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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Oral infection of mice and host cell invasion by *Trypanosoma cruzi* strains from Mexico

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Abstract

Oral infection by *Trypanosoma cruzi* has been responsible for frequent outbreaks of acute Chagas disease in the north of South America and in the Amazon region, where *T. cruzi* genetic group TcI predominates. TcI strains from different geographical regions have been used in oral infection in mice, but there is no information about strains from Mexico where TcI is prevalent. Here, we analyzed four Mexican strains as concerns the course of oral infection, the ability to invade host cells in vitro, and the profile of metacyclic trypomastigote surface molecules gp82 and gp90 that are implicated in parasite internalization. Oral infection of mice with metacyclic forms of all strains resulted in reduced blood and tissue parasitism, and mild to moderate inflammatory process in the heart/skeletal muscle. They expressed pepsin-resistant gp82 and gp90 molecules at high levels and invaded host cells poorly in full nutrient medium and efficiently under nutrient-deprived condition. The properties exhibited by Mexican strains were similar to those displayed by TcI strains from other geographical regions, reinforcing the notion that these features are common to the genetic group TcI as a whole.

Keywords *Trypanosoma cruzi* · Genetic group TcI · Oral infection · Mexican strains · Metacyclic trypomastigote · Surface molecules gp82 and gp90

Introduction

Transmission of *Trypanosoma cruzi* by the oral route has been responsible in the last 10–12 years for frequent outbreaks of acute Chagas disease in Brazil, Venezuela, and Colombia (Noya et al. 2015; Coura and Junqueira 2015). The source of infection has been attributed to contaminated food and beverages containing metacyclic trypomastigotes derived from insect triatomines. In the north of South America and in the Amazon region, where the outbreaks of orally transmitted Chagas disease have occurred more frequently, the predominant *T. cruzi* genetic group is TcI (Llewellyn et al. 2009; MILES et al. 2009). This genotype prevailed in parasites isolated from patients infected orally in outbreaks of Chagas disease in Venezuela and Colombia (Ramirez et al. 2013; Muñoz-Calderón et al. 2013). TcI predominates in Mexico (Bosseno et al. 2002, 2009; Connor et al. 2007; Monteón et al. 2016). Of 56 Mexican *T. cruzi* stocks analyzed, only two belonged to TcII lineage, and all others belonged to TcI and were isolated from both domestic and sylvatic cycles over a broad geographic area in Mexico (Bosseno et al. 2002). Of 24 *T. cruzi* strains, obtained primarily from humans in nine different geographical areas, including Sonora in the Northeast and Yucatán at the Southeast of Mexico, 20 human strains and 2 from insect vectors belonged to TcI, whereas only 2 strains, isolated from wild mammals, belonged to the TcII–TcVI groups (Martínez et al. 2013). As regards the triatomine-derived parasites characterized as TcII, TcIII, TcIV, and TcV strains, they were isolated from the main insect

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vector *Triatoma dimidiata* in central Veracruz, Mexico (Ramos-ligonio et al. 2012). What is not known is the number of people infected by different routes of transmission, and the actual prevalence of the disease is unknown because no official reporting of cases is performed (Carabarin-Lima et al. 2013).

Analysis of the course of infection in mice inoculated intraperitoneally with TcI strains, isolated from humans and triatomines in different geographic regions of Mexico, showed patent as well as subpatent parasitemias and tropism for heart/skeletal muscle (Gómez-Hernández et al. 2011). The outcome of oral infection with these strains is not known.

The referred study was performed with blood trypomastigotes inoculated intraperitoneally in mice. What is the outcome of infection with metacyclic trypomastigotes, which are the parasite forms responsible for orally transmitted infection, is still to be investigated. The mechanisms of oral *T. cruzi* infection in the mouse model have been partially elucidated. Metacyclic trypomastigote (MT) forms invade the gastric mucosal epithelium as the portal of entry into systemic infection (Hoft et al. 1996). Gp82, a MT-specific surface molecule, plays a critical role by promoting the parasite binding to the gastric mucin as the first step to traverse the mucus layer that protects the underlying target epithelial cells (Staquicini et al. 2010). Reaching the target cells, the parasites are probably internalized in a manner mediated by gp82, which in vitro promotes MT invasion of human epithelial cells (Yoshida 2006). In addition to gp82, the MT-specific surface molecule gp90 that functions as a negative regulator of host cell invasion (Málaga and Yoshida 2001) influence the course of infection by the oral route (Cortez et al. 2006). *T. cruzi* strains that express gp82 and pepsin-resistant gp90 at high levels are poorly invasive toward gastric epithelial cells and this is the case of TcI parasites (Maeda et al. 2016). Quite the opposite is the outcome of infection by parasite strains expressing pepsin-susceptible gp90 that efficiently enter target cells (Cortez et al. 2006; Covarrubias et al. 2007).

T. cruzi strains examined to date for oral infection studies include those isolated in Guatemala, Venezuela, and different geographical regions in Brazil (Cortez et al. 2003, 2006; Covarrubias et al. 2007; Maeda et al. 2016). Analysis of strains from Mexico would complement these studies and provide a broader view on the characteristics of infection by *T. cruzi* genetic group TcI as a whole.

Methods

Ethical considerations

The study was approved by the Ethics Committee on Animal Experimentation of Universidade Federal do Triângulo Mineiro (UFTM), protocol number 308. We have followed the guidelines that recommend the use of anesthetic agents, such as ketamine/xylazine, to euthanize animals.

Parasites and in vitro metacyclogenesis

Four *T. cruzi* strains from Mexico maintained in the Division of Parasitology, at UFTM, were used: CGH1 e CGH3 isolated *Triatoma longipennis,* KR1 isolated from *Triatoma picturata,* and NINOA obtained by xenodiagnosis from a patient with acute Chagas disease. The parasites were cultured at 28 °C in liver infusion tryptose medium supplemented with 10% fetal bovine serum. Metacyclic forms from cultures at the stationary growth phase were purified through passage in DEAE-cellulose column as described (Teixeira and Yoshida 1986).

Oral infection of mice and histopathological analysis

A total of 100 non-isogenic mice, 6–8 weeks old, were used. The animals were divided in four groups and each group received by the oral route $2{,}5 \times 10^4$ MT of a different strain. Parasitemia was evaluated every 2 days, from the first day of infection, for a period of 42 days, by examination of fresh blood samples under phase contrast microscope. Mice presenting subpatent parasitaemia were monitored using the microhematocrit method. The pre-patent period observed in the strains was 14 days. In addition, parasite DNA detection in tissues was performed by qPCR. The animals that survived after 42 days post-infection were euthanized using ketamine/xylazine and the diverse organs/tissues (heart, brain, stomach, esophagus, duodenum, and skeletal muscle) were collected, fixed in buffered 10% formalin, embedded in paraffin, and cut into 3- to 4-μm-thick sections. The sections were stained with hematoxylin/eosin, the presence of amastigote nests was examined, and the inflammatory processes were classified semiquantitatively.

DNA extraction and real-time PCR (qPCR)

DNA extraction from different tissues (skeletal muscle, esophagus, stomach, duodenum, heart, brain) was performed using the kit ReliaPrep™ gDNA Tissue Miniprep System (Promega, USA), according to the instructions of the manufacturer. For detection and quantification of *T. cruzi* we used the primers *Cruzi* 1 (ASTCGGCTGATCGTTTTCGA) and *Cruzi* 2 (AATTCCTCCAAGCAGCGGATA) that amplify a 166pb fragment of *T. cruzi* satellite DNA (Piron et al. 2007) and the probe *Cruzi* 3 (CACACACTGGACAC CAA) was stained with FAM 5′(6-carboxyfluorescein) and 3′BHQ1™ (Black Hole Quencher 1). As an internal control, we used the primers *Bact Fw* (AGCCATGT ACGTAGCCATCCA) and *Bact Rv* (TCTCCGGA GTCCATCACAATG) that amplify an 81pb fragment

of *Mus musculus β-actin* gene and the probe *Bact probe* (TGTCCCTGTATGCCTCTGGTCGTACCAC) was stained as above. The standard curves were obtained by serial 1:10 DNA dilutions of 10^5 parasites/ml of Ninoa strain to determine the amplification efficiency and as a control. The amplification was carried out in a Step One Plus - Real-Time PCR System (Applied Biosystems USA) according to the procedure by Piron et al. (2007): 2 min at 50 °C, denaturation at 95 °C for 10 min, and 40 cycles (15 s at 95 °C and 1 min at 58 °C). The sample was considered valid when the internal control (β-actin) was efficiently amplified and was considered positive for *T. cruzi* when the cycle threshold (Ct), which is the first cycle of the PCR reaction where fluorescence is detected (Piron et al. 2007), was < 40. To determine the parasite load, the sample normalization was carried using the formula $R = 2\text{-}^{\Delta Ct}$ ($R = 2$ - (Ct *T. cruzi* – Ct β-actina)) and thus generating a relative value for parasite DNA.

Host cell invasion assay

The human carcinoma-derived epithelial HeLa cells (Instituto Adolfo Lutz, São Paulo, SP, Brazil) were grown at 37 °C in Dulbecco's Minimum Essential Medium (DMEM), supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) in a humidified 5% CO_2 atmosphere. Cell invasion assays were performed by seeding purified MT onto each well of 24-well plates containing 13-mm diameter round glass coverslips coated with 1.2×10^5 HeLa cells, either in DMEM with 10% FCS (D10) or in PBS^{++} (PBS containing per liter: 140 mg $CaCl_2$, 400 mg KCl, 100 mg $MgCl_2.6H_2O$, 100 mg $MgSO_4.7H_2O$, 350 mg $NaHCO_3$). After 1 h incubation with parasites, the duplicate coverslips were fixed in Bouin solution, stained with Giemsa, and sequentially dehydrated in acetone, a graded series of acetone:xylol (9:1, 7:3, 3:7) and xylol. The number of intracellular parasites was counted in a total of 250 cells (Yoshida et al. 1989).

Flow cytometry

Metacyclic forms (1×10^7) were treated or not for 1 h with 2 mg/ml pepsin in citrate buffer at pH 3.5. Afterwards, the parasites were incubated at 4 °C for 1 h with monoclonal antibody 3F6 or 1G7, directed respectively to MT stage-specific surface molecule gp82 or gp90. Following fixation with 4% para-formaldehyde for 20 min and washings in PBS, the parasites were incubated for 1 h with fluorochrome R-phycoerythrin (PE) coupled to anti-mouse IgG (BD Pharmingen). Parasites were quantified using CellQuest program and BD FACSCalibur™ Flow Cytometer.

Statistical analysis

The normal distribution of quantitative variables was checked by the D'Agostino and Pearson test, and continuous variables were expressed as mean ± standard error. Mann-Whitney test was used for comparison of two independent groups. Differences were considered statistically significant when $p < 0.05$. Statistical analysis was performed using Excel 2013 for Windows (Microsoft, USA), StatView (ABACCUS, USA), and GraphPad Prism 5.0 (GraphPad Software, USA).

Results

Metacyclic forms of Mexican *T. cruzi* strains are poorly infective by the oral route and are poorly invasive toward HeLa cells in full nutrient medium

We evaluated the capacity of Mexican *T. cruzi* strains to infect mice by the oral route. Each group of 25 mice was infected orally with MT of strain CGH1, CGH3, KR1 or NINOA. The mice were considered to be infected when parasites were detected by microhematocrit and/or by microscopic examination of blood samples during the 42 day period of observation. Positive parasitism was found in 7 and 9 mice that received CGH1 and KR1 strains, respectively, whereas only 2 mice in each group infected with CGH3 and NINOA strains were positive (Fig. 1a). The survival rate was high: 96% for KR1 and CGH3 strains, 92% for CGH1 strain and 84% for NINOA strain. To determine whether the low infective capacity of these strains correlated with their ability to invade host cells, in vitro MT invasion assays were performed. Parasites were incubated with HeLa cells for 1 h in full nutrient DMEM (D10) or in nutrient-deficient PBS^{++}, which renders the cells more susceptible to invasion by TcI strains (Maeda et al. 2016). In full nutrient D10 medium, the number of KR1 strain MT that entered Hela cells was about 10 per 100 cells and the internalization of other strains was lower, whereas incubation in PBS^{++} resulted in efficient MT invasion (Fig. 1b).

Oral infection with Mexican *T. cruzi* strains results in low tissue parasitism

We determined the parasitism in diverse organs and tissues, using real-time PCR. *T. cruzi* DNA was detected in the highest amount in the skeletal muscle of a mouse infected with NINOA strain (Fig. 2). Although the parasite DNA content was much lower, skeletal muscle was also the preferential tissue for CGH1 and CGH3 strains, whereas KR1 strain

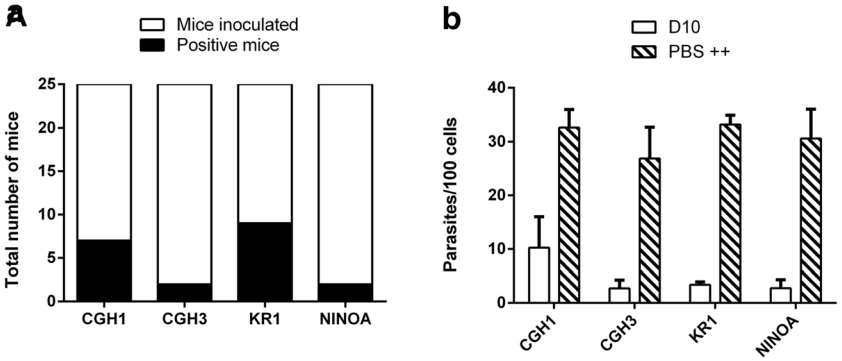

Fig. 1 Blood parasitism in mice infected orally with Mexican strains of *T. cruzi* and parasite invasion of host cells. **a**Four groups of 25 mice was infected by the oral route with MT ($2{,}5 \times 10^4$ parasites per mouse) of the indicated strains and parasitemia was monitored for 42 days by microhematocrit and contrast microscope examination of fresh blood samples. **b** HeLa cells were incubated with MT of the indicated strains for 1 h in full nutrient D10 medium or in nutrient-deprived PBS^{++}. After fixation and Giemsa staining, the number of intracelular parasites was counted in a total of 250 cells. Values are the means ± SD of three independent assays performed in duplicate

DNA was found in higher amounts in the heart than in skeletal muscle (Fig. 2).

Histological alterations and presence of amastigote nests correlate with poor parasitism

Mice with positive blood parasitism had their organs collected 42 days after oral administration of MT and processed for histological analysis. Inflammatory infiltrates of mild to moderate intensity were found in diverse organs, those of higher intensity predominating in the heart (Table 1). Amastigote nests were scarce (Table 1).

Mexican *T. cruzi* strains express pepsin-resistant MT-specific surface molecules gp90 and gp82 at high levels

We determined by western blot the expression of gp90, which negatively modulates host cell invasion in vitro and oral infection in mice (Málaga and Yoshida 2001; Cortez et al. 2006), as well as of gp82, which plays a crucial role in oral infection (Staquicini et al. 2010). Both gp90 and gp82 were

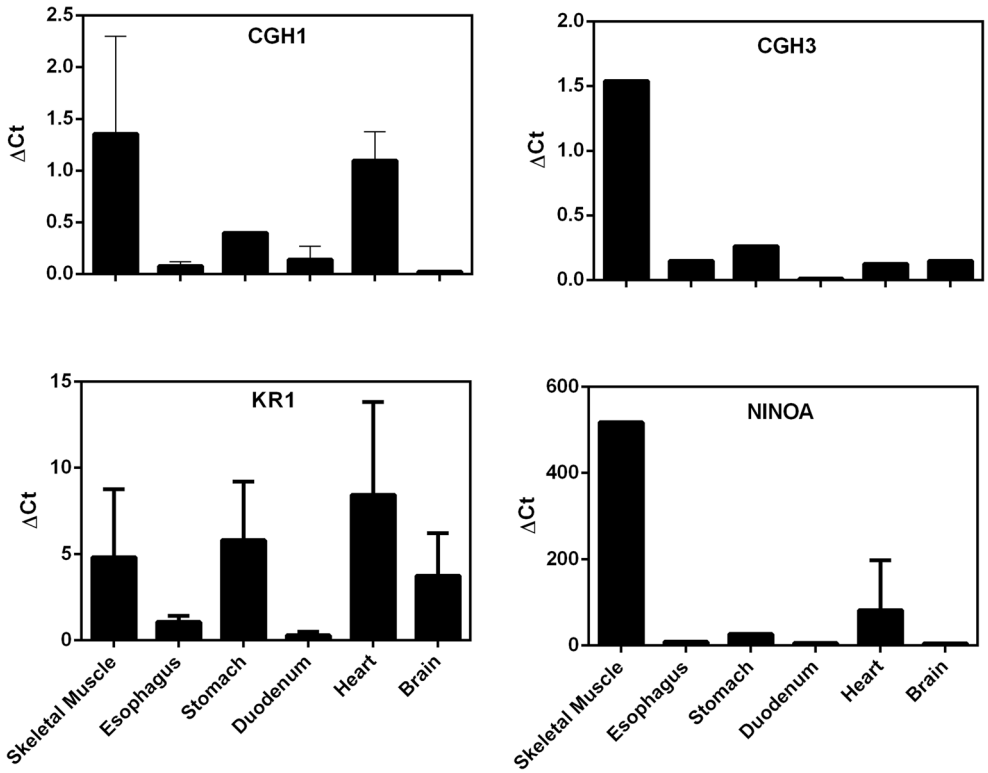

Fig. 2 Tissue parasitism in mice infected orally with Mexican strains of *T. cruzi*. Mice were infected with MT of the indicated strains and 42 days later the diverse organs were collected for analysis by qPCR to determine the parasite load. Sample normalization was carried using the formula $R = 2^{-\Delta Ct}$ ($R = 2^{-(Ct\ T.\ cruzi\ -\ Ct\ \beta\text{-}actina)}$) and thus generating a relative value for parasite DNA. Values are the means ± SD for all mice of each strain

Table 1 Inflammatory process in tissues of mice inoculated with *T. cruzi* MT

T. cruzi strain	Mouse	Skeletal muscle		Esophagus/stomach		Duodenum		Heart		Brain	
		II	AN	II	AN	II	AN	II	AN	II	AN
CGH1	1	+	+	+	−	+	−	++	+	+	−
	2	+	−	+	−	−	−	+	−	+	−
	3	+	−	+	−	−	−	+	−	−	−
	4	+	−	+	−	+	−	++	+	+	−
	5	+	−	+	−	−	−	+	−	−	−
CGH3	1	+	−	+	−	+	−	+	−	+	−
KR1	1	+	−	+	−	+		++	+	+	−
	2	+	−	+	+	+	−	++	−	−	−
	3	+	+	+	+	+	−	+	−	+	−
	4	+	−	+	−	+	−	+	−	+	−
	5	+	+	+	−	+	−	+	−	+	−
	6	−	−	+	−	+	−	+	−	−	−
NINOA	1	+	+	+	+	+	+	−	+	+	+
	2	+	−	−	−	+	−	−	+	−	−

II, intensity of inflammation; AN, amastigote nests

Classification of the inflammatory process: (−) absent, (+) 1–5 focus (mild), (++) 6–10 focus (moderate)

Classification of amastigote nests: (−) absent, (+) present

expressed at high levels in MT of all four strains (Fig. 3a). To confirm the presence of gp90 and gp82 on the parasite surface and also to determine their susceptibility to peptic digestion, flow cytometry analysis was performed with untreated and enzyme-treated MT. Parasites were treated or not with 2 mg/ml pepsin at pH 3.5 and then processed for flow cytometry analysis using monoclonal antibodies directed to gp90 or gp82. Parasites maintained without primary antibody was used as control for unspecific staining. Pepsin-resistant gp90 and gp82 were expressed on MT surface of all strains. Parasites maintained without primary antibodies were used as control for unspecific staining. CGH3, Ninoa, and KR1 strains presented pepsin-resistant GP90 and GP82, as demonstrated by similar MFI after pepsin treatment for both proteins, for gp90, CGH3—no pepsin (np), 88.3 ± 7.5/plus pepsin (pp), 74.6 ± 7; KR1—np, 55.3 ± 2.1/pp., 53.6 ± 5.5; Ninoa—np, 114.6 ± 20/pp., 93.1 ± 10, and for gp82, CGH-3—np, 323.3 ± 82/pp., 315 ± 21; KR1—np, 318.3 ± 12/pp., 307 ± 43; Ninoa—np, 346.6 ± 24/pp., 300.3 ± 76; Mean ± std. error; $p > 0.05$; Mann-Whitney test. Otherwise, CGH1 expressed pepsin-susceptible gp90 and gp82, for gp90, np, 44.1 ± 3/pp., 14.9 ± 1.5 and for gp82, np, 148.3 ± 6.3/pp., 84.3 ± 1.1; Mean ± std. error; $p < 0.05$; Mann-Whitney test (Fig. 3b).

Discussion

Our data have shown that metacyclic forms of Mexican *T. cruzi* strains are poorly infective in mice inoculated by the oral route. In most animals, the blood/tissue parasitism was either undetectable or very low. This outcome was similar to the course of oral infection with TcI strains from other geographical regions in Latin America (Maeda et al. 2016) and differs from TcII and TcVI strains analyzed previously, which led to patent parasitemia after oral infection and efficiently invaded host cells in vitro (Cortez et al. 2003, 2012a, 2012b; Cortez et al. 2012b; Covarrubias et al. 2007). As regards TcI subgenotypes, a large scale analysis of 105 *T. cruzi* TcI samples from 10 countries, including Mexico, identified TcIa, identified TcIa, TcIb, TcId, and a novel group TcIe (Cura et al. 2010). They found that TcIa was associated with domestic cycles in southern and northern South America and sylvatic cycles in Central and North America, TcIb was found in all transmission cycles from Colombia, TcId was identified in all transmission cycles from Argentina and Colombia, including Chagas cardiomyopathy patients, sylvatic Brazilian samples, and human cases from French Guiana, Panama, and Venezuela (Cura et al. 2010). Analyzing *T. cruzi* isolated from *Triatoma dimidiata* in the Yucatan Peninsula of Mexico and two strains away from that area, Monteón et al. (2016) found that they belonged to TcIa. The strains we analyzed belong to TcIa subgenotype (unpublished), which appears to be the most prevalent in Mexico (Coura and Borges-Pereira 2010; Monteón et al. 2016) and may be involved in oral transmission in Mexico. Although there is no report on outbreaks of Chagas disease by oral infection in Mexico, this route may be of relevance for *T. cruzi* transmission and deserves attention by health authorities. Monitoring of oral infection is also important in other countries of the region. Food as source of *T. cruzi* infection has been reported in areas with occurrences of

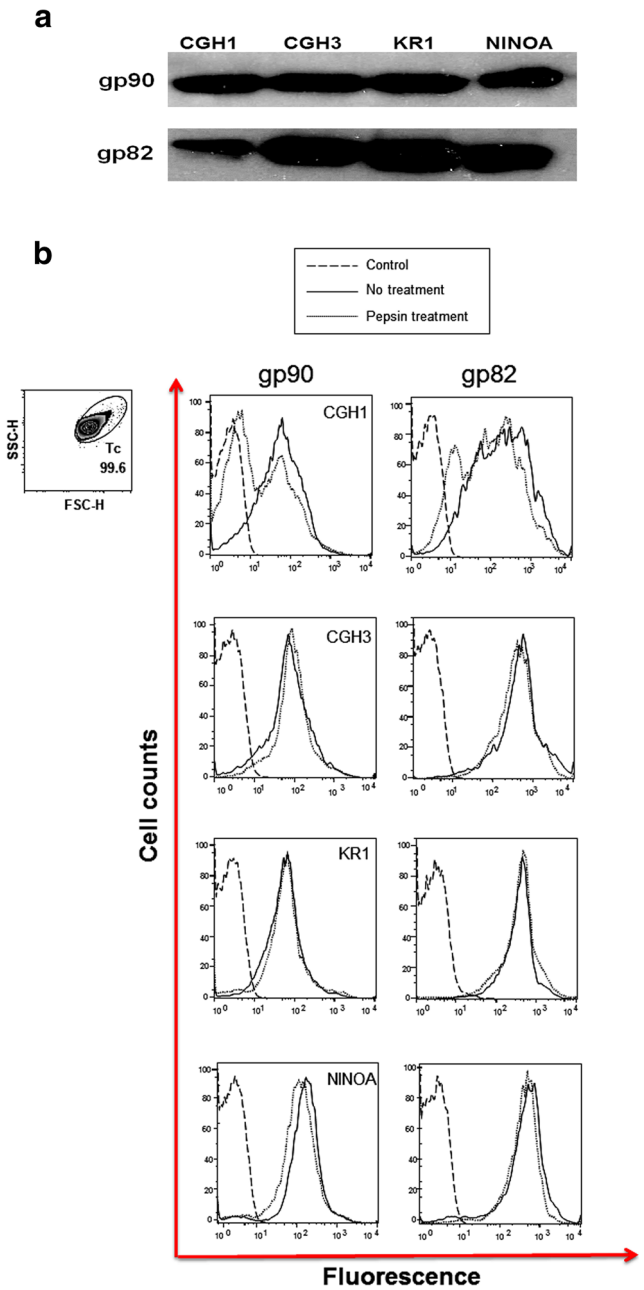

Fig. 3 Expression of *T. cruzi* surface molecules gp90 and gp82. **a** Detergent soluble extracts of metacyclic forms were analyzed by immunoblotting using monoclonal antibodies directed to gp90 and gp82. **b** Metacyclic forms were treated or not for 1 h with 2 mg/ml pepsin, at pH 3.5 and processed for flow cytometry analysis, using the same antibodies as in **a**. Representative data of three independent experiments are shown

Chagas disease (Coura and Borges-pereira 2010; Coura et al. 2014; de Noya et al. 2015; Coura and Junqueira 2015).

An earlier analysis of 135 samples from across the geographic distribution of TcI has shown that Tc populations of sylvatic triatomine vectors and mammalian reservoir hosts are extraordinarily genetically diverse, whereas the diversity of most human strains is reduced (Llewellyn et al. 2009). Notwithstanding that genetic diversity, what emerges from the present and previous studies with TcI strains, either from the sylvatic transmission cycle or from patients with Chagas disease, is the reduced ability of MT to invade host cells and to infect mice (Yoshida 2006; Maeda et al. 2016). This is compatible with the fact that metacyclic forms of all TcI strains examined to date express high levels of gp90, which negatively regulates target cell invasion. Differently from some TcII strains that have their infectivity increased, when inoculated by the oral route, due to the peptic digestion of gp90 in the gastric juice (Cortez et al. 2006; Covarrubias et al. 2007), such an event does not occur in TcI strains expressing pepsin-resistant gp90.

The fact that acute infection of mice with TcI strains is characterized by low or undetectable prasitemia/tissue parasitism does not imply that the outcome is always benign. In a murine model of chronic *T. cruzi* infection with a TcI clone from a human patient, it was shown that infection in C3H/HePAS mouse strain progresses chronically and results in intense cardiac inflammatory lesions (Marinho et al. 2004). There is also a report on TcI parasites isolated from endomyocardial biopsies of a chronic chagasic patient with end-stage heart failure (Teixeira et al. 2006). In a large urban outbreak of orally transmitted Chagas disease in Venezuela, in which the isolated parasites were identified as TcI (Muñoz-Calderón et al. 2013), 75% were symptomatic, 20.3% required hospitalization, and a child died of acute chagasic myocarditis (Alarcón de Noya et al. 2010). In Jalisco, Mexico, an anatomopathological analysis of 47 necropsies from patients with positive serology for *T. cruzi* revealed amastigote nests in the heart (Lozano-Kasten et al. 2008). Another study carried out in Merida, Mexico, reported cardiac involvement compatible with acute or chronic stages of Chagas' disease in 36 patients, who suffered from cardiopathy, cardiomegaly, or conduction abnormalities (Zavala-Castro et al., 1995). Cardiopathy is the clinical manifestation prevalent in countries north of the Amazon, and the rarity of megaesophagus and megacolon could be ascribed to the local predominant endemicity of TcI (Miles et al. 2009). In Mexico, a few cases of megaesophagus and megacolon were registered (Lozano-Kasten et al. 2008).

We infer from the present study with Mexican strains and previous studies with strains from Brazil, Guatemala, and Venezuela, isolated from patients with Chagas diseases, *Didelphis marsupialis,* or the insect vector (Yoshida 2006; Maeda et al. 2016), that metacyclic forms of *T. cruzi* genetic group TcI share several features not displayed for instance by TcII and TcVI strains: the reactivity with monoclonal antibody 1G7 directed to gp90, reduced cell invasion capacity in full nutrient medium and low infectivity in mice. Thus, as regards the properties of metacyclic trypomastigotes, great diversity among TcI strains is not observed.

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Compliance with ethical standards

The study was approved by the Ethics Committee on Animal Experimentation of Universidade Federal do Triângulo Mineiro (UFTM), protocol number 308. We have followed the guidelines that recommend the use of anesthetic agents, such as ketamine/xylazine, to euthanize animals.

Conflict of interest The authors declare that they have no conflict of interest.

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