Apoptosis (2013) 18:653–663
DOI 10.1007/s10495-013-0822-x

ORIGINAL PAPER

Trypomastigotes and amastigotes of *Trypanosoma cruzi* induce apoptosis and STAT3 activation in cardiomyocytes in vitro

Philipp Stahl · Volker Ruppert · Thomas Meyer · Jörg Schmidt · Marco A. Campos · Ricardo T. Gazzinelli · Bernhard Maisch · Ralph T. Schwarz · Françoise Debierre-Grockiego

Published online: 24 February 2013
© Springer Science+Business Media New York 2013

Abstract The haemoflagellate *Trypanosoma cruzi* is the causative agent of Chagas' disease that occurs in approximately 8 million people in Latin America. Patients infected with *T. cruzi* frequently suffer of cardiomegaly and may die of myocardial failure. Here we show that *T. cruzi* trypomastigotes (extracellular form) increased in vitro apoptosis of rat cardiomyocytes. Additionally, we demonstrated that amastigotes (intracellular form), for which a method for purification was established, were also able to induce cardiomyocyte apoptosis. Increase of apoptosis was associated with up-regulation of the apoptotic gene *bax* by trypomastigotes, while expression of the anti-apoptotic gene *bcl-2* was down-regulated by amastigotes. The transcription factor STAT3 but not STAT1 was activated in cardiomyocytes by trypomastigotes. In addition, *tlr7* gene expression was up-regulated in cardiomyocytes incubated with trypomastigotes, suggesting that this Toll-like receptor is involved in the intracellular recognition after host cell invasion by *T. cruzi*. Glycosylphosphatidylinositols purified from trypomastigotes did not induce cardiomyocyte apoptosis and STAT activation but down-regulated *tlr7* gene expression. In conclusion, cardiomyopathy observed in Chagas' disease might be in part due to apoptosis of cardiomyocytes induced directly by the parasite.

Keywords *Trypanosoma cruzi* · Trypomastigote · Amastigote · Cardiomyocyte · STAT3 · Glycosylphosphatidylinositol

Philipp Stahl and Volker Ruppert contributed equally to this work. A part of this work is presented as a partial fulfilment of the requirements of the doctoral thesis (Dr. rer. nat.) of Philipp Stahl.

P. Stahl · J. Schmidt · R. T. Schwarz · F. Debierre-Grockiego
Laboratory of Parasitology, Institute for Virology, Philipps University, 35043 Marburg, Germany

V. Ruppert · T. Meyer · B. Maisch
Department of Cardiology, Philipps University, 35033 Marburg, Germany

T. Meyer
Department of Psychosomatic Medicine and Psychotherapy, Georg August University, 37075 Göttingen, Germany

M. A. Campos · R. T. Gazzinelli
Research Centre René Rachou, FIOCRUZ, Belo Horizonte, MG 30190-002, Brazil

R. T. Gazzinelli
Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA

R. T. Gazzinelli
Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG 31270-910, Brazil

R. T. Schwarz
CNRS-UMR 8576, Unit of Structural and Functional Glycobiology, Univ Lille Nord de France, Lille 59000, France

F. Debierre-Grockiego (✉)
Université François Rabelais de Tours, UMR 1282 Infectiologie et Santé Publique, Tours 37200, France
e-mail: francoise.debierre@univ-tours.fr

F. Debierre-Grockiego
INRA, UMR 1282 Infectiologie et Santé Publique, Nouzilly 37380, France

Introduction

The protozoan *Trypanosoma cruzi* is the etiological agent of Chagas' disease (or American trypanosomiasis) that affects approximately eight million people in Latin America. The parasite is transmitted by endemic blood-sucking insects and congenitally. International migration is a factor, which can confront non-endemic countries with the Chagas' disease through blood transfusion and organ transplantation. Chronic Chagas' disease occurs in about one-third of those who are infected after 10–20 years [1]. The heart is one of the most affected organs and cardiac disorders develop in over 90 % of cases [2]. Myocardial involvement in chronic Chagas' disease includes arrhythmias, conduction defects, cardiomegaly, congestive heart failure, and thromboembolic events [3]. Histopathology reveals widespread areas of cellular infiltration and inflammation. Cellular hypertrophy and myocardial fibrosis are usually associated with cardiac symptoms, and death frequently results from either rhythm disturbances or congestive heart failure [4]. The histological examination of the infected heart shows pseudocysts within the muscle fibres but the number of parasites is low in relation to the intensity of the myocarditis. *T. cruzi* antigens were detected in nearly all hearts from chronic chagasic patients that died due to heart failure [5].

In the case of Chagas' heart disease, cardiomyocyte apoptosis in response to *T. cruzi* trypomastigotes is a complex field where conflicting results have been found. Apoptosis has been identified in cardiomyocytes from chagasic patients suffering from heart failure [6]. Apoptosis of infected cells seems to be a critical factor in the course of infection, since it can favour pathogen control. In contrast, pathogen-induced cell death leads to the elimination of key immune cells. De Souza et al. [7] reported that cardiomyocytes become apoptotic after infection with different strains of *T. cruzi*, whereas Aoki et al. [8] showed a protective effect of *T. cruzi* due to cruzipain that acts as a survival factor for cardiomyocytes. Trypomastigotes invade cells and differentiate into amastigotes that divide by binary fission and differentiate again into trypomastigotes, which are released after host cell lysis. Besides this classical process, active invasion of amastigotes into cells has been also reported. Amastigotes obtained by extracellular and intracellular differentiation from trypomastigotes entered in monocytes, divided with similar replication times, and developed into trypomastigotes [9]. When injected intraperitoneally into mice, amastigotes were as infective as trypomastigotes [9]. In the present study, we have investigated the effect of *T. cruzi* trypomastigotes and their glycosylphosphatidylinositols (GPIs) on apoptosis of primary rat cardiomyocytes and for the first time, the role of *T. cruzi* amastigotes on cardiomyocyte death.

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway has been implicated in myocardial infarction, cardiac hypertrophy, myocarditis and dilated cardiomyopathy [10]. Cardiomyocytes express several cytokine receptors, and locally secreted cytokines activating the JAK-STAT pathway might be responsible for cardiomyocyte apoptosis and the resulting cardiomyopathy. In particular, the balance between STAT1 and STAT3 activation might determine the death or survival of cardiomyocytes during infection with *T. cruzi*. For this reason, we have explored STAT activation in cardiomyocytes in the presence of *T. cruzi* trypomastigotes. Toll-like receptors play an important role in the recognition of pathogens and several groups have demonstrated the role of TLR2, TLR4, TLR7, and TLR9 and their adaptor MyD88 in the clearance of *T. cruzi* and in the survival of mice [11–14]. Furthermore, *tlr7* and *tlr9* gene were overexpressed in patients with myocardis [15, 16]. We wondered whether genes coding for MyD88 and for the intracellular receptors TLR7 and TLR9 were modulated by *T. cruzi* in cardiomyocytes.

Materials and methods

Parasite culture and GPI purification

The Brazilian Y strain of *T. cruzi* was grown in Vero cells in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 5 % fetal calf serum. Trypomastigotes were collected from the culture supernatant while amastigotes were released from host cells by using glass beads and the Mixer Mill homogenizer (Retsch). Subsequently, amastigotes were purified by glass wool filtration. Briefly, 20 ml syringe was filled with 4.5 g of glass wool in the first 10 ml volume, and with 3 g of glass wool in the second 10 ml volume. This column was equilibrated with DMEM prior to the passage of the suspension containing free amastigotes and Vero cell debris. The column was washed with 150 ml of medium to recover all amastigotes. Trypomastigote glycolipids were extracted with chloroform–methanol–water (10:10:3, by volume), partitioned between water and water-saturated *n*-butyl alcohol, and the different GPI-related molecules were then separated by thin-layer chromatography with [^{3}H]glucosamine (Hartmann) labelled *T. cruzi* GPIs used as tracers. GPIs were scraped off, re-extracted with chloroform–methanol–water (10:10:3, by volume), and recovered in the butanol phase after water-saturated *n*-butyl alcohol/water partition. Absence of endotoxin was checked with the Limulus Amebocyte Lysate kit QCL-100 (Bio-Whittaker).

Isolation of cardiomyocytes

Adult Sprague–Dawley rats, purchased from Charles River (Sulzfeld), were anaesthetized with 110 mg/kg body weight

pentobarbital sodium. The heart was quickly excised and placed in ice cold Ca^{2+}-free Minimal Essential Medium (MEM, Gibco). The aorta was cannulated and retrogradly perfused at constant pressure of 100 cm H_2O at 37 °C with medium I, containing Ca^{2+}-free MEM, 5 mM taurine and 5 mM creatine. Then, perfusion for enzymatic digestion was performed with medium II (medium I with 0.5 mg/ml collagenase, 0.1 % BSA and 50 μM Ca^{2+}) for about 40 min according to the size of the heart and the possible coronary perfusion. Afterwards the heart was sliced and incubated in medium II for 10 min at 37 °C. The cells were then filtered through a 200 μm nylon mesh, washed with medium III (medium I with 1 % BSA) and centrifuged for 1 min at $90\times g$. The pellet was suspended in 10 ml medium III and again incubated for 10 min at 37 °C. The cardiomyocytes were washed and gradually adapted by raising the Ca^{2+} concentration from 125 to 1,000 μM in five steps. After centrifugation through an ''Optiprep'' gradient, viable cells accumulated in the interphase. These cells (99 % purity) were then washed and cultured in M199 medium (Gibco) containing 5 mM taurine, 5 mM creatine, 2 mM carnitine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 % BSA, 0.1 μM insulin and 20 % fetal calf serum. For further experiments, cardiomyocytes were cultured in 6-well plates in RPMI 1640 medium (Gibco) containing 2 % glutamine, 5 % fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Detection of apoptosis

Rat cardiomyocytes (7×10^4) were cultivated for 24 or 48 h with medium alone, with *T. cruzi* trypomastigotes or amastigotes at a cell-to-parasite ratio of 1:1, 1:10, 1:20, and 1:100, or with trypomastigote GPIs (purified from 5×10^8 parasites). In some experiments, cardiomyocytes were pre-incubated for 18 h with 1 μM dexamethasone prior to infection with trypomastigotes (1:100). Apoptosis was detected in rat cardiomyocytes by terminal desoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), according to the manufacturer's instructions (Roche Diagnostics). Cardiomyocytes were applied after labelling onto a 10-cup slide (Menzel) and protected with mount medium (FluorSave, Merck) and cover slide. For each culture condition, the percentage of apoptotic cardiomyocytes was determined on at least 200 cells with a Zeiss Axiophot microscope (Carl Zeiss MicroImaging). In some experiments, DNA was stained with Hoechst 33342 for 1 h at 37 °C to show cell penetration of amastigotes. Caspase-3/7, caspase-8, and caspase-9 activities were measured in rat cardiomyocytes by luminescent assays according to the manufacturer's instruction (Caspase-Glo Assays, Promega) after 24 h incubation with *T. cruzi* trypomastigotes or amastigotes at a cell-to-parasite ratio of 1:10.

Measurement of STAT activation

Rat cardiomyocytes (5×10^3) were incubated for 30 min, 1 h, or 4 h with medium alone, with *T. cruzi* trypomastigotes at cell-to-parasite ratio of 1:10 or 1:100, or with GPIs extracted from 5×10^8 trypomastigotes. Nuclear proteins were then extracted and analyzed for STAT1 and STAT3 activation using the Trans-AM™ transcription factor assay kit, according to the manufacturer's protocol (Active Motif). This assay is based on a sandwich ELISA method. Activated STAT1 or STAT3 binds to its specific coated oligonucleotide and is recognized by an antibody raised against activated transcription factors. STAT1/3 binding results in a colorimetric reaction measured at 450 nm.

Analysis of mRNA expression

Rat cardiomyocytes (3.5×10^4) were incubated for 4, 8, or 24 h with medium alone, with *T. cruzi* trypomastigotes, or with trypomastigote GPIs. Total RNA was extracted from the rat cardiomyocytes by using the PeqGold Trifast kit (Peqlab), according to the manufacturer's instructions. The Verso™ cDNA kit (Thermo Fisher) was used to convert mRNA into cDNA at 42 °C for 30 min followed by a denaturation step at 95 °C for 2 min. Amplification was then performed by polymerase chain reaction (PCR) in a Mastercycler (Eppendorf) by using the Absolute™ Blue QPCR SYBR Green Mix Plus ROX Vial (Thermo Fisher) and the primer pairs 5′-GAGAAACCTGCCAAGTATGATG and 5′-TTTCTTACTCCTTGGAGGCCAT for *gapdh*, 5′-AGGTATCACCAGCCGCATAC and 5′-TGCACAATCCTTTTCATCCA for *apaf-1*, 5′-CGAGCTGATCAGAACCATCA and 5′-CTCAGCCCATCTTCTTCCAG for *bax*, 5′-ATACCTGGGCCACAAGTGAG and 5′-TGATTTGACCATTTGCCTGA for *bcl-2*, 5′-CCCCTGACATGCCTATCACT and 5′-TGTCCCAAAGGAAACACACA for *myd88*, 5′-TGTGCACCAAGAGGCTACAG and 5′-TGGCCCAGGTAGAGTGATTC for *tlr7*, and 5′-TGCAGGAGCTGAACATGAAC and 5′-GAGAGCTGGGGTGAGACTTG for *tlr9*, and 5′- GCTGCAACAGATGAGGATGA and 5′-CTATCTCGATGTCCCCTCCA for *irf-1*. The following PCR program was applied: 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. A melting curve analysis was run after final amplification via a temperature gradient from 55 to 94 °C in 0.5 °C increment steps measuring fluorescence at each temperature for a period of 10 s. All reactions were carried out in duplicate for each sample. The relative expression of a transcript was calculated as the ratio between the specific transcript level and the level of *gapdh* determined for each sample. Using the Eppendorf Realplex 1.5 software, the threshold (Ct) at which the cycle numbers were measured was adjusted to areas of

exponential amplification of the traces. The delta-Ct method was used to compare expression levels of two samples by applying the formula $2^{-(\Delta Ct\ target - \Delta Ct\ control)}$.

Statistical analysis

Means and standard deviations were calculated. The Wilcoxon's test, the one-way ANOVA test followed by the Bonferroni's multiple comparison test, or the unpaired Student's t test were adopted for statistical evaluation and $P < 0.05$ was considered significant (GraphPad Prism 5 software).

Results

T. cruzi trypomastigotes and amastigotes increase apoptosis of rat cardiomyocytes in vitro

Studies on cardiomyocyte apoptosis have shown apoptotic or anti-apoptotic effects of *T. cruzi* trypomastigotes [7, 8], while no study was conducted on the effect of amastigotes on the death of cardiac cells. In order to investigate this point, trypomastigotes and amastigotes were obtained from in vitro culture of *T. cruzi* (Fig. 1a). Extracellular trypomastigotes were collected from culture supernatant. Intracellular amastigotes were purified by using a method developed to improve yield and purity. For this, Vero cells highly infected by amastigotes were detached from the flask with a scraper and mixed with glass beads to liberate the parasites. The next step consisted in filtration on a glass wool column to obtain parasites free of cell debris (compare Fig. 1b, c, before and after filtration). By this method, more than 99 % of amastigotes were recovered. Cardiomyocytes were isolated from rat heart and incubated for 24 or 48 h with trypomastigotes or amastigotes at cell-to-parasite ratio of 1:1, 1:10, 1:20, and 1:100. Apoptotic cardiomyocytes were counted after labelling of fragmented nuclei by using the TUNEL assay (Fig. 2a). As shown in Fig. 2b (left panel), trypomastigotes of *T. cruzi* significantly induced apoptosis of rat cardiomyocytes in vitro from cell-to-parasite ratio of 1:10 at 24 h, and from cell-to-parasite ratio of 1:1 at 48 h ($P < 0.001$, ANOVA/Bonferroni). Percentages of apoptotic cardiomyocytes increased with the number of parasites ($P < 0.001$ at 24 and 48 h, ANOVA/Bonferroni) and with time ($P < 0.01$ between 24 and 48 h, Wilcoxon's test). As for trypomastigotes, apoptosis of rat cardiomyocytes was significantly increased in the presence of *T. cruzi* amastigotes compared to medium alone (Fig. 2b, right panel) from cell-to-parasite ratio of 1:10 at 24 h ($P < 0.001$, ANOVA/Bonferroni), and from cell-to-parasite ratio of 1:1 at 48 h ($P < 0.01$, ANOVA/Bonferroni). This increase also occurred in a parasite number-dependent manner ($P < 0.001$ at 24 h and $P < 0.01$ at 48 h, ANOVA/Bonferroni) and in a time-dependent manner ($P < 0.01$ between 24 and 48 h, Wilcoxon's test). Staining of nuclei with Hoechst showed the presence of amastigotes in the cytoplasm of cardiomyocytes, indicating that amastigotes are able to invade these cells in vitro (Fig. 2c). To confirm that the DNA degradation was due to an apoptotic process, activity of caspases in cardiomyocytes incubated during 24 h with trypomastigotes or amastigotes at the cell-to-parasite ratio of 1:10 was evaluated by the cleavage of their specific substrates. The results shown in Fig. 2d indicate that both trypomastigotes and amastigotes strongly activated caspase-8 compared to cardiomyocytes alone ($P < 0.001$, ANOVA/Bonferroni) and in a lesser extent caspase-3/7 and caspase-9 ($P < 0.01$, ANOVA/Bonferroni).

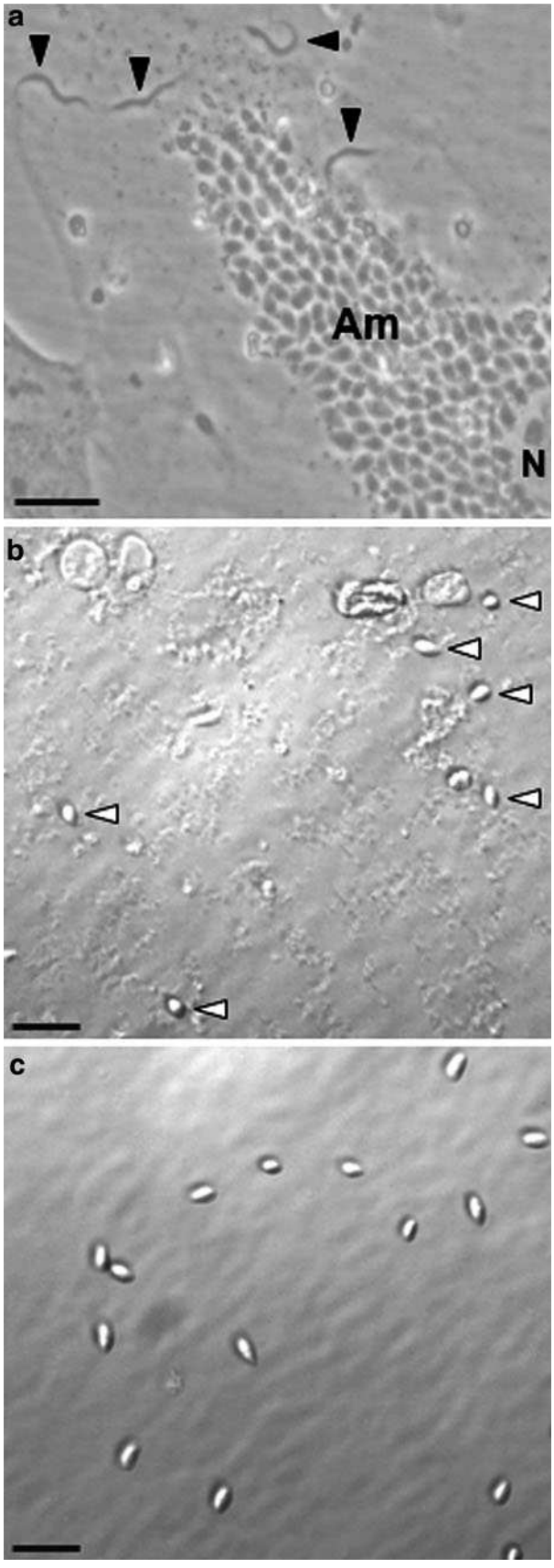
T. cruzi GPIs have no apoptotic effect and dexamethasone does not protect cardiomyocytes against trypomastigote-induced apoptosis

The surface of *T. cruzi* is covered with mucin-like structures that are anchored by glycosylphosphatidylinositols (GPIs). These glycolipids are implicated in the pathogenicity of protozoan diseases by causing inflammatory responses and other clinical symptoms [17]. It was previously shown that GPIs of *Plasmodium falciparum*, the agent of malaria, enhance apoptosis of rat cardiomyocytes in vitro [18]. In order to understand the role of GPIs in the apoptotic cell death induced by the whole parasite, GPIs were extracted from *T. cruzi* trypomastigotes by organic solvents, purified by thin layer chromatography, and subsequently tested on cells. Incubation of cardiomyocytes during 24 h with *T. cruzi* GPIs did not lead to an increase of apoptosis (Fig. 3a). In contrast to GPIs of *P. falciparum*, prolongation of the culture to 48 h did not change the result (not shown).

Endogenous glucocorticoids are protective during acute *T. cruzi* infection [19]. In addition, it has been described that pre-incubation with dexamethasone, a synthetic glucocorticoid, protects cardiomyocytes from apoptosis induced by doxorubicin [20]. Here, 18 h pre-incubation of cardiomyocytes with 1 μM dexamethasone slightly but not significantly ($P = 0.069$) reduced the trypomastigote-induced apoptosis after 24 additional hours of incubation (Fig. 3a).

T. cruzi trypomastigotes and amastigotes differently regulate the expression of apoptotic genes in cardiomyocytes

To understand the mechanisms involved in cardiomyocyte apoptosis in response to *T. cruzi*, the cells were incubated



◀ **Fig. 1** Separation of trypomastigotes and amastigotes from an in vitro culture of *T. cruzi*. **a** Free trypomastigotes (marked with *black arrowheads*) were collected from the cell culture supernatant (culture flask observed with a Zeiss Primovert microscope). Numerous amastigotes (Am) were detected in the cytoplasm of Vero host cells (*N* nucleus). **b** Free amastigotes (*white arrowheads*) scattered throughout cell debris were obtained by breaking of Vero cells with glass beads. **c** Clean suspension of intact amastigotes was obtained by elimination of Vero cell debris on a glass wool column (Malassez slide observed with a Leitz Laborlux S microscope, *bars* 20 μm)

for 2, 4, 8, or 24 h with trypomastigotes and then cardiomyocyte mRNA was extracted and transcription of regulatory genes measured by quantitative RT-PCR. A significant ($P < 0.04$) up-regulation of the pro-apoptotic gene *bax* was observed after 2 h of stimulation (Fig. 3b). This increase was still observed after 4 and 8 h but was not significant. In the same way, expression of the pro-apoptotic gene *apaf-1* was slightly increased by trypomastigotes at 2, 4, and 8 h but this increase was not significant. At each time point, the anti-apoptotic gene *bcl-2* was not modulated by trypomastigotes (Fig. 3b). In the presence of *T. cruzi* amastigotes, *bax* gene expression in cardiomyocytes was also up-regulated and the increase was significant ($P < 0.002$) at 4 h (Fig. 3c). As with trypomastigotes, *apaf-1* expression increased at 2 and 4 h but not significantly. In contrast, *bcl-2* gene expression was first significantly down-regulated ($P < 0.01$ at 2 h) and then significantly up-regulated ($P < 0.03$ at 4 h). According to the absence of apoptotic effect, GPIs of *T. cruzi* trypomastigotes did not modulate *bcl-2*, *bax*, and *apaf-1* genes (Fig. 3d).

STAT3 is activated by *T. cruzi* trypomastigotes in cardiomyocytes

Since the balance between STAT1 and STAT3 activation might determine the death or survival of cardiomyocytes during infection with *T. cruzi*, these factors of transcription were studied in early infection. For this, cardiomyocytes isolated from rat were incubated for 30 min, 1 h, or 4 h with *T. cruzi* trypomastigotes, and nuclear proteins were then extracted. Activation of STAT1 and STAT3 was determined by using a STAT transcription factor assay kit. Trypomastigotes at cell-to-parasite ratio of 1:10 or 1:100 induced a significant ($P < 0.01$, ANOVA/Bonferroni) increase of STAT3 activation in comparison with the basal STAT3 activation in cardiomyocytes in medium alone (Fig. 4a). STAT1 activation was also increased in cardiomyocytes exposed to trypomastigotes in this very early stage of infection (30 min) and also after 4 h at the 1:100 cell-to-parasite ratio, although this did not reach statistical significance (Fig. 4a). In addition, the expression of *irf-1*, a

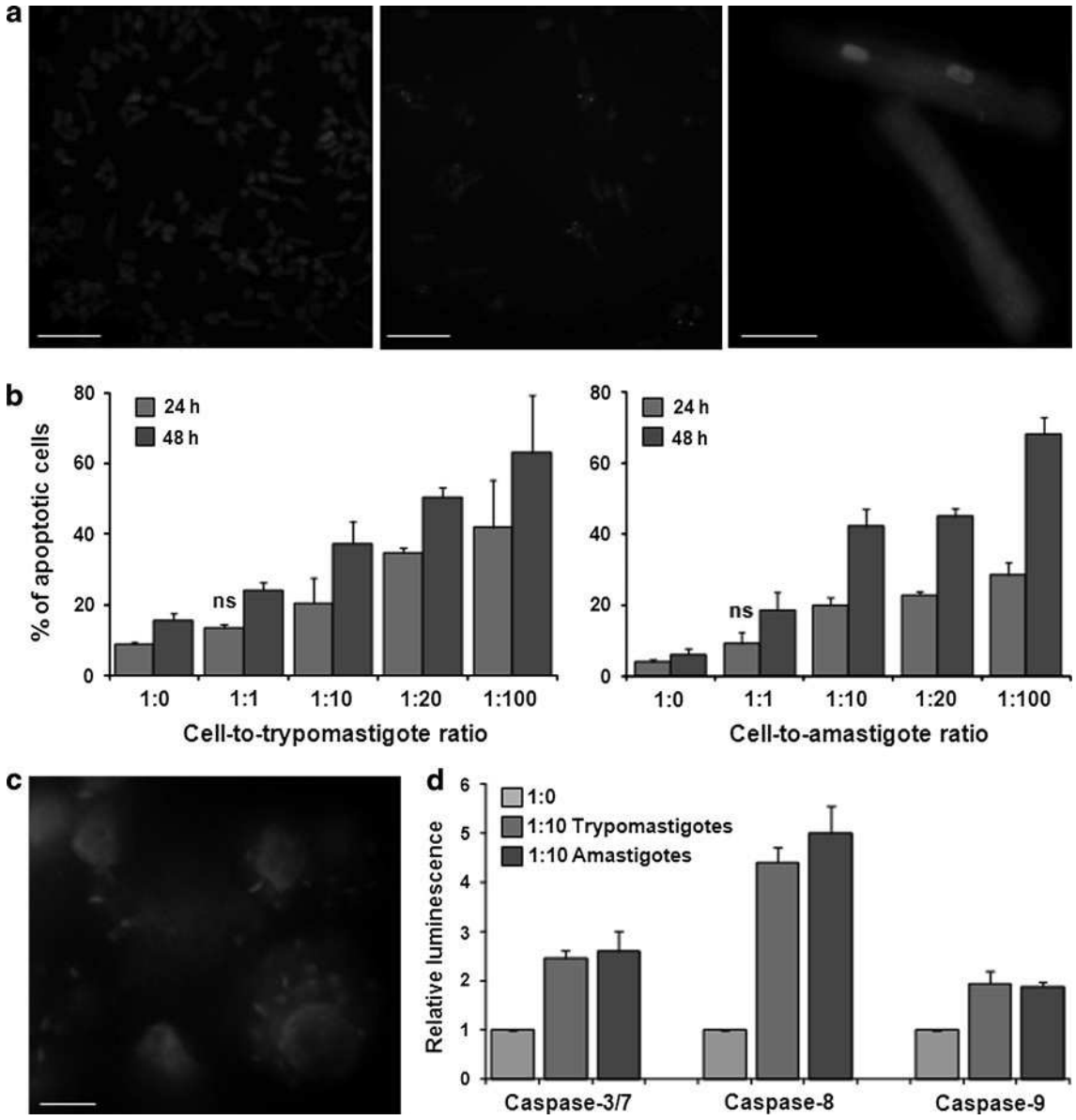
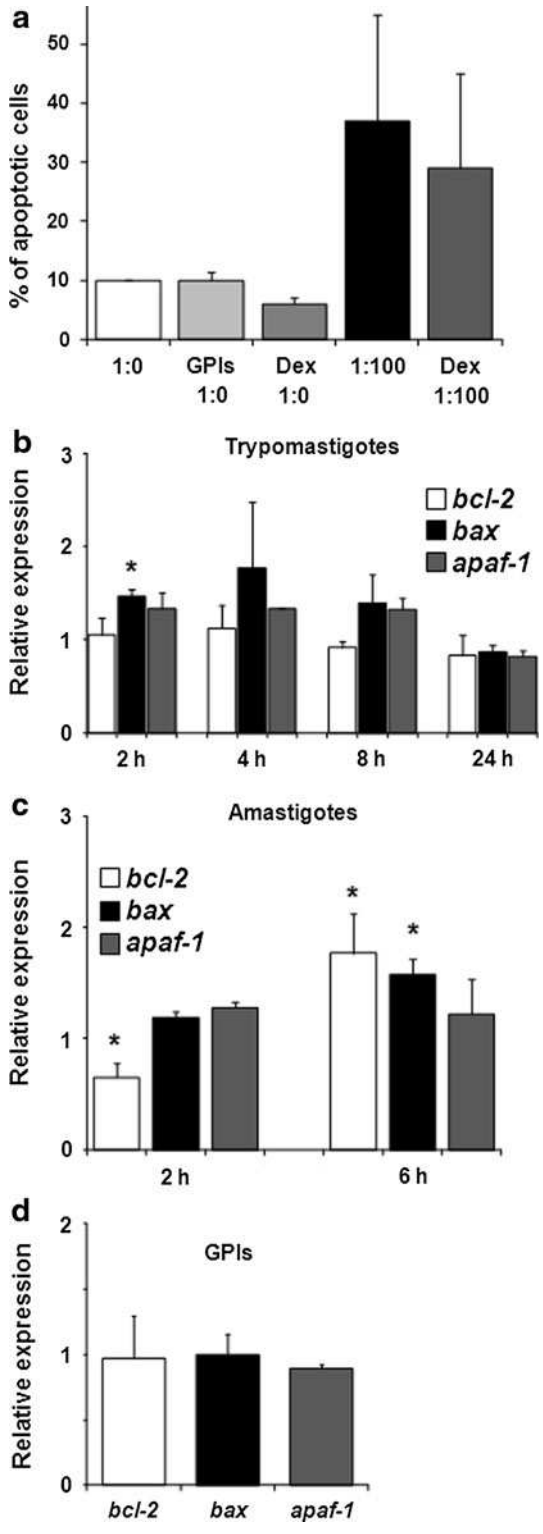


Fig. 2 *T. cruzi* trypomastigotes and amastigotes induce apoptosis and caspase activity in rat cardiomyocytes in vitro. **a** Fluorescence microscopic analysis (Zeiss AxioPhot microscope) of cardiomyocytes labelled with TUNEL reagent after 24 h incubation with cell-to-parasite ratio of 1:0 (*left panel*, *bar* 300 μm) or 1:10 (*middle* and *right panels*, *bar* 300 and 50 μm, respectively). **b** Percentage of cardiomyocyte apoptosis induced at 24 and 48 h by *T. cruzi* trypomastigotes (*left*) and amastigotes (*right*) at different cell-to-parasite ratios. Results are presented as mean ± SD from four independent experiments (*ns* non significant with ANOVA/Bonferroni). **c** Fluorescence microscopic analysis (Zeiss AxioPhot microscope) with Hoechst stain demonstrates amastigotes in the cytoplasm of cardiomyocytes (*bar* 20 μm). **d** Caspase-3/7, caspase-8, and caspase-9 activities measured in rat cardiomyocytes in vitro after 24 h incubation with *T. cruzi* trypomastigotes or amastigotes at a cell-to-parasite ratio of 1:10 relative to the caspase activities (=1) in cardiomyocytes alone (cell-to-parasite ratio of 1:0). Results are presented as mean ± SD from 3 independent experiments

gene regulated by STAT1 was not modulated in cardiomyocytes incubated for 4 and 8 h with trypomastigotes (Fig. 4b). GPIs of trypomastigotes neither affected the level of STAT3 activation (Fig. 4a) nor induced *irf-1* gene expression (Fig. 4b).

Expression of genes coding for molecules involved in the response of the innate immune system against pathogens was studied. Interestingly, *tlr7* coding for the TLR7 endosomal toll-like receptor was significantly ($P < 0.04$) up-regulated in cardiomyocytes incubated for 8 h with trypomastigotes (Fig. 4c). The expression of *tlr9* coding for another endosomal receptor was not significantly increased. These results suggest that TLR7 might be involved in the intracellular recognition of the parasite after cardiomyocyte invasion by *T. cruzi*. Expression of *myd88* occurs downstream the expression of *tlr7* and only after



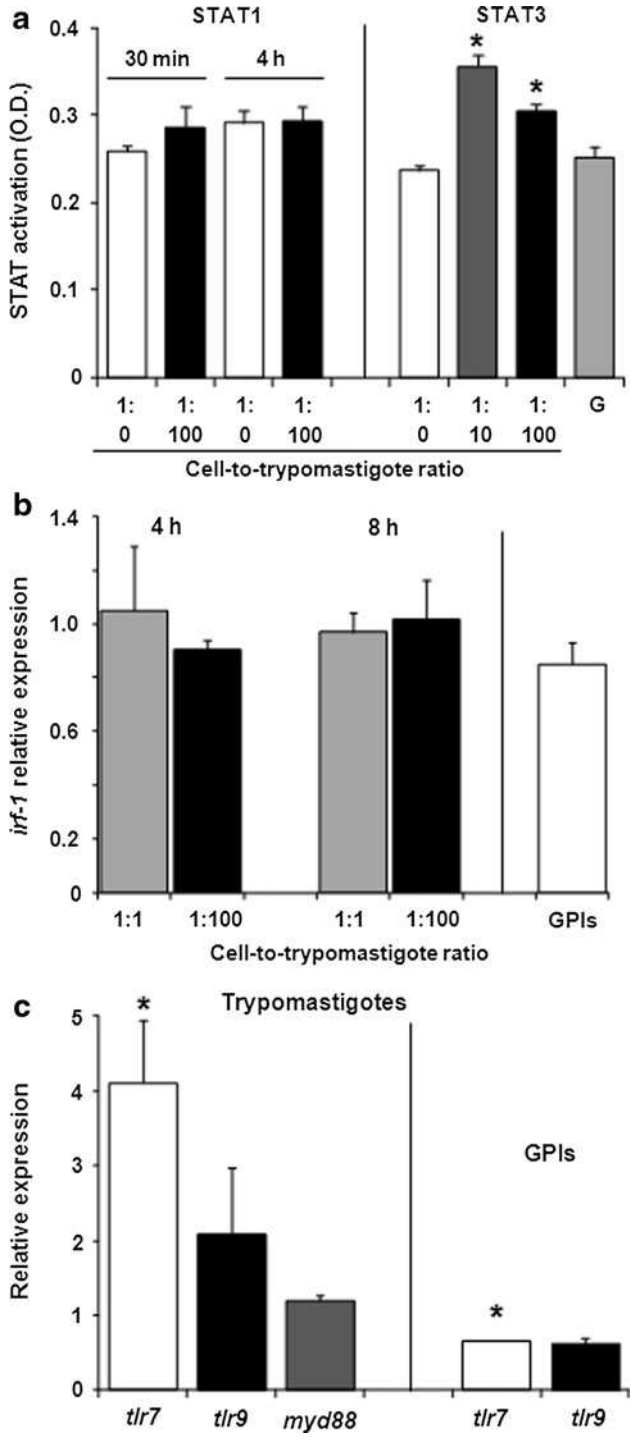
TLR7 is translated and directed to endosomes. This could explain why *tlr7* expression is higher than those of *myd88* (Fig. 4c). Trypomastigote GPIs significantly ($P < 0.03$) reduced the expression of *tlr7* but did not significantly affect *tlr9* expression (Fig. 4c), indicating that the GPIs are not responsible for the up-regulation of these two genes in response to entire trypomastigotes.

◀ **Fig. 3 a** Effect of GPIs or dexamethasone on cardiomyocyte apoptosis. Cardiomyocytes were incubated for 24 h with GPIs extracted from 5×10^8 trypomastigotes without entire parasite (GPIs 1:0), or for 42 h with trypomastigotes at cell-to-parasite ratio of 1:100 (1:100) compared to 1:0 (1:0), or for 18 h with 1 μM of dexamethasone followed by 24 h with trypomastigotes at cell-to-parasite ratio of 1:100 (Dex 1:100) compared to 1:0 (Dex 1:0). Apoptosis was determined using TUNEL reaction. Results are presented as mean ± SD from 2 independent experiments. **b–d** *T. cruzi* trypomastigotes and amastigotes but not GPIs modulate expression of *bcl-2* and *bax*. Expression of apoptosis regulating genes (*bcl-2*, *bax*, *apaf-1*) after: 2, 4, 8, and 24 h incubation with trypomastigotes at cell-to-parasite ratio of 1:100 (**b**); 2 and 6 h incubation with amastigotes at cell-to-parasite ratio of 1:20 (**c**); 8 h incubation with GPIs extracted from 5×10^8 trypomastigotes (**d**). Expression was calculated as the ratio between the specific transcript level and the level of *gapdh*; expression is relative to gene expression in cardiomyocytes cultured without parasite. Results are presented as mean ± SD from three (**b**, **c**) and two (**d**) independent experiments

Discussion

It is at present accepted that during Chagas' cardiomyopathy, heart cells can die by necrosis and also by apoptosis, and infection by *T. cruzi* parasites may result in myocardial apoptosis [21, 22]. Mouse cardiomyocyte apoptosis in response to trypomastigotes was studied by different groups giving opposite results. De Souza et al. [7] showed cardiomyocyte apoptosis after infection with different strains of *T. cruzi* at a cell-to-parasite ratio of 1:20. In contrast Aoki et al. [8] demonstrated a protective effect of *T. cruzi* due to cruzipain that acts as a survival factor for cardiomyocytes. Petersen et al. [23] showed that *T. cruzi* infection itself does not trigger apoptosis of neonatal rat ventricular myocytes. Furthermore, *T. cruzi* reduced staurosporine-, tumor necrosis factor (TNF)α-, or serum depletion-induced apoptosis of myocytes [23]. However, these two last studies did not indicate the cell-to-parasite ratio. In our study, both trypomastigotes and amastigotes at a cell-to-parasite ratio from 1:1 increased the rate of apoptosis in cardiomyocytes after 24 and 48 h of infection. Our finding of direct apoptosis is consistent with the current dogma suggesting that in the course of Chagas' disease the presence of the parasite is required to initiate the whole process from myocarditis to the development of late-stage cardiomyopathy [24].

In the study of de Souza et al. [7] the quantitative analysis obtained from TUNEL assay showed that the majority (70 %) of the apoptotic cells were infected but they also noted the presence of apoptotic uninfected cells, as well as highly infected cells not engaged in the apoptosis stage. Aoki et al. [8] reported that cardiac cells bearing



◀ **Fig. 4** STAT activation and *tlr*/*Myd88* gene expression in cardiomyocytes incubated with trypomastigotes or GPIs. **a** Cardiomyocytes were incubated with trypomastigotes with a cell-to-parasite ratio of 1:10 (dark grey) or 1:100 (*black*) compared to 1:0 (*white*), or with GPIs extracted from 5×10^8 trypomastigotes (G, *light grey*). STAT activation was detected in nuclear extracts using ELISA on oligonucleotide-bound STAT1 or STAT3 dimers. Mean ± SD from 3 independent experiments are shown. **b** Cardiomyocytes were incubated with trypomastigotes with a cell-to-parasite ratio of 1:1 (*white*) or 1:100 (*black*) or with GPIs extracted from 5×10^8 trypomastigotes. Expression of the STAT1-regulated gene *irf-1* was calculated as the ratio between the *irf-1* transcript level and the level of *gapdh* and is relative to gene expression in cardiomyocytes cultured in medium alone. Mean ± SD from three independent experiments are shown. **c** Cardiomyocytes were incubated 8 h with trypomastigotes with a cell-to-parasite ratio of 1:100 (*left*) or with GPIs extracted from 5×10^8 trypomastigotes (*right*). Expression of *tlr7*, *tlr9*, and *myd88* was calculated as the ratio between the specific transcript level and the level of *gapdh* and is relative to gene expression in cardiomyocytes cultured in medium alone. Mean ± SD from three independent experiments are shown

T. cruzi amastigotes did not exhibit apoptotic nuclei. During infection in vivo, apoptosis was observed only very rarely in myocytes that contained clusters of amastigotes [21]. In these studies, amastigotes differentiated in the cells after invasion by trypomastigotes. In our experiments, amastigotes were isolated from the cytoplasm of host cells and added as extracellular parasites to cardiomyocytes. One day later, also intracellular amastigotes were observed by microscopy. This observation indicates that amastigotes are able to invade this cell type. It was already shown that amastigotes at cell-to-parasite ratios from 1:30 to 1:65 invade cardiomyocytes through host cell heparan sulfate proteoglycans [25]. However, the authors have investigated amastigotes derived from trypomastigotes in vitro and these parasites might differ from intracellular amastigotes. Ferreira et al. [26] reviewed signalling events that occur after invasion of different host cell types by extracellular amastigotes.

The balance between pro-apoptotic and anti-apoptotic Bcl-2 family members determines the fate of a cell exposed to an apoptotic stimulus. Bax is responsible for pore formation in the mitochondria membrane through which cytochrome c is liberated. This molecule associates with Apaf-1 to activate caspase-9 that in turn activates effector caspases -3 and -7. The increase of *bax* and *apaf-1* gene expression observed in the present work tends to support the hypothesis that the mitochondrial pathway of apoptosis participates in cardiomyocyte apoptosis induced by *T. cruzi* trypomastigotes. In addition, we observed the down-regulation of the expression of *bcl-2* induced by amastigotes. These results suggest that cardiomyocyte apoptosis induced by trypomastigotes and amastigotes of *T. cruzi* might be due to distinct balance between members of the bcl-2 family. Manque and colleagues have recently studied the differential expression of 353 genes in mouse cardiomyocytes infected with trypomastigotes (cell-to-parasite ratio of 1:10) at different time points (1 to 48 h) [27]. Two pro-apoptotic genes (*bid*, *fas*) and two genes associated with cell death or survival (*gadd45b*, *phlda1*) were up-regulated in the early and intermediate phases of infection (1–12 h), while other genes associated with cell survival were also up-regulated in the late phase of the cardiomyocyte infection (24–48 h). It is thus very difficult to understand the full mechanism of survival or

apoptosis of *T. cruzi*-infected cardiomyocytes. In the report of Aoki et al. [8] the survival of myocytes by cruzipain was associated with an increase of Bcl-2 and Bcl-xL protein expression. Different corticoids including dexamethasone protect rat cardiomyocytes from apoptosis induced by doxorubicin [20]. Here we show that dexamethasone slightly but not significantly reduced trypomastigote-induced cardiomyocyte apoptosis. Activation of NF-κB and phosphorylation of p53 are increased during apoptosis induced by doxorubicin in rat cardiomyocytes [28, 29]. The anti-apoptotic effect of dexamethasone in cardiomyocytes treated with doxorubicin might be due to an inhibition of p53 phosphorylation and NF-κB activation.

In this study, we found that the transcription factor STAT3 was activated in cardiomyocytes in the very early stage of infection with trypomastigotes. The very low increase in STAT1 activation may be due to the short time of measurement after the addition of parasites. It was demonstrated that different STAT3-activating cytokines, such as leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), and IL-6, promote cardiomyocyte survival by increasing expression of cardioprotective genes including *bcl-2* and *bcl-x* [30–32]. Thus, a transcription factor other than STAT3 should be responsible for *bax* and *apaf-1* expression in *T. cruzi*-infected cardiomyocytes.

Toll-like receptors play an important role in the recognition of pathogens and several groups have studied the survival of mice lacking TLRs or the adaptor MyD88 after inoculation with *T. cruzi*. Parasitemia was dramatically increased in MyD88$^{-/-}$ mice infected with *T. cruzi* and associated with accelerated and enhanced mortality, reaching 100 % in about three weeks [11]. Except for days 8 and 9 post-infection, parasitemia was almost not increased in TLR2$^{-/-}$ mice [11]. The TLR4-mutant C3H/HeJ mice died 16 days post-infection with the number of trypomastigotes dramatically increased compared to wild type C3H/HeN mice that typically survived an additional 3–7 days [12]. TLR9$^{-/-}$ animals presented elevated parasite numbers in the blood, which increased in TLR2/9$^{-/-}$ double knock-out mice. The TLR2/9$^{-/-}$ mice were even more susceptible than the TLR9$^{-/-}$ mice [13]. The enhanced susceptibility to *T. cruzi* infection was associated with decreased serum levels of IL-12 and IFN-γ in the TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice. TLR2/9$^{-/-}$ and MyD88$^{-/-}$ mice displayed similar numbers of circulating parasites in the blood, but Myd88$^{-/-}$ mice succumbed by day 30 post-infection, while only 40–50 % of the TLR2/9$^{-/-}$ animals died in a 50-day period [13]. The authors argue for the involvement of additional MyD88-dependent TLR/IL-1R family member(s) in the control of mortality due to *T. cruzi* infection. In a more recent study, TLR9$^{-/-}$ and TLR7$^{-/-}$ mice presented similar susceptibility to *T. cruzi* with 100 % mortality after less than 35 days post-infection [14]. A critical role for TLR7 in induction of protective IL-12 during *T. cruzi* infection was found [14]. The authors suggested that parasites internalized by professional phagocytic cells (dendritic cells and macrophages) are destroyed, releasing RNA, which activates TLR7 in the phagolysosomes. At a transcriptional level, we have observed here an up-regulation of *tlr7* expression and to a lesser extent of *tlr9* expression in cardiac myocytes in response to infection with *T. cruzi* trypomastigotes. This suggests that TLR7 might be involved in the intracellular recognition of trypomastigotes in this cell type.

Different studies were performed in order to identify the PAMPs (pathogen-associated molecular patterns) of *T. cruzi* that trigger TLR signalling. Tc52 is a *T. cruzi* protein that binds to dendritic cells and signals via TLR2 in these cells [33]. All stages of *T. cruzi* contain unmethylated CpG motifs in genomic DNA whose pro-inflammatory activity has been shown to be mediated by TLR9 [13]. *T. cruzi* trypomastigotes synthesize GPIs containing unsaturated alkylacylglycerols recognized by TLR2/6 complexes, while epimastigotes have GIPLs containing ceramides, recognized by TLR4 [12, 34]. TLR2 is essential for the induction of IL-12, TNF-α, and nitric oxide (NO) by macrophages in response to *T. cruzi* GPIs [34]. Furthermore, GPIs of *T. cruzi* act in synergy with the DNA of the parasite (recognized by TLR9) in the induction of cytokines by macrophages [13]. GPIs of *P. falciparum* increase apoptosis of rat cardiomyocytes in vitro [18]. Cardiomyocytes treated with GPIs of *P. falciparum* showed an up-regulation of *apaf-1* and *bax* and a higher expression of these two apoptotic genes in relation to *bcl-2* [18]. In contrast, we have shown here that GPIs of *T. cruzi* are not able to induce cardiomyocyte death and to modulate the expression of *bcl-2*, *bax*, and *apaf-1* genes in these cells. The biological activity of the GPIs was checked by the capacity to induce TNF-α production by macrophages (not shown). A strong TNF-α secretion could contribute to cardiomyocyte death in vivo through activating the TNFR/caspase-8/-10 extrinsic pathway of apoptosis. Even though GPIs of *T. cruzi* do not have per se apoptotic effects in isolated cardiomyocytes, there is the possibility that they participate in apoptosis through the induction of inflammatory cytokines by cells of the innate immune system. GPIs neither activated STAT1 nor STAT3 in cardiomyocytes but down-regulated *tlr7* gene expression. Thus, we can conclude that parasite molecules other than GPIs are responsible for the effects obtained with entire parasites.

Acknowledgments This work was supported by the Deutsche Stiftung für Herzforschung (to F.D.-G., R.T.S., and B.M.), Alumni Medizin Marburg e.V. (to P.S.), Roland und Elfriede Schauer-Stiftung (to P.S.), Studienstiftung des Deutschen Volkes (to P.S.), BMBF BRA 07/046 (to R.T.S. and M.A.C.), NIH 1RO1AI071319-01 (to R.T.G.), INCTV/CNPq 573547/2008-4/FAPEMIG CBB—APQ-00077-09 (to R.T.G. and M.A.C.), FAPEMIG CBB—APQ-01603-09 (to M.A.C.), and the program PROBRAL of DAAD/CAPES (to R.T.S. and R.T.G.).

References

1. Rossi MA, Bestetti RB (1995) The challenge of chagasic cardiomyopathy. The pathologic roles of autonomic abnormalities, autoimmune mechanisms and microvascular changes, and therapeutic implications. Cardiology 86:1–7
2. Parada H, Carrasco HA, Anez N, Fuenmayor C, Inglessis I (1997) Cardiac involvement is a constant finding in acute Chagas' disease: a clinical, parasitological and histopathological study. Int J Cardiol 60:49–54
3. Rassi A Jr, Rassi A, Little WC (2000) Chagas' heart disease. Clin Cardiol 23:883–889
4. Higuchi Mde L, Benvenuti LA, Martins Reis M, Metzger M (2003) Pathophysiology of the heart in Chagas' disease: current status and new developments. Cardiovasc Res 60:96–107
5. Palomino SA, Aiello VD, Higuchi ML (2000) Systematic mapping of hearts from chronic chagasic patients: the association between the occurrence of histopathological lesions and *Trypanosoma cruzi* antigens. Ann Trop Med Parasitol 94:571–579
6. Tostes S Jr, Bertulucci Rocha-Rodrigues D, de Araujo Pereira G, Rodrigues V Jr (2005) Myocardiocyte apoptosis in heart failure in chronic Chagas' disease. Int J Cardiol 99:233–237
7. de Souza EM, Araujo-Jorge TC, Bailly C, Lansiaux A, Batista MM, Oliveira GM, Soeiro MN (2003) Host and parasite apoptosis following *Trypanosoma cruzi* infection in in vitro and in vivo models. Cell Tissue Res 314:223–235
8. Aoki MP, Guinazu NL, Pellegrini AV, Gotoh T, Masih DT, Gea S (2004) Cruzipain, a major *Trypanosoma cruzi* antigen, promotes arginase-2 expression and survival of neonatal mouse cardiomyocytes. Am J Physiol Cell Physiol 286:C206–C212
9. Ley V, Andrews NW, Robbins ES, Nussenzweig V (1988) Amastigotes of *Trypanosoma cruzi* sustain an infective cycle in mammalian cells. J Exp Med 168:649–659
10. Barry SP, Townsend PA, Latchman DS, Stephanou A (2007) Role of the JAK-STAT pathway in myocardial injury. Trends Mol Med 13:82–89
11. Campos MA, Closel M, Valente EP, Cardoso JE, Akira S, Alvarez-Leite JI, Ropert C, Gazzinelli RT (2004) Impaired production of proinflammatory cytokines and host resistance to acute infection with *Trypanosoma cruzi* in mice lacking functional myeloid differentiation factor 88. J Immunol 172:1711–1718
12. Oliveira AC, Peixoto JR, de Arruda LB, Campos MA, Gazzinelli RT, Golenbock DT, Akira S, Previato JO, Mendonca-Previato L, Nobrega A, Bellio M (2004) Expression of functional TLR4 confers proinflammatory responsiveness to *Trypanosoma cruzi* glycoinositolphospholipids and higher resistance to infection with *T. cruzi*. J Immunol 173:5688–5696
13. Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A (2006) Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in *Trypanosoma cruzi* infection. J Immunol 177:3515–3519
14. Caetano BC, Carmo BB, Melo MB, Cerny A, dos Santos SL, Bartholomeu DC, Golenbock DT, Gazzinelli RT (2011) Requirement of UNC93B1 reveals a critical role for TLR7 in host resistance to primary infection with *Trypanosoma cruzi*. J Immunol 187: 1903–1911
15. Ruppert V, Meyer T, Pankuweit S, Moller E, Funck RC, Grimm W, Maisch B (2008) Gene expression profiling from endomyocardial biopsy tissue allows distinction between subentities of dilated cardiomyopathy. J Thorac Cardiovasc Surg 136(360–369):e361
16. Heidecker B, Kittleson MM, Kasper EK, Wittstein IS, Champion HC, Russell SD, Hruban RH, Rodriguez ER, Baughman KL, Hare JM (2011) Transcriptomic biomarkers for the accurate diagnosis of myocarditis. Circulation 123:1174–1184
17. Debierre-Grockiego F, Schwarz RT (2010) Immunological reactions in response to apicomplexan glycosylphosphatidylinositols. Glycobiology 20:801–811
18. Wennicke K, Debierre-Grockiego F, Wichmann D, Brattig NW, Pankuweit S, Maisch B, Schwarz RT, Ruppert V (2008) Glycosylphosphatidylinositol-induced cardiac myocyte death might contribute to the fatal outcome of *Plasmodium falciparum* malaria. Apoptosis 13:857–866
19. Roggero E, Perez AR, Tamae-Kakazu M, Piazzon I, Nepomnaschy I, Besedovsky HO, Bottasso OA, del Rey A (2006) Endogenous glucocorticoids cause thymus atrophy but are protective during acute *Trypanosoma cruzi* infection. J Endocrinol 190:495–503
20. Chen QM, Alexander D, Sun H, Xie L, Lin Y, Terrand J, Morrissy S, Purdom S (2005) Corticosteroids inhibit cell death induced by doxorubicin in cardiomyocytes: induction of antiapoptosis, antioxidant, and detoxification genes. Mol Pharmacol 67:1861–1873
21. Zhang J, Andrade ZA, Yu ZX, Andrade SG, Takeda K, Sadirgursky M, Ferrans VJ (1999) Apoptosis in a canine model of acute chagasic myocarditis. J Mol Cell Cardiol 31:581–596
22. Henriques-Pons A, Oliveira GM, Paiva MM, Correa AF, Batista MM, Bisaggio RC, Liu CC, Cotta-De-Almeida V, Coutinho CM, Persechini PM, Araujo-Jorge TC (2002) Evidence for a perforin-mediated mechanism controlling cardiac inflammation in *Trypanosoma cruzi* infection. Int J Exp Pathol 83:67–79
23. Petersen CA, Krumholz KA, Carmen J, Sinai AP, Burleigh BA (2006) *Trypanosoma cruzi* infection and nuclear factor kappa B activation prevent apoptosis in cardiac cells. Infect Immun 74:1580–1587
24. Tarleton RL, Zhang L (1999) Chagas disease etiology: autoimmunity or parasite persistence? Parasitol Today 15:94–99
25. Bambino-Medeiros R, Oliveira FO, Calvet CM, Vicente D, Toma L, Krieger MA, Meirelles MN, Pereira MC (2012) Involvement of host cell heparan sulfate proteoglycan in *Trypanosoma cruzi* amastigote attachment and invasion. Parasitology 138:593–601
26. Ferreira ER, Bonfim-Melo A, Mortara RA, Bahia D (2012) *Trypanosoma cruzi* extracellular amastigotes and host cell signaling: more pieces to the puzzle. Front Immunol 3:363
27. Manque PA, Probst CM, Pereira MC, Rampazzo RC, Ozaki LS, Pavoni DP, Silva Neto DT, Carvalho MR, Xu P, Serrano MG, Alves JM, Meirelles Mde N, Goldenberg S, Krieger MA, Buck GA (2011) *Trypanosoma cruzi* infection induces a global host cell response in cardiomyocytes. Infect Immun 79:1855–1862
28. Wang S, Kotamraju S, Konorev E, Kalivendi S, Joseph J, Kalyanaraman B (2002) Activation of nuclear factor-kappaB during doxorubicin-induced apoptosis in endothelial cells and myocytes is pro-apoptotic: the role of hydrogen peroxide. Biochem J 367: 729–740
29. Liu J, Mao W, Ding B, Liang CS (2008) ERKs/p53 signal transduction pathway is involved in doxorubicin-induced apoptosis in H9c2 cells and cardiomyocytes. Am J Physiol Heart Circ Physiol 295:H1956–H1965
30. Fujio Y, Kunisada K, Hirota H, Yamauchi-Takihara K, Kishimoto T (1997) Signals through gp130 upregulate bcl-x gene expression via STAT1-binding cis-element in cardiac myocytes. J Clin Invest 99:2898–2905
31. Harada M, Qin Y, Takano H, Minamino T, Zou Y, Toko H, Ohtsuka M, Matsuura K, Sano M, Nishi J, Iwanaga K, Akazawa H, Kunieda T, Zhu W, Hasegawa H, Kunisada K, Nagai T, Nakaya H, Yamauchi-Takihara K, Komuro I (2005) G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. Nat Med 11:305–311
32. Ponce NE, Cano RC, Carrera-Silva EA, Lima AP, Gea S, Aoki MP (2012) Toll-like receptor-2 and interleukin-6 mediate

cardiomyocyte protection from apoptosis during *Trypanosoma cruzi* murine infection. Med Microbiol Immunol 201:145–155

33. Ouaissi A, Guilvard E, Delneste Y, Caron G, Magistrelli G, Herbault N, Thieblemont N, Jeannin P (2002) The *Trypanosoma cruzi* Tc52-released protein induces human dendritic cell maturation, signals via Toll-like receptor 2, and confers protection against lethal infection. J Immunol 168:6366–6374
34. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procopio DO, Travassos LR, Smith JA, Golenbock DT, Gazzinelli RT (2001) Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol 167:416–423