves, M. J. and Gadelha, F. R. (2004). *Trypanosoma cruzi* response to the oxidative stress generated by hydrogen peroxide. *Molecular and Biochemical Parasitology* **133**, 37–43.

Flohé, L. (2012). The trypanothione system and its implications in the therapy of trypanosomatid diseases. *International Journal of Medical Microbiology* **302**, 216–220.

Gallagher, S., Winston, S. E., Fuller, S. A. and Hurrell, J. G. (2008). Immunoblotting and immunodetection. *Current Protocols in Molecular Biology* **83**, III:10·8: 10·8·1–10·8·28.

Garcia, E. S., Castro, D. P., Figueiredo, M. B., Genta, F. A. and Azambuja, P. (2009). *Trypanosoma rangeli*: a new perspective for studying the modulation of immune reactions of *Rhodnius prolixus*. *Parasites & Vectors* **2**, 1–10.

Goes, G. R., Rocha, P. S., Diniz, A. R. S., Aguiar, P. H. N., Machado, C. R. and Vieira, L. Q. (2016). *Trypanosoma cruzi* needs a signal provided by reactive oxygen species to infect macrophages. *PLOS Neglected Tropical Diseases* **10**, e0004555.

Graça-Souza, A. V., Maya-Monteiro, C., Paiva-Silva, G. O., Braz, G. R. C., Paes, M. C., Sorgine, M. H., Oliveira, M. F. and Oliveira, P. L. (2006). Adaptations against heme toxicity in blood-feeding arthropods. *Insect Biochemistry and Molecular Biology* **36**, 322–335.

Gretes, M., Poole, L. B. and Karplus, P. A. (2012). Peroxiredoxins in parasites. *Antioxidants & Redox Signaling* **17**, 608–633.

Grisard, E. C., Steindel, M., Guarneri, A. A., Eger-Mangrich, I., Campbell, D. A. and Romanha, A. J. (1999). Characterization of *Trypanosoma rangeli* strains isolated in Central and South America: an overview. *Memórias do Instituto Oswaldo Cruz* **94**, 203–209.

Guhl, F. and Vallejo, G. A. (2003). *Trypanosoma* (Herpetosoma) *rangeli* Tejera, 1920: an updated review. *Memórias do Instituto Oswaldo Cruz* **98**, 435–442.

Hellemond, J. J. V., Opperdoes, F. R. and Tielens, A. G. M. (2005). The extraordinary mitochondrion and unusual citric acid cycle in *Trypanosoma brucei*. *Biochemical Society Transactions* **33**, 967–971.

Igoillo-Esteve, M., Maugeri, D., Stern, A. L., Beluardi, P. and Cazzulo, J. J. (2007). The pentose phosphate pathway in *Trypanosoma cruzi*: a potential target for the chemotherapy of Chagas disease. *Anais da Academia Brasileira de Ciências* **79**, 649–663.

Irigoín, F., Cibils, L., Comini, M. A., Wilkinson, S. R., Flohé, L. and Radi, R. (2008). Insights into the redox biology of *Trypanosoma cruzi*: Trypanothione metabolism and oxidant detoxification. *Free Radical Biology & Medicine* **45**, 733–742.

Jockers-Scherübl, M. C., Schirmer, R. H. and Krauth-Siegel, R. L. (1989). Trypanothione reductase from *Trypanosoma cruzi*. Catalytic properties of the enzyme and inhibition studies with trypanocidal compounds. *European Journal of Biochemistry* **180**, 267–272.

Kelly, J. M., Taylor, M. C., Smith, K., Hunter, K. J. and Fairlamb, A. H. (1993). Phenotype of recombinant *Leishmania donovani* and *Trypanosoma cruzi* which over-express trypanothione reductase. Sensitivity towards agents that are thought to induce oxidative stress. *European Journal of Biochemistry* **218**, 29–37.

Koerich, L. B., Emmanuelle-Machado, P., Santos, K., Grisard, E. C. and Steindel, M. (2002). Differentiation of *Trypanosoma rangeli*: high production of infective trypomastigote forms *in vitro*. *Parasitology Research* **88**, 21–25.

Krauth-Siegel, R. L. and Comini, M. A. (2008). Redox control in trypanosomatids, parasitic protozoa with trypanothione-based thiol metabolism. *Biochimica et Biophysica Acta* **1780**, 1236–1248.

https://doi.org/10.1017/pao.2016.14 Published online by Cambridge University Press

Krieger, S., Schwarz, W., Ariyanayagam, M. R., Fairlamb, A. H., Krauth-Siegel, R. L. and Clayton, C. (2000). Trypanosomes lacking trypanothione reductase are avirulent and show increased sensitivity to oxidative stress. *Molecular Microbiology* **35**, 542–552.

Leirião, P., Rodrigues, C. D., Albuquerque, S. S. and Mota, M. M. (2004). Survival of protozoan intracellular parasites in host cells. *EMBO Reports* **5**, 1142–1147.

Leroux, A. E., Maugeri, D. A., Opperdoes, F. R., Cazzulo, J. J. and Nowicki, C. (2010). Comparative studies on the biochemical properties of the malic enzymes from *Trypanosoma cruzi* and *Trypanosoma brucei*. *FEMS Microbiology Letters* **314**, 25–33.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. *Methods* **25**, 402–408.

Macleod, E. T., Maudlin, I., Darby, A. C. and Welburn, S. C. (2007). Antioxidants promote establishment of trypanosome infections in tsetse. *Parasitology* **134**, 827–831.

Maugeri, D. and Cazzulo, J. J. (2004). The pentose phosphate pathway in *Trypanosoma cruzi*. *FEMS Microbiology Letters* **234**, 117–123.

Mielniczki-Pereira, A. A., Chiavegatto, C. M., López, J. A., Colli, W., Alves, M. J. and Gadelha, F. R. (2007). *Trypanosoma cruzi* strains, Tulahuen 2 and Y, besides the difference in resistance to oxidative stress, display differential glucose-6-phosphate and 6- Phosphogluconate dehydrogenases activities. *Acta Tropica* **101**, 54–60.

Nogueira, N. P., Saraiva, F. M. S., Sultano, P. E., Cunha, P. R. B. B., Laranja, G. A. T., Justo, G. A., Sabino, K. C. C., Coelho, M. G. P., Rossini, A., Atella, G. C. and Paes, M. C. (2015). Proliferation and differentiation of *Trypanosoma cruzi* inside its vector have a new trigger: redox status. *PLoS ONE* **10**, e0116712.

Paiva, C. N. and Bozza, M. T. (2014). Are reactive oxygen species always detrimental to pathogens? *Antioxidants & Redox Signaling* **20**, 1001–1012.

Paiva, C. N., Feijó, D. F., Dutra, F. F., Carneiro, V. C., Freitas, G. B., Alves, L. S., Mesquita, J., Fortes, G. B., Figueiredo, R. T., Souza, H. S. P., Fantappié, M. R., Lannes-Vieira, J. and Bozza, M. T. (2012). Oxidative stress fuels *Trypanosoma cruzi* infection in mice. *Journal of Clinical Investigation* **122**, 2531–2542.

Peloso, E. F., Vitor, S. C., Ribeiro, L. H., Piñeyro, M. D., Robello, C. and Gadelha, F. R. (2011). Role of *Trypanosoma cruzi* peroxiredoxins in mitochondrial bioenergetics. *Journal of Bioenergetics and Biomembranes* **43**, 419–424.

Peloso, E. F., Gonçalves, C. C., Silva, T. M., Ribeiro, L. H., Piñeyro, M. D., Robello, C. and Gadelha, F. R. (2012). Tryparedoxin peroxidases and superoxide dismutases expression as well as ROS release are related to *Trypanosoma cruzi* epimastigotes growth phases. *Archives of Biochemistry and Biophysics* **520**, 117–122.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, 2002–2007.

Piacenza, L., Peluffo, G., Alvarez, M. N., Kelly, J. M., Wilkinson, S. R. and Radi, R. (2008). Peroxiredoxins play a major role in protecting *Trypanosoma cruzi* against macrophage- and endogenously-derived peroxynitrite. *Biochemical Journal*, **410**, 359–368.

Piacenza, L., Alvarez, M. N., Peluffo, G. and Radi, R. (2009*a*). Fighting the oxidative assault: the *Trypanosoma cruzi* journey to infection. *Current Opinion in Microbiology* **12**, 415–421.

Piacenza, L., Zago, M. P., Peluffo, G., Alvarez, M. N., Basombrio, M. A. and Radi, R. (2009*b*). Enzymes of the antioxidant network as novel determiners of *Trypanosoma cruzi* virulence. *International Journal for Parasitology* **39**, 1455–1464.

Piacenza, L., Peluffo, G., Alvarez, M. N., Martínez, A. and Radi, R. (2013). *Trypanosoma cruzi* antioxidant enzymes as virulence factors in Chagas disease. *Antioxidants & Redox Signaling* **19**, 723–734.

Piñeyro, M. D., Parodi-Talice, A., Arcari, T. and Robello, C. (2008). Peroxiredoxins from *Trypanosoma cruzi*: virulence factors and drug targets for treatment of Chagas disease? *Gene* **408**, 45–50.

Räz, B., Iten, M., Grether-Bühler, Y., Kaminsky, R. and Brun, R. (1997). The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. *Acta Tropica* **68**, 139–147.

Romão, P. R., Fonseca, S. G., Hothersall, J. S., Noronha-Dutra, A. A., Ferreira, S. H. and Cunha, F. Q. (1999). Glutathione protects macrophages and *Leishmania major* against nitric oxide-mediated cytotoxicity. *Parasitology* **118**, 559–566.

Romero, I. C., Saraiva, N. and Walker, J. (2005). Selective action of fluoroquinolones against intracellular amastigotes of *Leishmania* (*Viannia*) *panamensis in vitro*. *Journal of Parasitology* **91**, 1474–1479.

Romero, I., Téllez, J., Yamanaka, L. E., Steindel, M., Romanha, A. J. and Grisard, E. C. (2014). Transsulfuration is an active pathway for cysteine biosynthesis in *Trypanosoma rangeli*. *Parasites & Vectors* **7**, 197.

Rossato, F. A., Zecchin, K. G., La Guardia, P. G., Ortega, R. M., Alberici, L. C., Costa, R. A. P., Catharino, R. R., Graner, E., Castilho, R. F. and Vercesi, A. E. (2014). Fatty acid synthase inhibitors induce apoptosis in non tumorigenic melan-a cells associated with inhibition of mitochondrial respiration. *PLoS ONE* **9**, e101060.

Sambrook, J., Fritch, E. F. and Maniatis, T. (2001). *Molecular Cloning: A Laboratory Manual*, Vol. **3**, 2nd Edn. Cold Spring Harbor, New York, USA.

Schwende, H., Fitzke, E., Ambs, P. and Dieter, P. (1996). Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *Journal of Leukocyte Biology* **59**, 555–561.

Shames, S. L., Fairlamb, A. H., Cerami, A. and Walsh, C. T. (1986). Purification and characterization of trypanothione reductase from *Crithidia fasciculata* newly discovered member of the family of disulphide containing flavoprotein oxidoreductase. *Biochemical* **25**, 3519–3526.

Silva, M., Peloso, E. F., Vitor, S. C., Ribeiro, L. H. and Gadelha, F. R. (2011). O_2 consumption rates along the growth curve: new insights into *Trypanosoma cruzi* mitochondrial respiratory chain. *Journal of Bioenergetics and Biomembranes* **43**, 409–417.

Stoco, P. H., Wagner, G., Talavera-Lopez, C., Gerber, A., Zaha, A., Thompson, C. E., Bartholomeu, D. C., Lückemeyer, D. D., Bahia, D., Loreto, E., Prestes, E. B., Lima, F. M., Rodrigues-Luiz, G., Vallejo, G. A., Filho, J. F., Schenkman, S., Monteiro, K. M., Tyler, K. M., de Almeida, L. G., Ortiz, M. F., Chiurillo, M. A., de Moraes, M. H., de Cunha, O. L., Mendonça-Neto, R., Silva, R., Teixeira, S. M., Murta, S. M., Sincero, T. C., Mendes, T. A., Urmenyi, T. P., Silva, V. G., DaRocha, W. D., Andersson, B., Romanha, A. J., Steindel, M., de Vasconcelos, A. T. and Grisard, E. C. (2014). Genome of the avirulent human-infective Trypanosome - *Trypanosoma rangeli*. *PLOS Neglected Tropical Diseases* **8**, e3176.

Souza, A. V. G., Petretski, J. H., Demasi, M., Bechara, E. J. H. and Oliveira, P. L. (1997). Urate protects a blood-sucking insect against hemin-induced oxidative stress. *Free Radical Biology & Medicine* **22**, 209–214.

Taylor, M. C., Kelly, J. M., Champman, C. J., Fairlamb, A. H. and Miles, M. A. (1994). The structure, organization, and expression of the *Leishmania donovani* gene encoding trypanothione reductase. *Molecular and Biochemical Parasitology* **64**, 293–301.

Teixeira, S. M. R. and Darocha, W. D. (2003). Control of gene expression and genetic manipulation in the Trypanosomatidae. *Genetics and Molecular Research* **2**, 148–158.

Temperton, N. J., Wilkinson, S. R., Meyer, D. J. and Kelly, J. M. (1998). Overexpression of superoxide dismutase in *Trypanosoma cruzi* results in increased sensitivity to the trypanocidal agents gentian violet and benznidazole. *Molecular and Biochemical Parasitology* **96**, 167–176.

Tovar, J. and Fairlamb, A. H. (1996). Extrachromosomal, homologous expression of trypanothione reductase and its complementary mRNA in *Trypanosoma cruzi*. *Nucleic Acids Research* **24**, 2942–2949.

Tovar, J., Cunningham, M. L., Smith, A. C., Croft, S. L. and Fairlamb, A. H. (1998). Downregulation of *Leishmania donovani* trypanothione reductase by heterologous expression of a trans-dominant mutant homologue: effect on parasite intracellular survival. *Proceedings of the National Academy of Sciences* **95**, 5311–5316.

Wang, H. and Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology & Medicine* **27**, 612–616.

https://doi.org/10.1017/pao.2016.14 Published online by Cambridge University Press