Catalase expression impairs oxidative stress-mediated signalling in *Trypanosoma cruzi*

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SUMMARY

Trypanosoma cruzi is exposed to oxidative stresses during its life cycle, and amongst the strategies employed by this parasite to deal with these situations sits a peculiar trypanothione-dependent antioxidant system. Remarkably, *T. cruzi*'s antioxidant repertoire does not include catalase. In an attempt to shed light on what are the reasons by which this parasite lacks this enzyme, a *T. cruzi* cell line stably expressing catalase showed an increased resistance to hydrogen peroxide (H_2O_2) when compared with wild-type cells. Interestingly, preconditioning carried out with low concentrations of H_2O_2 led untransfected parasites to be as much resistant to this oxidant as cells expressing catalase, but did not induce the same level of increased resistance in the latter ones. Also, presence of catalase decreased trypanothione reductase and increased superoxide dismutase levels in *T. cruzi*, resulting in higher levels of residual H_2O_2 after challenge with this oxidant. Although expression of catalase contributed to elevated proliferation rates of *T. cruzi* in *Rhodnius prolixus*, it failed to induce a significant increase of parasite virulence in mice. Altogether, these results indicate that the absence of a gene encoding catalase in *T. cruzi* has played an important role in allowing this parasite to develop a shrill capacity to sense and overcome oxidative stress.

Key words: *Trypanosoma cruzi*, catalase, oxidative stress, oxidative signalling.

INTRODUCTION

Catalase, one of the most efficient enzymes known, probably evolved when an oxygenated atmosphere first appeared on Earth, requiring organisms to handle and neutralize toxic oxygen radical by-products. This antioxidant enzyme, found in nearly all aerobic organisms, decomposes hydrogen peroxide (H_2O_2) into water and oxygen (Chelikani *et al.* 2004). H_2O_2 represents a two-electron reduction state of molecular oxygen and originates mainly from the enzymatic dismutation catalysed by superoxide dismutase (SOD) isoforms. Despite its low reactivity, H_2O_2 can easily diffuse across biological membranes and generate hydroxyl radicals ($^{\bullet}OH$), which can react with biomolecules and cause damage (Novo and Parola, 2008; Winterbourn, 2008).

Surprisingly, a catalase homologous sequence has not been identified in *Trypanosoma cruzi* genome (El-Sayed *et al.* 2005). *Trypanosoma cruzi* is the etiologic agent of Chagas Disease, which is endemic in Latin America and is now getting disseminated to non-endemic areas due to human emigration (Hotez *et al.* 2012). Given the particularities of its complex life cycle, this parasite is certainly required to deal with situations of oxidative stress (Piacenza *et al.* 2009*a*). In fact, hydroperoxide antioxidant defences in *T. cruzi* consist of a sophisticated system of linked pathways in which reducing equivalents from NADPH – mostly derived from the pentose phosphate pathway – are delivered to a variety of enzymatic detoxification systems through the dithiol trypanothione [$T(SH)_2$, N1, N8-bisglutathionylspermidine] and the thioredoxin homologue tryparedoxin (TXN) or glutathione (Irigoín *et al.* 2008).

Five distinct peroxidases have been identified in *T. cruzi*, which differ in their subcellular location and substrate specificity. Two of these, namely cytosolic (cTXNPx) and mitochondrial (mTXNPx) tryparedoxin peroxidases, detoxify H_2O_2, peroxynitrite (Piñeyro *et al.* 2011), and small-chain organic

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hydroperoxides (Wilkinson *et al*. 2000). Another peroxidase, the ascorbate-dependent haeme-peroxidase (APX), is located in endoplasmic reticulum and confers resistance against H_2O_2 challenge, using ascorbate as reducing substrate. Indeed, parasites overexpressing APX display an increased resistance to exogenous H_2O_2 (Wilkinson *et al*. 2002*a*). Finally, the two remaining peroxidases show extensive similarity to glutathione-dependent peroxidases: Glutathione-dependent peroxidase I (GPXI) is present in both cytosol and glycosome, and the glutathione-dependent peroxidase II (GPXI) is found in the endoplasmic reticulum. GPXI and GPXII seem to play specific roles in cellular oxidative defence since both are able to detoxify fatty acid and phospholipid hydroperoxides, although they do not exhibit activity against H_2O_2 (Wilkinson *et al*. 2002*b*, *c*). In addition, *T. cruzi* harbours a repertoire of four iron superoxide dismutase (Fe-SODs) – which are located in different subcellular compartments – to detoxify $O_2^{\bullet-}$ (Villagrán *et al*. 2005; Mateo *et al*. 2008).

In order to establish infection in the vertebrate host, *T. cruzi* metacyclic trypomastigotes must invade macrophages and overcome the highly oxidative conditions generated inside the phagosome. Several antioxidant enzymes, including the mitochondrial Fe-SOD, mTXNPx, cTXNPx, trypanothione synthetase and APX, are upregulated during the transformation of the insect-derived, non-infective epimastigotes into the infective metacyclic trypomastigotes. These biochemical changes may pre-adapt metacyclic forms, allowing them to develop the ability of detoxify reactive oxygen and nitrogen species (ROS and RNS, respectively) generated by macrophages during the *T. cruzi*-mammalian host–cell interactions (Atwood *et al*. 2005; Piacenza *et al*. 2009*b*).

Given the importance of *T. cruzi* antioxidant system regarding the infection process, and also considering that kinetoplastids which parasitize insects do have catalase, the lack of this antioxidant enzyme in the aetiologic agent of Chagas Disease is puzzling. In this work, we generated a *T. cruzi* cell line expressing catalase from *Escherichia coli*, and investigated how this transfected parasite responds to oxidative stress. We found that catalase heterologous expression in *T. cruzi* affects both the oxidative stress-induced signalling and the parasite fitness in different oxidant environments.

MATERIALS AND METHODS

Ethics statement

This study was conducted in strict accordance with recommendations found in Guide for Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br/) and in Federal Law 11.794 (8 October 2008). All animals were handled in absolute conformity with good animal practice as defined by the Internal Ethics Committee in Animal Experimentation (CETEA) from Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil (protocol number 214/11). This project was also approved by the National Technical Biosafety Commission (CTNBio) under the process number 01200.003883/97-02.

Plasmids

In order to generate a *T. cruzi* cell line that stably expresses catalase, the gene *katE* from *E. coli* (GenBank: M55161.1) was inserted into the integrative expression vector *pROCK_HYGRO* (DaRocha *et al*. 2004). *katE* encodes catalase – also known as hydroperoxidase II (HPII) –, which is very similar to eukaryotic catalases (Von Ossowski *et al*. 1991). *katE* sequence was amplified through polymerase chain reaction (PCR) using the following primers: 5′– TCT AGA ATG TCG CAA CAT AAC GAA AAG AAC C–3′ (forward) and 5′–CTC GAG TCA GGC AGG AAT TTT GTC AAT CTT AG –3′ (reverse), and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The resulting pGEM-T_*katE* plasmid was then digested using *Xba*I and *Xho*I to release double-digested *katE* fragment, which was then ligated to *pROCK_HYGRO* – previously incubated with the same restriction enzymes – to allow the generation of *pROCK_katE_HYGRO* vector.

Parasite growth and transfection

Trypanosoma cruzi strain CL Brener was used for all experiments. Epimastigotes were grown in liver infusion tryptose (LIT) medium (pH 7·4) supplemented with 10% fetal bovine serum (FBS, GibcoBRL, Invitrogen, Carlsbad, CA, USA) and 1% streptomycin/penicillin, at 28 °C. Parasite transfection was performed using electroporation as described elsewhere (DaRocha *et al*. 2004). Transfected parasites were cultured for 6 weeks in the presence of hygromycin (200 μg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) to select parasites having *pROCK_katE_HYGRO* stably incorporated. Cells overexpressing MTH, or expressing heterologous *MutT*, were previously generated by our group (Aguiar *et al*. 2013). Cells were cultured until logarithmic growth phase before all experiments. Trypomastigotes were obtained from the supernatant from monolayers of infected LLCMK2 cell cultures growth in 2% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine-supplemented Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich). Released trypomastigotes were purified by centrifuging culture

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supernatants at 2000 ***g***, for 5 min. The undisturbed pellet was incubated for 2 h at 37 °C, and motile trypomastigotes were collected from the supernatant.

RNA purification and RT–PCR

Total RNA was purified from 1×10^8 *T. cruzi* epimastigotes using the TRIzol (Invitrogen, Life technologies, CA, USA) method, followed by the treatment with DNAse (Invitrogen) for removal of contaminant DNA, according to the manufacturer's instructions. Purified RNA was then used to perform a cDNA synthesis reaction with 500 ng oligo (dT), using the SuperScript III First-strand Synthesis System for RT–PCR (Invitrogen). The subsequent specific amplification of *katE* fragment was performed using the primers previously described. Negative control was processed in the same conditions as for the other samples, except for the absence of reverse transcriptase.

Total peroxidase activity

Total peroxidase activity was measured in the supernatant of 2×10^7 parasites lysed in 50 μL of ice-cold 50 mM NaCl, 20 mM Tris–HCl (pH 7·4) containing 1% Triton X-100. The activity was determined after 30 min of incubation with 2 μM Amplex Red (Invitrogen) and 2 mM H_2O_2, at 25 °C (Augusto *et al*. 2015), using the UV–Vis–fluorescent microplate reader SpectraMax M3 Reader® (Molecular Devices, Sunnyvale, CA, USA), operating at 530 nm (excitation) and 590 nm (emission).

Parasite cloning

Trypanosoma cruzi trypomastigotes transfected with *pROCK_katE_HYGRO* were grown in solid blood agar medium [48·4% LIT, 0·75% low melting agarose and 2·5% defibrinated rabbit blood, and inactivated complement (Gomes *et al*. 1991)] supplemented with 200 μg mL^{-1} hygromycin (Sigma-Aldrich). After 30 days of selection at 28 °C, plates were examined, and six clones were selected from different plates, which were grown in liquid medium for further characterization.

Epimastigote growth and survival curves

Parasite growth curves were initiated at the density of 1×10^7 cells mL^{-1}. In a daily basis, aliquots were withdrawn and cells were counted until the stationary phase was reached. To test the resistance to H_2O_2, parasite cultures containing 1×10^7 cells mL^{-1} were treated with 75 or 100 μM H_2O_2 in phosphate-buffered saline (PBS; pH 7·4). After 20 min, parasites were centrifuged and resuspended in LIT medium. The number of cells was determined after 72 h. Alternatively, survival curves were performed by incubating the cells in the presence of 25 μM H_2O_2 – i.e. H_2O_2-preconditioning – for 24 or 96 h before subsequent treatment with H_2O_2. Results were expressed as the percentage of growth in relation to control. In these experiments, the cell number was determined through a cytometry chamber using erythrosine vital stain to differentiate living from dead cells. Three independent experiments were performed in triplicate.

H_2O_2 detoxyfication

Hydrogen peroxide detoxification was determined by incubating 1×10^7 parasites mL^{-1} in PBS (pH 7·4), in the presence of 1 U mL^{-1} horseradish peroxidase and 2 μM AmplexRed (Invitrogen). After 30 min, parasites were centrifuged and the resulting fluorescence was measured in the supernatants using the SpectraMax M3 Reader® (Molecular Devices), operating at 530 nm (excitation) and 590 nm (emission). Three independent experiments were carried out in triplicates.

Preparation of antibodies, protein extracts and Western blotting analysis

Anti-*T. cruzi* iron superoxide dismutase B (anti-Fe-SOD B) and anti-*T. cruzi* trypanothione reductase (anti-TR) polyclonal antibodies were obtained as previously described (Piñeyro *et al*. 2008; Peloso *et al*. 2012). For Western blot analysis, parasites (1×10^8 cells mL^{-1}) were incubated in the absence or in the presence of H_2O_2 (100 μM, PBS) for 30 min. Cells were then harvested by centrifugation (2500 ***g***, 10 min), resuspended in 80 μL of PBS containing 1 mM $MgCl_2$, and mixed with an equal volume of lysis buffer [50 mM Tris–HCl (pH 7·4), 1% Tween 20, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 0·1 mM PMSF, aprotinin 1 mg mL^{-1}; leupeptin 1 mg mL^{-1}]. The resulting suspension was sonicated in a Sonopuls Ultrasonic Homogenizer (Bandelin, Berlin, Germany) for 10 cycles of 1 s, with an interval of 1 s and 30% max amperage. The material was kept for 2 h on ice, and then centrifuged (13 000 ***g***, 4 °C, 15 min). Protein concentration was determined using the Bradford method (Bradford, 1976). An equal volume of loading buffer was added to the protein extract [100 mM Tris–HCl, (pH 6·8), 4% SDS, 0·02% bromophenol blue, 20% glycerol, 200 mM β-mercaptoethanol], and samples were heated at 96 °C for 4 min. Protein extracts (30 μg) were analysed by SDS–PAGE and electroblotted onto a polyvinylidene difluoride membrane using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Irvine, CA, USA). Membranes were blocked by incubation with 5% instant non-fat dry milk in PBS containing 0·05% Tween 20 (PBS-T) for 1 h, washed and incubated for 2 h in the presence of antibodies. After washing the membranes with

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PBS-T (3 × 10 min each), incubation with HRP-linked anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) was carried out at room temperature, for 1 h, at the end of which membranes were washed with PBS (3 × 10 min each) (Morales *et al*. 2009; Peloso *et al*. 2011). Bands were revealed using ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences, Issaquah, WA, USA). Data were obtained from three independent experiments, analysed using the ImageJ software (National Institute of Health, Bethesda, MD, USA), and normalized by anti-tubulin signal (T9026, Sigma-Aldrich), used as loading control.

In vitro *infection*

Four to 8 weeks old male and female C57BL/6 mice were obtained from the CEBIO (Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil), kept in controlled dark–light cycle and temperature conditions, and fed *ad libitum* with a commercial diet for rodents (Labina, Purina, SP, Brazil). Peritoneal macrophages were isolated 4 days after the injection of 2 mL of thioglycollate 3% medium (BD, Le Pont de Claix, France) into the peritoneal cavity. Mice were then euthanized, and the peritoneal cells were harvested by repeated cycles of aspiration and re-injection with 10 mL of cold PBS using a 10 mL-syringe with a 24 G needle. More than 80% of cells harvested were macrophages. Cells were centrifuged at 1500 ***g*** for 10 min at 4 °C, and resuspended in DMEM supplemented with 10% FBS (Cultilab, Campinas, SP, Brazil), 1% penicillin/streptomycin and 2 mM glutamine. Macrophages were counted in a hemocytometer prior to seeding 5×10^5 cells into each well from a 24-well plate and incubated with 5% CO_2 at 37 °C, for 2 h. Purified parasites were diluted in DMEM medium, and infection was performed for 2 h. In some experiments catalase–polyethylene glycol (CAT, 40 U well^{-1}, Sigma-Aldrich) was added to the cells 2 h before the infection. Immediately after macrophage infection, cells were washed four times with PBS (pH 7·4) to remove extracellular parasites. Cells were reincubated with medium for 72 h before fixation with methanol. Coverslips with attached macrophages were stained with Panótico stain (Laborclin, Pinhais, PR, Brazil), and a minimum of 300 macrophages per coverslip was counted. Results were expressed as infection index (% infected macrophages × number of amastigotes/total number of macrophages) and the values correspond to means ± S.D. of three independent experiments.

Mouse infection

Trypanosoma cruzi trypomastigotes were maintained by blood passage in IFN-γ KO mice (Dalton *et al*. 1993) every 7 days, and obtained from heparinized blood, counted, and used to infect naïve animals. Experimental infection was performed by injection of 1×10^6 trypomastigotes into the peritoneal cavity. Parasitaemia was assessed by counting the trypomastigotes in 5 μL of tail vein blood from the third day post infection until parasites became undetectable. The number of parasites per mL of blood was calculated as previously described (Brener, 1962).

Insect infection

Fifth-stage nymphs of *Rhodnius prolixus* were reared at 26 ± 1 °C and relative humidity of 65 ± 5%, with natural illumination. They were fed on chicken and mice anesthetized with an intraperitoneal injection of a ketamine (150 mg kg^{-1}; Cristália, Itapira, SP, Brazil)/xylazine (10 mg kg^{-1}; Bayer, São Paulo, SP, Brazil) mixture. Experimental infections were carried out using uninfected insects, which were artificially fed with heat-inactivated rabbit blood containing 1×10^7 epimastigotes mL^{-1} (Garcia *et al*., 1975). Control insects were fed on the same heat-inactivated rabbit blood at the same conditions, except for the parasite presence (Elliot *et al*. 2015). In order to obtain the rectum, each infected triatomine was dissected 30 days after feeding with blood containing parasites. Samples were homogenized in PBS and examined by direct microscopic observation. *Trypanosoma cruzi* population density in the intestine was quantified using a Neubauer chamber. Three independent experiments were performed.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA). Data are presented as mean ± S.D.. Results were analysed for significant differences using either ANOVA or Student's *t*-test, as indicated in figures legends. Minimum significance level was set at $P < 0{\cdot}05$.

RESULTS

Catalase-expressing T. cruzi *cell line*

In order to better understand how *T. cruzi* handles oxidative stress, and the reasons why this parasite lacks catalase, we generated a *T. cruzi* cell line, which stably maintains the expression of catalase from *E. coli* (KatE), which is encoded by the gene *katE*. After effectively cloning *katE* into the expression vector pROCK_HYGRO, we transfected and selected a *T. cruzi* clone capable of growing in the presence of hygromycin. Then, aiming to verify if the selected *T. cruzi* cell line was indeed able to transcript *katE*, we isolated total RNA from both

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transfected (*T. cruzi*^{katE}) and untransfected (*T. cruzi*^{WT}) epimastigotes, and performed a RT–PCR. The expected 2262-bp DNA fragment of *katE* was successfully amplified from the cDNA of *T. cruzi*^{katE} (Fig. 1A, *T. cruzi*^{katE}/+RT); in addition, the expression of KatE in *T. cruzi*^{katE} population, as well as in clones #1 and #2, was further confirmed by Western blotting (Fig. 1B, lanes 2–4). We then sought to verify whether or not KatE was active in the parasite environment through the measurement of total peroxidase activity present in the supernatant of *T. cruzi*^{katE} whole cell lysates. We then were able to verify that transfected parasites exhibited significant higher levels of peroxidase activity when compared with untransfected ones, demonstrating that KatE is biologically active in *T. cruzi* (Fig. 1C).

T. cruzi^{katE} *exhibit similar growth rates to* T. cruzi^{WT}*, but shows increased resistance to* H_2O_2

Next, we determined the *in vitro* growth rate of *T. cruzi*^{katE} epimastigotes in order to verify if the expression of *katE* could exert an impact in cellular replication. We found that such expression did not change the parasite *in vitro* proliferation rates under standard culture conditions as *T. cruzi*^{katE} exhibits a similar growth rate to *T. cruzi*^{WT} (Fig. 2A). However, we observed that *T. cruzi*^{katE} does present increased survival rates when compared with those from *T. cruzi*^{WT} upon exposure to increased H_2O_2 concentrations (Fig. 2B). This indicates that parasites expressing *katE* are more resistant to oxidative damage induced by H_2O_2 than those ones in which this enzyme is not found.

Expression of catalase abolishes H_2O_2*-mediated resistance in* T. cruzi

Previous studies have shown that wild-type *T. cruzi* cells treated with sub-lethal concentrations of H_2O_2 become preconditioned, and present an increased resistance to higher concentrations of this ROS, showing that H_2O_2 is capable of mediating improved resistance against oxidant insults in this parasite (Finzi *et al*. 2004). In this sense, we sought to determine if the expression of *katE* would abolish the adaptation mediated by H_2O_2 in *T. cruzi*, since this enzyme decomposes this mediator molecule into water and oxygen. First, we verified that, under our conditions, 25 μM H_2O_2 acts as a sub-lethal concentration capable of preconditioning and increasing survival rates in *T. cruzi*^{WT} and *T. cruzi*^{katE} further exposed to both 75 and 100 μM H_2O_2 (Fig. 3A). Next, we observed that, when the oxidative challenge was carried out with 75 μM H_2O_2, the presence of KatE did not prevent increased survival rates in H_2O_2-preconditoned *T. cruzi*^{katE} when compared with those observed in H_2O_2-preconditoned

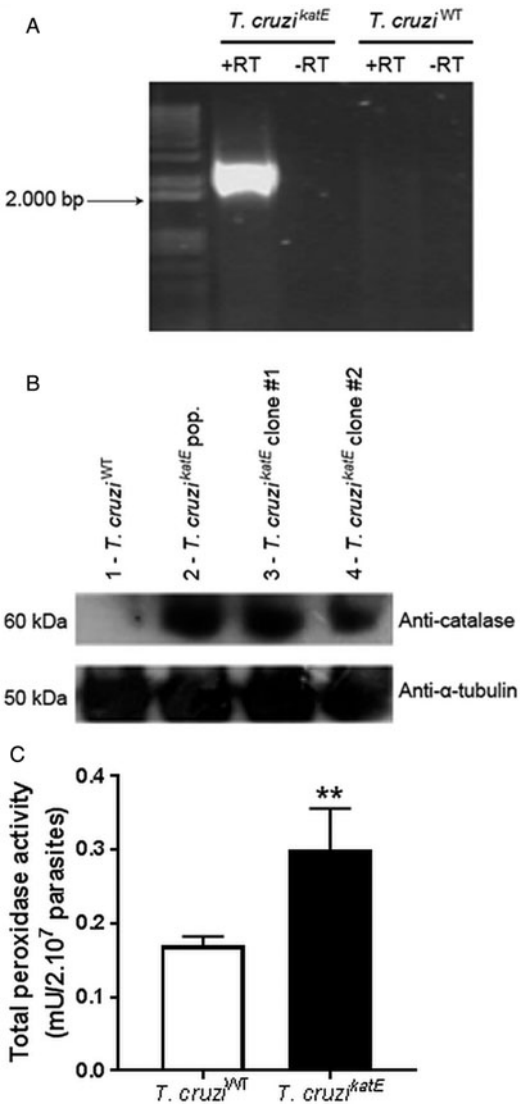

Fig. 1. Expression of *katE* in *T. cruzi*. (A) RT–PCR products obtained from total RNA extracted from *T. cruzi*^{WT} or *T. cruzi*^{katE}, with (+RT) or without (+RT) reverse transcriptase; (B) Catalase blot in extracts from *T. cruzi*^{WT}, from a selected population (*T. cruzi*^{katE} pop.), and from two clones of *T. cruzi*^{katE} epimastigotes (*T. cruzi*^{katE} clone #1 and *T. cruzi*^{katE} clone #2). Tubulin was used as loading control; (C) Total peroxidase activity from soluble extracts of 2×10^7 *T. cruzi*^{WT} or *T. cruzi*^{katE} parasites. Data are presented as mean ± S.D.. $^{**}P < 0{\cdot}01$ *vs* *T. cruzi*^{WT} (Unpaired Student's *t*-test, $n = 3$).

T. cruzi^{WT} (Fig. 3A, 75 μM H_2O_2, +H_2O_2 pretreat., *T. cruzi*^{katE} *vs* *T. cruzi*^{WT}). However, when the oxidative challenge was conducted with 100 μM H_2O_2, H_2O_2-preconditoned *T. cruzi*^{katE} parasites exhibited a similar survival rate when compared with H_2O_2-preconditoned *T. cruzi*^{WT} cells (Fig. 3A, 100 μM H_2O_2, +H_2O_2 pretreat., *T. cruzi*^{katE} *vs* *T. cruzi*^{WT}), despite having elevated acquired ability in handling oxidative insults (Fig. 1C). This observation demonstrates the existence of an oxidative condition in which H_2O_2-preconditoned *T. cruzi*^{WT} is able to present the same level of survival

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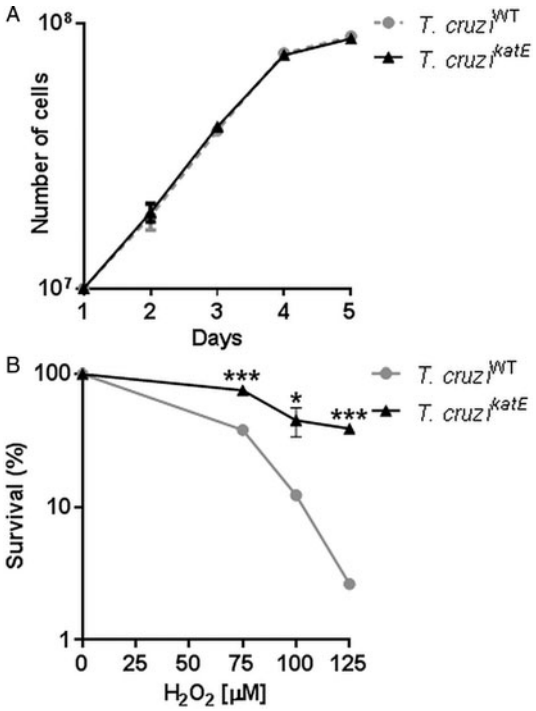

Fig. 2. KatE promotes increased resistance to H_2O_2. (A) *T. cruzi*^{WT} and *T. cruzi*^{katE} epimastigotes were grown in LIT medium, and the number of cells was determined every day until parasites reach the stationary phase. No significant difference was found between the cells (unpaired Student's *t*-test, $n = 3$); (B) *T. cruzi*^{WT} and *T. cruzi*^{katE} epimastigotes were incubated with or without 75, 100 and 125 μM H_2O_2 for 20 min in PBS, centrifuged, and resuspended in LIT medium. After 72 h at 28 °C, the percentage of live cells was determined. Data are presented as mean ± S.D.. $*P < 0{\cdot}05$ and $***P < 0{\cdot}001$ *vs* *T. cruzi*^{WT} (Unpaired Student's *t*-test, $n = 3$).

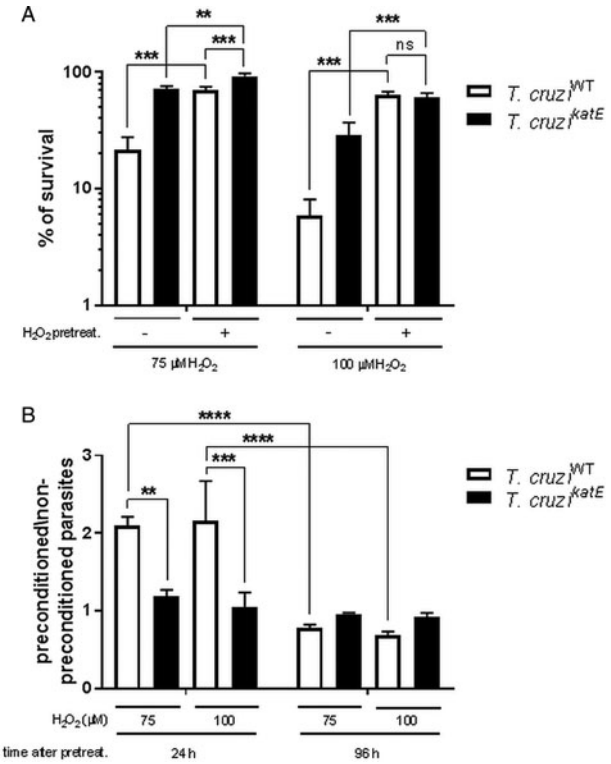

Fig. 3. *KatE* expression impairs *T. cruzi's* adaptation to oxidative stress. (A) *T. cruzi*^{WT} and *T. cruzi*^{katE} epimastigotes preconditioned or not with 25 μM H_2O_2 for 24 h were challenged with 75 or 100 μM H_2O_2 for 20 min in PBS (pH 7·4), and then were allowed to grow in LIT medium. After 72 h, cell viability was determined. Percentages of survival were determined in relation to non-preconditioned cells. Data are presented as mean ± S.D.. $**P < 0{\cdot}005$ and $***P < 0{\cdot}001$; ns = no statistical significance (one-way ANOVA/Bonferroni, $n = 3$); (B) *T. cruzi*^{WT} and *T. cruzi*^{katE} epimastigotes were preconditioned or not with 25 μM H_2O_2 for either 24 or 96 h before being exposed to higher concentrations of H_2O_2 for 20 min. Cells were plated, and after 72 h, viability was determined. Results represent the ratio between the number of preconditioned and non-preconditioned parasites. Data are presented as mean ± S.D.. $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ and $****P < 0{\cdot}0001$ (One-way ANOVA/Bonferroni, $n = 3$).

of H_2O_2-preconditoned *T. cruzi*^{katE}, suggesting a concentration-dependent role of H_2O_2 in cellular adaptation of wild-type *T. cruzi* in response to oxidative stress. Then, in order to confirm that preconditioning with H_2O_2 is able to induce an adaptive advantage, and not simply acts selecting the most resistant cells to oxidation, we decided to pretreat *T. cruzi* cells with 25 μM H_2O_2 and challenge the cells (with either 75 or 100 μM H_2O_2) 24 or 96 h after the final of pretreatment. We then observed that, after 24 h, the ratios between preconditioned and non-preconditioned alive cells was higher in *T. cruzi*^{WT} when compared with cells expressing *katE* (Fig. 3B, 24 h), showing that the absence of catalase increases the cellular capacity of *T. cruzi* to adapt to oxidant insults. We further verified that, after 96 h, *T. cruzi*^{WT} cells were unable to exhibit the adaptive advantage verified in earlier times after H_2O_2 pretreatment (Fig. 3, open bars). Altogether, these results demonstrate that the presence of *katE* impairs the adaptive response triggered by oxidative stress in *T. cruzi*, and the oxidative signalling promoted by H_2O_2 is transient as viability assessment after 96 h did not exhibit increased survival rates observed in *T. cruzi*^{WT} after 24 h (Fig. 3B).

KatE impairs T. cruzi's *ability in maintaining basal* H_2O_2 *levels*

Since both resistance and adaptation to oxidative stress generated by H_2O_2 differ between *T. cruzi*^{WT} and *T. cruzi*^{katE} (Figs 2B and 3), we decided to check whether or not these dissimilarities could be related to changes in cellular ability to decompose H_2O_2. We then assessed the capacity of transfected and untransfected parasites in detoxifying H_2O_2 by measuring the amount of H_2O_2 present in the systems after challenging intact cells,

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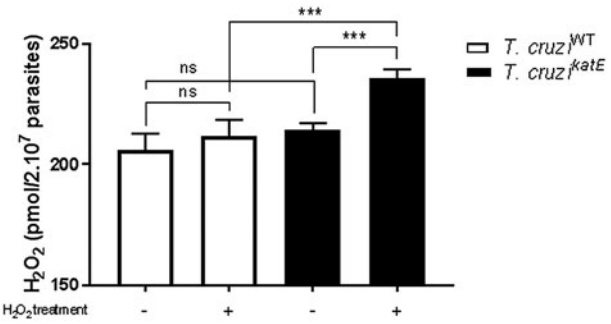

Fig. 4. Maintenance of basal H_2O_2 levels in *T. cruzi* is hindered by *katE* expression. H_2O_2 levels were measured by incubating 2×10^7 *T. cruzi*^{WT} and *T. cruzi*^{katE} epimastigotes mL^{-1} in PBS in the presence of 1 U mL^{-1} horseradish peroxidase and 2 μM Amplex Red for 30 min ($-H_2O_2$ treatment). Alternatively, parasites were treated with 100 μM H_2O_2 for 20 min in PBS before the assay ($+H_2O_2$ treatment). Parasites were centrifuged and the resulting fluorescence was measured from supernatants using a fluorescence spectrophotometer operating at 530 nm (excitation) and 590 nm (emission). Data are presented as mean ± S.D.. ***$P < 0{\cdot}001$; ns = no statistical significance (One-way ANOVA/Bonferroni, $n = 3$).

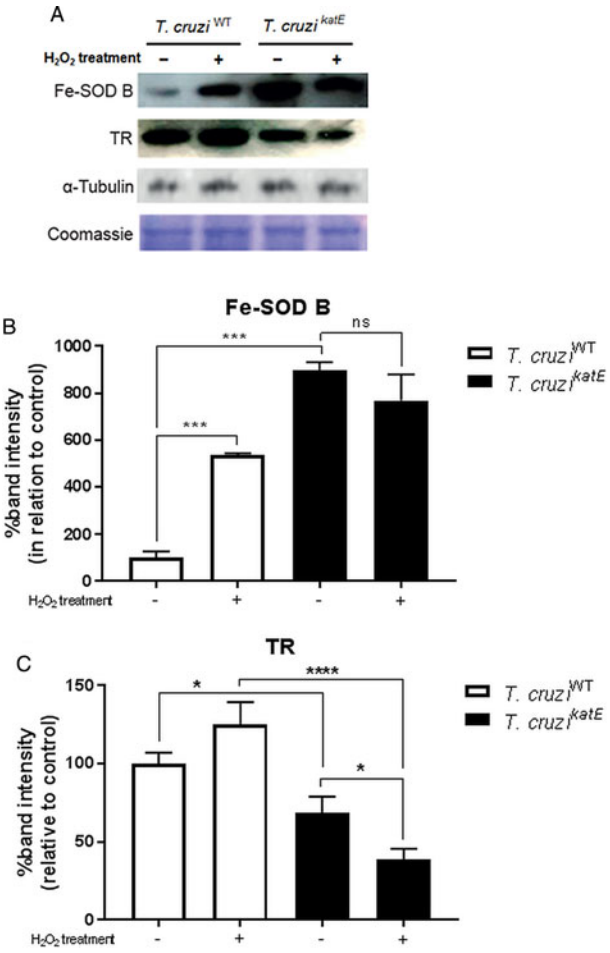

Fig. 5. *KatE* expression increases Fe-SOD B and reduces trypanothione reductase levels in *T. cruzi*. (A) Western blots of extracts obtained from *T. cruzi*^{WT} and *T. cruzi*^{katE} with or without 100 μM H_2O_2 treatment for 20 min. Blots were probed with anti-iron superoxide dismutase B (SOD B), trypanothione reductase (TR), or anti-α-tubulin antibodies and a similar gel stained with Coomassie Brilliant blue. In (B) and (C), each band had its signal normalized by the *T.cruzi*^{WT}/$-H_2O_2$ band (set as 100). Data are presented as mean ± S.D.. For (B) and (C), *$P < 0{\cdot}05$, ***$P < 0{\cdot}001$ and ****$P < 0{\cdot}0001$; ns = no statistical significance (One-way ANOVA/Bonferroni, $n = 3$).

with this oxidant. We found that both non-H_2O_2-treated *T. cruzi*^{WT} and *T. cruzi*^{katE} showed similar residual H_2O_2 levels (Fig. 4). However, *T. cruzi*^{katE} exhibited increased levels of residual H_2O_2 after the oxidant challenge compared with *T. cruzi*^{WT} (Fig. 4, *T. cruzi*^{katE}/$+H_2O_2$ treatment *vs* *T. cruzi*^{WT}/$+H_2O_2$ treatment). Interestingly, these results elicit the fact that *katE* expression not only fails to provide *T. cruzi* cells an additional ability to decompose H_2O_2, but also impairs the parasite capacity to detoxify H_2O_2 (Fig. 4). Altogether, these data suggest that cellular H_2O_2 are maintained at basal levels in *T. cruzi* (Fig. 4, empty bars; *T. cruzi*^{katE}/$-H_2O_2$ treatment), possibly due to the physiological requirement of this ROS in adequate quantities to properly signal cellular adaptation when the parasite faces oxidative environments.

Catalase expression in T. cruzi *alters the levels of antioxidant enzymes*

The unexpected finding that *katE* expression hinders *T. cruzi*'s ability in maintaining physiological levels of H_2O_2 (Fig. 4), led us to investigate if other enzymes involved in antioxidant defence could be involved in the decreased capacity in detoxifying this oxidant. We then sought to determine the levels of Fe-SOD B and trypanothione reductase (TR) in both transfected and untransfected *T. cruzi* cells, challenged or not with 100 µM H_2O_2 for 20 min. We verified that, when there is no exposure to H_2O_2, *T. cruzi*^{katE} showed increased Fe-SOD B levels in relation to *T. cruzi*^{WT} (Fig. 5A and 5B, *T. cruzi*^{katE}/$-H_2O_2$ treatment *vs* *T. cruzi*^{WT}/$-H_2O_2$ treatment). Interestingly, we also observed that challenge with H_2O_2 increased Fe-SOD B levels in *T. cruzi*^{WT} parasites in relation to wild-type unchallenged cells (Fig. 5B, open bars), but did not alter the levels of this enzyme in *T. cruzi*^{katE} (Fig. 5B, solid bars). Moreover, we verified that unchallenged *T. cruzi*^{katE} exhibited a reduced expression of TR when compared with unchallenged *T. cruzi*^{WT} (Fig. 5C, *T. cruzi*^{katE}/$-H_2O_2$ treatment *vs* *T. cruzi*^{WT}/$-H_2O_2$ treatment), indicating that *katE* expression in *T. cruzi* decreases TR levels in this parasite. Interestingly, while H_2O_2-challenged *T. cruzi*^{WT} cells showed a tendency to exhibit increased TR levels (Fig. 5C, empty bars), H_2O_2-challenged *T. cruzi*^{katE} parasites did exhibit reduced levels of this enzyme (Fig. 5C, solid bars),

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indicating that the presence of KatE in fact interferes in proper antioxidant signalling in *T. cruzi*.

The role of catalase in the in vitro *infection of macrophages by* T. cruzi

We showed that oxidative signalling, which is required to prepare *T. cruzi* parasites to deal with host conditions, is impaired by the presence of KatE (Figs 3 and 4). Therefore, we asked if the expression of *KatE* could, in addition, interfere with both infectivity and growth of *T. cruzi* inside macrophages. In fact, several studies have associated *T. cruzi*-induced oxidative stress during macrophage infection with stimulation of *T. cruzi* infection (Nogueira and Cohn, 1978; Nathan *et al*. 1979; Nogueira *et al*. 2011; Paiva *et al*. 2012; Nogueira *et al*. 2015;). Thus, we used *T. cruzi*^{WT} and *T. cruzi*^{katE} trypomastigotes to infect macrophages previously treated – or not – with commercially-available catalase in order to suppress the cellular availability of H_2O_2, since this ROS has proven to be a molecular mediator which triggers antioxidant signalling in *T. cruzi*. Therefore, we promoted infection using *T. cruzi* harbouring the gene *mutT* from *E. coli* (*T. cruzi*^{mutT}), which encodes *MutT*, a protein previously shown to increase *T. cruzi* resistance to oxidative stress (Aguiar *et al*. 2013). *MutT* – or MTH1 in *T. cruzi* – hydrolyses 8-oxo-dGTP in the nucleotide pool, converting it to the monophosphate form so that it cannot be incorporated into DNA by polymerases (Nakabeppu *et al*. 2006; Setoyama *et al*. 2011). Interestingly, both *T. cruzi*^{katE} and *T. cruzi*^{mutT} parasites display increased proliferation capacity when compared with *T. cruzi*^{WT} in macrophages without catalase supplementation (Fig. 6A). Added-catalase decreases macrophage infection capacity in *T. cruzi*^{mutT} (*T. cruzi*^{mutT} + CAT *vs* *T. cruzi*^{mutT} −CAT; $P = 0{\cdot}006$, unpaired Student's *t*-test) but does not alter *T. cruzi*^{katE} infectivity capacity. Altogether, these observations demonstrate that *T. cruzi*^{KatE} parasites exhibit the maximum infection index amongst all the cells studied, i.e. *T. cruzi*^{WT}, *T. cruzi*^{KatE} and *T. cruzi*^{mutT} (Fig. 6A); in effect, KatE plays a key role in increasing the infection by 300% in relation to the wild-type cell (Fig. 6A), in a manner independent of the presence of the oxidative mediator molecule as supplementation with commercially available catalase does not alter the infection index (*T. cruzi*^{katE}/+CAT *vs* *T. cruzi*^{katE}/−CAT, $P = 0.971$, unpaired Student's *t*-test). However, as discussed above, the same supplementation plays a crucial role in decreasing the infection index of *T. cruzi* expressing *MutT* (Fig. 6, *T. cruzi*^{mutT}/+CAT *vs* *T. cruzi*^{mutT}/−CAT), eliciting the fact that parasite infectivity is modulated by *MutT* in a manner dependent on the presence of oxidants. Since *MutT* expression is able, *per se*, to promote a two-fold increase in *T. cruzi*'s infectivity (Fig. 6A, *T. cruzi*^{mutT} *vs* *T. cruzi*^{WT}), we conclude that oxidative environment of infected macrophages increases *T. cruzi* infectivity through the parasite's ability to properly signal antioxidant response in consequence of oxidative stress.

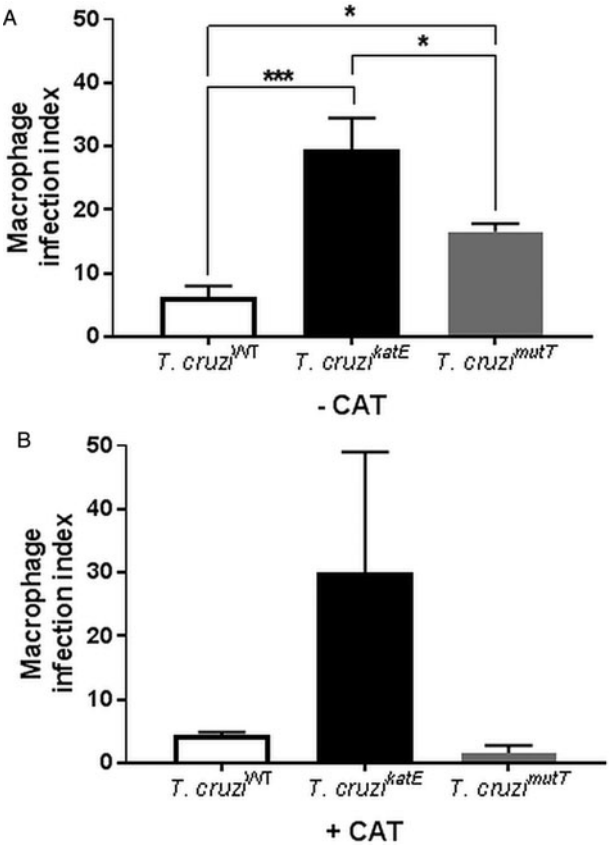

Fig. 6. Treatment with catalase reduces the proliferation of *T. cruzi*^{mutT} in macrophages. (A) Inflammatory macrophages obtained from the peritoneal cavity of C57BL/6 mice were incubated with *T. cruzi* bloodstream trypomastigotes – *T. cruzi*^{WT}, *T. cruzi*^{katE} or *T. cruzi*^{mutT}; (B) Alternatively, macrophages were treated with catalase-polyethylene glycol (CAT) 2 h before the infection (+CAT). Cells were washed to remove extracellular parasites and fixed after 72 h. Slides were stained and counted to determine the number of parasites per macrophage. A minimum of 200 macrophages were counted per group. Data are presented as mean ± S.D.. $^*P < 0{\cdot}05$ and $^{***}P < 0{\cdot}001$ (One-way ANOVA/ Bonferroni, $n = 3$).

T. cruzi^{katE} *exhibits increased parasitaemia compared with* T. cruzi^{WT}

Once determined that *T. cruzi*^{katE} exhibits an increased macrophages infectivity in a manner independent of the presence of the mediator molecule H_2O_2, as well as ability to trigger antioxidant response through *MutT* plays a key role in the macrophage infection process, we sought to examine the *T. cruzi*^{WT}, *T. cruzi*^{katE} and *T. cruzi*^{MTH} – a *T. cruzi*

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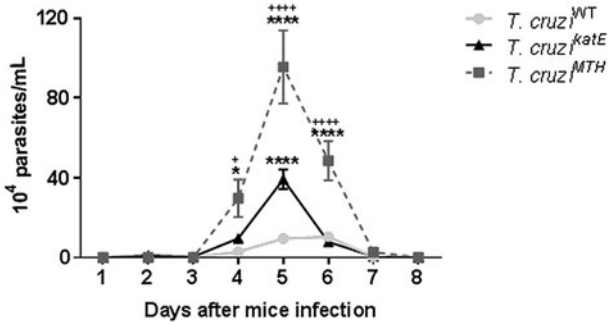

Fig. 7. Mice infected with *T. cruzi*^{katE} show a reduced parasitaemia in relation to *T. cruzi*^{MTH}-infected mice. C57BL/6 mice were infected via intraperitoneal route with 10^6 *T. cruzi* blood stream trypomastigotes (*T. cruzi*^{WT}, *T. cruzi*^{katE} and *T. cruzi*^{MTH}). Parasitsemia was assessed by counting the parasites in the tail vein blood of the infected mice. Data are presented as mean ± S.D.. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $****P < 0{\cdot}0001$ *vs T. cruzi*^{WT}; $^{++++}P < 0{\cdot}0001$ *vs T. cruzi*^{katE} (two-way ANOVA/ Bonferroni, $n = 5$).

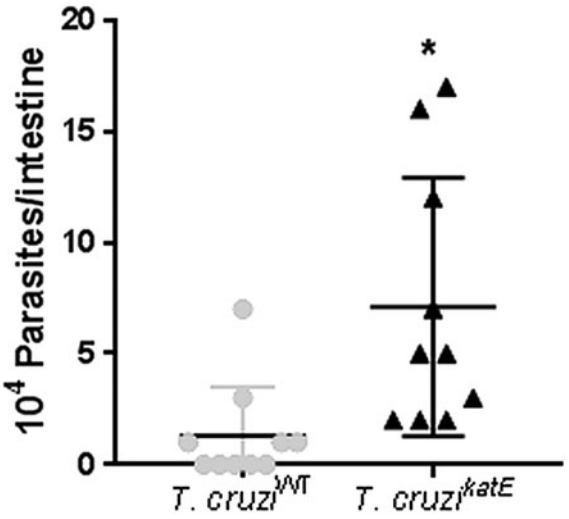

Fig. 8. KatE increases *T. cruzi* proliferation in the invertebrate host intestine. Fifth-stage nymphs of *R. prolixus* were fed with heat-inactivated rabbit blood containing 10^7 *T. cruzi* epimastigotes mL^{-1}. Thirty days after infection, intestines were obtained through insects dissection, and parasites were quantified using a Neubauer chamber. Data are presented as mean ± S.D.. $*P < 0{\cdot}05$ *vs T. cruzi*^{WT} (unpaired Student's *t*-test, $n = 3$).

cell line overexpressing *MTH*, the *T. cruzi* homologue for *E. coli*'s *MutT* – abilities in infecting mice. Trypomastigotes from wild-type *T. cruzi* and from the two aforementioned variants were injected into the peritoneal cavity of 4–8 weeks-old mice. We then observed that *T. cruzi*^{katE} cells showed higher parasitaemia levels in relation to *T. cruzi*^{WT} parasites on day 4 (Fig. 7), and that *T. cruzi*^{MTH} cells exhibited the highest parasitaemia levels among all *T. cruzi* cells studied on days 3–5 (Fig. 7). Interestingly, as previously discussed, overexpression of *MTH* in *T. cruzi* is related to an increased resistance to oxidative stress similarly to *MutT* (Aguiar *et al*. 2013). Altogether, these results show that capacity to mediate oxidative signalling modulates *T. cruzi*'s ability to parasitize its vertebrate host.

KatE increases T. cruzi *proliferation in the invertebrate host intestine*

After studying the behaviour of *T. cruzi*^{katE} and *T. cruzi*^{MTH} cells in the vertebrate host, we went on to verify the ability of these cells in infecting the invertebrate host. Then, fifth-stage nymphs of *R. prolixus* were infected with either *T. cruzi*^{WT} or *T. cruzi*^{katE} cells. Thirty days after infection, the number of parasites found in the intestines of the invertebrate host was higher when the infection had been carried out with *T. cruzi*^{katE} cells (Fig. 8). This result indicates that acquired antioxidant ability increases *T. cruzi*'s infectivity in *R. prolixus*.

DISCUSSION

ROS produced *in vivo* used to be regarded almost exclusively as deleterious molecules for eukaryotic cells (Balaban *et al*. 2005; Wallace, 2005). However, it has been becoming plausible that $O_2^{\bullet-}$ and H_2O_2 can act as critical intermediates in cellular signalling pathways (Buetler, 2004; Hamanaka and Chandel, 2010). During normal cell growth, a tight balance between these two ROS species is maintained by cellular antioxidant systems, whose impairment generates a pro-oxidant environment (Mittra and Andrews, 2013). In fact, H_2O_2 may act as a messenger molecule since its stability and membrane diffusibility present selective reactivity towards cysteine residues, which provides an advantage with regard signaling capacity. The best characterized mechanism by which H_2O_2 acts as a signalling molecule is the oxidation of critical cysteine residues within redox-sensitive proteins (D'Autréaux and Toledano, 2007). It has been shown that mammalian cells produce H_2O_2 to mediate diverse physiological responses such as cellular proliferation, differentiation and migration (Sundaresan *et al*. 1995; Rhee *et al*. 2000). Besides that, high levels of H_2O_2 are involved in the activation of signalling pathways to induce proliferation, migration, and invasion in cancer cells, which correlates with low levels of catalase expression (Picardo *et al*. 1996; Subapriya *et al*. 2002; Yoo *et al*. 2008; Sen *et al*. 2012).

The antioxidant system of *T. cruzi* has been considered a target for chemotherapeutic approaches to treat Chagas disease due to its importance regarding the adaptation towards the oxidative environment to which this parasite gets exposed during its life cycle (Krauth-Siegel *et al*. 2005; Irigoín *et al*. 2008). In order to cope with oxidative stress and

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establish infection in hosts, *T. cruzi* exhibits an efficient and peculiar antioxidant machinery (Piacenza *et al*. 2009*a*); remarkably, an intriguing fact is that *T. cruzi* antioxidant system does not include catalase (El-Sayed *et al*. 2005), which is found in virtually all aerobic organisms. Aiming to better understand the absence of catalase in this parasite, we generated a *T. cruzi* cell line which stably expresses catalase from *E. coli* (KatE). Such approach led us to verify that cells expressing *katE* show increased resistance against oxidative stress generated by exposure to exogenous H_2O_2, and exhibit the same growth rate from untransfected cells (Fig. 2A). In fact, we previously found that *T. cruzi* either expressing *E. coli*'s MutT – an enzyme responsible for the removal of the oxidation product 7,8-dihydro-8-oxoguanine (8-oxoG) from the nucleotide pool –, or overexpressing the MutT homologue in *T. cruzi*, MTH, also exhibit increased resistance to H_2O_2, and same growth rate observed in *T. cruzi* wild-type cells (Aguiar *et al*. 2013).

Previous works have shown that, at low concentrations, H_2O_2 acts both improving the proliferation and increasing the resistance against oxidative stress in *T. cruzi* (Finzi *et al*. 2004). Similarly, *Leishmasodnia chagasi* promastigotes, when incubated with either sub-lethal concentrations of menadione (an $O_2^{\bullet-}$ generator), or H_2O_2 itself, develops an increased resistance to H_2O_2 toxicity. It was also observed that a sub-lethal dose of menadione stimulates promastigotes to become more virulent in a BALB/c mouse model of leishmaniasis (Wilson *et al*. 1994). On top of that, another work involving *Leishmania* suggested that low concentrations of ROS, including H_2O_2, regulate proliferation and differentiation by modulating the activity of cellular targets by oxidation (Mittra and Andrews, 2013) – this is also observed in bacteria (Demple and Halbrook, 1983), yeast (Davies *et al*. 1995) and mammalian cells (Wiese *et al*. 1995). Altogether, these observations suggest that adaptation to oxidative stress ensues when cells are first submitted to low concentrations of H_2O_2 and are afterwards exposed to higher concentrations of the oxidative agent. In this work, our results show that a pretreatment with H_2O_2 for 24 h before exposure to higher H_2O_2 concentrations induces an adaptive response in *T. cruzi* (Fig. 3). Although these findings are in contrast with those reported by Finzi *et al*. (2004), we hypothesize that either the use of distinct *T. cruzi* strains, or the use of different methodologies, or even both, may explain the different outcomes observed.

The fact that the expression of KatE reduced the *T. cruzi* ability to adapt to low doses of H_2O_2 (Fig. 3A) suggests that the expression of this enzyme have an effect in the adaptation promoted by the pretreatment with oxidants. Besides that, we verified that the persistent oxidative signalling promoted by H_2O_2 is not effective in mediating increased cellular resistance against oxidative insults (Fig. 3B), suggesting that there is a gradual loss of adaptability, as observed in mammalian and yeast cells (Demple and Halbrook, 1983; Wiese *et al*. 1995). In fact, our data also suggest that these observed differences concerning parasite resistance and adaptation to oxidative stress might be related to changes in the ability in detoxifying H_2O_2: *T. cruzi* cells expressing *katE* previously exposed to H_2O_2 exhibited increased levels of H_2O_2 after being challenged with this oxidant when compared with wild-type cells (Fig. 4), suggesting that *katE* expression impairs the cellular ability to cope with H_2O_2 toxicity.

Since KatE impaired the capacity of transfected parasites to overcome H_2O_2 toxicity, we decided to verify if other antioxidant enzymes were somehow affected by the expression of catalase. In fact, we found that Fe-SOD B levels are increased by heterologous expression of *katE* and pretreatment with H_2O_2 (Fig. 5). Strikingly, the presence of KatE prevents adaptation to oxidative stress in *T. cruzi* (Fig. 3). Interestingly, alterations in antioxidant enzymes levels were also found in cancer cells: Picardo *et al*. (1996) observed that the SOD levels are elevated in melanoma cells, whereas catalase levels are reduced. Besides that, catalase overexpression in a cancer breast model is able to decrease the tumour invasiveness, suggesting the overexpression of this enzyme could reduce the oxidative stress signalling, which may be important to determine tumour invasiveness (Goh *et al*. 2011). Based on these findings, it has been suggested that tumour cells reduce their catalase levels to allow the H_2O_2 to signal an oxidative stress situation (Goh *et al*. 2011).

We also observed that *T. cruzi* expressing *katE* exhibited a reduced level of TR (Fig. 5C), which is indirectly involved in the cellular redox metabolism. Additionally, wild-type parasites treated with H_2O_2 had their of TR levels increased in relation to untreated cells, while the same was not observed in cells expressing *katE*. The influence of H_2O_2 treatment on antioxidant enzymes involved in H_2O_2 metabolism was shown by Finzi *et al*. (2004), who observed that incubation of *T. cruzi* epimastigotes with H_2O_2 also increased the cTXNPx levels, which probably occurred to promote cell detoxification (Finzi *et al*. 2004). Also, treatment of *T. cruzi* trypomastigotes with increasing concentrations of H_2O_2 is related to modulations of cTXNPx and mTXNPx levels in a dose-dependent way (Gadelha *et al*. 2013). The fact that TR levels are increased after H_2O_2 exposure in wild-type parasites, but not in cells expressing *katE*, strongly suggests that catalase expression in *T. cruzi* changes the parasite ability to signal oxidative stress.

It is remarkable that wild-type *T. cruzi* is able to maintain the basal levels of H_2O_2 in situations of

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either excess or absence of this oxidant (Fig. 4). This observation suggests that there is a buffering system able to maintain a certain level of H_2O_2, and that such system is deregulated in the presence of catalase.

Infection of macrophages with *T. cruzi* induces oxidative stress, which stimulates parasite replication (Bergeron *et al*. 2008; Alvarez *et al*. 2011; Paiva *et al*. 2012). Here, we verified that expression of *katE* in *T. cruzi* increases parasitism in macrophages (Fig. 6A), and that treatment with exogenous catalase reduces MutT-expressing *T. cruzi* capacity to parasite these immune cells (Fig. 6B). In line with these data, it is known that treatment with antioxidants inhibits epimastigote proliferation *in vitro* (Nogueira *et al*. 2011), and reduces *T. cruzi* parasitaemia (Figueiredo *et al*. 2000). Taken together, these findings corroborate the hypothesis that oxidant signalling is needed for *T. cruzi* proliferation in a manner prevented by the expression of catalase.

When in the intestine of *R. prolixus*, *T. cruzi* has to handle with a highly oxidative environment. In fact, blood digestion results in the release of extremely high concentrations of haeme, the prosthetic group of haemoglobin (Graça-Souza *et al*. 2006). Haeme toxicity is often related to ROS generation through Fenton reaction, promoting lipid, protein and DNA oxidations (Aft and Mueller, 1983; Gutteridge and Smith, 1988). Parasites expressing catalase could be more resistant to this environment by degrading H_2O_2. This fact, together with the finding that a group of monoxenous parasites, such as *Crithidia fasciculata*, *Crithidia acanthocephali*, *Crithidia luciliae*, *Leptomonas pyrrhocoris*, *Leptomonas seymouri*, *Lotmaria passim*, *Novymonas esmeraldas* (trypanosomatids which only infects insects), express catalase (Alcolea *et al*. 2014; Kraeva *et al*. 2016), suggest that this enzyme is very important to confer resistance to the oxidative stress in the invertebrate host. With regard to the vertebrate host, however, catalase expression might impair oxidative stress signalling triggered by H_2O_2 – it has been already shown that this molecule is found in reduced levels in this environment (Mueller *et al*. 1997).

Phylogenetic hypotheses suggest that, during evolution, a free-living kinetoplastid ancestral may have been ingested by insects, and have been adapted itself to the intestinal habitat, originating the monoxenous trypanosomatids. When the haematophagy appeared in nature, it is possible that the parasites were inoculated into vertebrates. Thus, parasites which were adapted to this new environment started to alternate between insects and vertebrates, originating the dixenous trypanosomatids (Simpson *et al*. 1980, 2006; Hamilton *et al*. 2004; Stevens, 2008). As postulated by Kraeva *et al*. (2016), a group of monoxenous trypanosomatids received the catalase gene by horizontal transfer from some unknown bacterium. In this sense, it is possible that dixenous trypanosomatids, as *T. cruzi*, have never received this gene by horizontal transfer. Noteworthy is the fact that *Leishmania* does not have the catalase gene, even belonging to the catalase-positive branch phylogenetic tree (Kraeva *et al*. 2016); this finding reinforces the idea that the presence of catalase impairs oxidative signalling in dioxenous kinetoplastids (Kraeva *et al*. 2016). Catalase expression could impair the oxidative stress signal originated in an oxidant environment which is required for cellular differentiation, and, to some extent, allowed protection to the parasite against oxidative damage. In this context, our findings suggest that the absence of catalase gene could improve signalling capacity triggered by oxidative mediator molecules in *T. cruzi*.

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