Original Article

Trypanosoma cruzi down-regulates mechanosensitive proteins in cardiomyocytes

Tatiana G. Melo[a*], Daniel Adesse[b], Maria de Nazareth Meirelles[a†], Mirian Claudia S. Pereira[a]

a) Laboratório de Ultraestrutura Celular, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro RJ/ Brasil.
b) Laboratório de Biologia Estrutural, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro RJ/Brasil

†: *in memorian*

*: Corresponding author: tatybio@ioc.fiocruz.br

https://orcid.org/0000-0003-2831-4004

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ABSTRACT

BACKGROUND Cardiac physiology depends on coupling and electrical and mechanical coordination through the intercalated disc. Focal adhesions offer mechanical support and signal transduction events during heart contraction-relaxation processes. Talin links integrins to the actin cytoskeleton and serves as a scaffold for the recruitment of other proteins, such as paxillin in focal adhesion formation and regulation. Chagasic cardiomyopathy is caused by infection by *Trypanosoma cruzi* and is a debilitating condition comprising extensive fibrosis, inflammation, cardiac hypertrophy and electrical alterations that culminate in heart failure. *OBJECTIVES* Since mechanotransduction coordinates heart function, we evaluated the underlying mechanism implicated in the mechanical changes, focusing especially in mechanosensitive proteins and related signaling pathway during infection of cardiac cells by *T. cruzi*. *METHODS* We investigated the effect of *T. cruzi* infection on the expression and distribution of talin/paxillin and associated proteins in mouse cardiomyocytes *in vitro* by western blotting, immunofluorescence and quantitative RT-PCR.

FINDINGS Talin and paxillin spatial distribution in *T. cruzi*-infected cardiomyocytes *in vitro* were altered, associated with a downregulation of these proteins and mRNAs levels at 72 hours post-infection (hpi). Additionally, we observed an increase in the activation of the focal adhesion kinase (FAK) concomitant with increase in □-1-integrin at 24 hpi. Finally, we detected a decrease in the activation of FAK at 72 hpi in *T. cruzi*-infected cultures. *MAIN CONCLUSION* The results suggest that these changes may contribute to the mechanotransduction disturbance evidenced in chagasic cardiomyopathy.

Keywords: Cardiomyocytes; *Trypanosoma cruzi*; focal adhesion; talin; paxillin; mechanotransduction.

INTRODUCTION

Trypanosoma cruzi is the etiological agent of Chagas' disease, also known as American trypanosomiasis, a disorder that affects 7 million people worldwide. Even after 109 years of its discovery, Chagas' disease remains neglected and a serious public health problem. Currently, this disease, found mainly in endemic areas in Latin America, spreads worldwide due to migrating populations [(1)]. The parasite displays a transmission cycle that involves both an invertebrate and a vertebrate host. An infected hematophagous triatomine vector releases the infective metacyclic trypomastigote forms of *T. cruzi* in its feces in the vertebrate host during its blood meal. The parasite rapidly invades local host cells and transforms into the replicative amastigote form. After intense replication within the host cell cytoplasm, the parasites differentiate back to trypomastigotes and promote cell lysis, reaching the bloodstream and disseminating the infection. Acute *T. cruzi* infection leads to focal myocarditis with accompanying necrosis of infected myocytes and reparative interstitial fibrosis. During the chronic disease, parasitemia drastically decreases and parasites are barely detected. However, 30-40% of individuals can develop chronic chagasic cardiomyopathy (CCC), a debilitating condition comprising extensive fibrosis, inflammation, heart enlargement and arrhythmias that culminate in heart failure [(2)].

Arrhythmogenic cardiomyopathies, such as CCC, can also be considered intercalated disc disorders, since cardiac physiology depends on the integrity of such structures for synchronized firing and contraction. Cardiac myocytes are coupled and coordinated through an intercalated disc (ID), a junctional platform of structural and signaling molecules, such as gap and adherens junction proteins [3]. In addition, cardiac physiology mechanobiology also involves mechanical focal adhesion properties, providing force, elasticity and signal transduction [4]. Focal adhesions are points where extracellular matrix (ECM) components associate to the actin cytoskeleton through surface receptors, mainly integrin, and are regulated by the focal adhesion kinase (FAK) signaling pathway. FAK is a nonreceptor protein tyrosine kinase, activated mainly by integrin-dependent manner, containing a central kinase domain flanked by N- and C-terminal extensions. FAK presents an autoinhibitory FERM domain, located within the N-terminal region of FAK, which associates with the plasma membrane via its interaction with different receptors. The C-terminal region of FAK comprises the focal adhesion targeting (FAT) domain that binds directly to paxillin and talin, which in turn binds to the cytoplasmic tail of β_1-integrins, modulating bi-directional signal transduction [5].

Focal adhesion sites offer mechanical support and signal transduction events during heart contraction-relaxation processes. A set of mechanosensitive adapter proteins regulates cell–ECM and cell-cell adhesions during mechanical force transmission [4]. Talin, a 50 kDa mechanosensing protein, has been shown to provide the primary link between integrin and actin cytoskeleton, as well as to participate as a scaffold for the recruitment of other proteins in focal adhesion formation[6]. Talin structure consists of a globular N-terminal head and a large C-terminal rod that form an antiparallel homodimer. A FERM domain at the N-terminal head comprises a high-affinity binding site for the integrin β cytoplasmic domain while the rod domain (220 kDa) links to actin, vinculin and also paxillin. However, talin binding to paxillin depends on paxillin phosphorylation and their association plays an important role in focal adhesion regulation [7]. Paxillin (68 kDa), a focal adhesion protein that was

earlier observed in nascent focal adhesions at the cell periphery, is a molecular adaptor or multi-domain scaffold protein that can be phosphorylated on Tyr-31 and Tyr-118 in a FAK/Src-dependent manner. Paxillin localizes to the intracellular surface of focal adhesion sites that interact with multiple signaling pathways, recruiting diverse structural and regulatory proteins. The C-terminal half of paxillin contains four double-zinc finger motifs, called the LIM domain, important in protein-protein interactions. The phosphorylation of LIM domains (LIMs 2 and 3) is required for targeting paxillin to focal adhesion. The N-terminal paxillin domain presents five leucine and aspartate rich-LD motifs that mediate proteins interactions, such as focal adhesion kinase and vinculin, and contains tyrosine, serine and threonine phosphorylation sites that coordinate signaling [8]. Moreover, the N-terminal domain also comprises a proline-rich region that interacts with the vinculin-binding protein ponsin and contributes to the formation of costameres in cardiac muscle [9]. One striking fact is that a disturbance in cardiac structural components [10], including costameres [11] and junctional complexes, such as gap and adherens junctions [12, 13], have been evidenced in *T. cruzi* infection and may contribute to the severity of the cardiomyopathy. Given the mechanosensitivity of talin and paxillin and their participation in cardiac mechanotransduction, we evaluated the expression and distribution of talin/paxillin and proteins associated during infection of cardiac cells by *T. cruzi*. Changes in this mechanosensing induced by the infection may alter the force balance across cell-extracellular matrix interaction.

METHODS

1. Cell culture and T. cruzi infection

Cardiac muscle cells isolated from 18-day-old mouse embryos in a collagenase/trypsin solution, were plated into 24-wells for immunofluorescence or in 60 mm^2 culture dishes for biochemical analyses. Cells were maintained in DMEM with 10% fetal bovine serum (FBS; Cultilab, São Paulo, Brazil), 2.5 mM $CaCl_2$, 1 mM L-glutamine (Sigma), 2% chicken embryo extract and 1% Penicillin/Streptomycin solution (Life Technologies, São

Paulo, Brazil) at 37 ºC in a 5% CO_2 atmosphere. All procedures with animals were approved by the Oswaldo Cruz Institute Committee for the Use of Laboratory Animals (license LW-37/13, Oswaldo Cruz Foundation).

Trypomastigote forms of *T. cruzi*, Y strain (MHOM/BR/00/Y), were obtained from Swiss Webster mice at the parasitemia peak, as described in. Cardiac cells were infected at a multiplicity of 10 parasites per host cell (10:1). After 24 h of interaction, free trypomastigotes were removed by washes with Ringer's solution (154 mM NaCl, 56 mM KCl, 225 mM $CaCl_2$ pH 7.0). The infection was interrupted after 24 and 72 hours post infection (hpi).

2. Indirect Immunofluorescence

Cells were fixed for 20 min at 4 °C with 4% paraformaldehyde (Sigma Aldrich, São Paulo, Brazil) in PBS. Primary antibodies were incubated overnight at 4 ºC anti-talin (Santa Cruz Biotechnology, Dallas, TX 1:20), anti-paxillin (Santa Cruz Biotechnology, 1:100), anti-FAK (Santa Cruz Biotechnology, 1:50), or anti-phosphorylated FAK (Invitrogen, 1:200), followed by incubation with secondary anti-mouse IgG-AlexaFluor555 (ThermoFisher). F-actin was visualized with AlexaFluor 488-labeled Phalloidin (ThermoFisher) and DNA was detected with To-PRO-3 Iodide (Life Technologies, São Paulo, Brazil) or DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma Aldrich). Slides were mounted and analyzed using a confocal laser scanning microscope LSM 510 META (Zeiss) or Zeiss AxioImage M2 microscope with the Apotome system.

3. Protein extraction and immunoblotting assay

Proteins were extracted in 50 mM Tris-HCl containing 1% Triton x-100, protease inhibitors: 10 µM E-64 (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma) and 1 µM pepstatin (Sigma), pH 8.0. A total of 20 µg of protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad, California, USA) with a transfer buffer

(25 mM Tris, 192 mM glycine and 10% methanol, pH 8.3). Membranes were incubated overnight with anti-talin (1:200), anti-paxillin (1:1000), anti-FAK (1:500), anti-pFAK (1000) or anti-β1-integrin (1:1000) antibodies diluted in blocking solution (5% nonfat dry milk or 1% BSA). Anti-GAPDH antibody (Ambion) was used as the loading control. Secondary anti-rabbit IgG or anti-mouse IgG HRP-labeled antibodies (1:5000) were incubated, revealed by chemoluminescence (PIERCE) and exposed to X-ray films. Densitometric analyses were performed using the ImageJ software. The immunoblotting experiments were performed independently three times.

4. Real time PCR analysis

Total RNA was extracted with Trizol (Life Technologies, São Paulo, Brazil). One microgram of RNA was reversely transcribed into cDNA using Superscript III kit (Life Technologies). DNA contamination was excluded by prior treatment with DNase I (Qiagen). Real-time PCR was performed using Taqman gene expression assays (Life Technologies) for talin and paxillin (Mm00659397_m1, Mm00448533_m1). Gapdh (Mm99999915_g1) was used as the normalizing control. A total of 0.5 □l of cDNA was used in triplicate for each primer assay. Relative quantitative analyses were performed using the $2^{-\Delta\Delta Ct}$ method.

5. Statistical analyses

Student's t-test was used to determine the significance of differences between mean values from at least three independent assays. A p value ≤ 0.05 was considered significant.

RESULTS

The distribution of focal adhesion proteins was analyzed in cardiomyocytes at the early (24 h) and late (72 h) stages of *T. cruzi* infection *in vitro*. Terminally differentiated cardiac

myocytes were identified by abundant striation as shown by F-actin staining (Figures 2, 4 and 5). First, we evaluated the spatial localization of talin and paxillin, mechanosensing and mechanosignaling proteins respectively, in non-infected cardiomyocytes. The immunofluorescence analyses revealed both talin and paxillin located at the sites of cell-substrate adhesion (Figures 1 and 2). In addition, talin immunostaining appears as a striated pattern in cardiomyocytes (Figure 1). Double labelling of paxillin and actin filament also revealed paxillin anchoring the ends of myofibrils in cardiomyocytes (Figure 2). Few intracellular parasites were visualized in the host cell cytoplasm by To-PRO-3 Iodide or DAPI staining during the initial times of infection (24 hpi), (Figure 1 and 2). At this time, no alterations were noted concerning focal adhesion protein distribution. Striated talin labelling, linking the myofibrils to sarcolemma, and also its location at the focal adhesion sites, were clearly seen at 24 hpi., Paxillin staining was also unaltered showing intense fluorescence signal at the ends of actin filaments, near the edges of the cells (Figure 2). On the other hand, with the progression of the intracellular *T. cruzi* cycle (72 hpi), both talin and paxillin demonstrated changes in their spatial distribution. Highly infected cells showed a drastic reduction of these focal adhesion proteins at attachment sites (Figures 1 and 2). Interestingly, a strong positive reactivity was observed in intracellular amastigotes by the anti-paxillin antibody. Additionally, no costameric talin staining was observed at 72 hpi (Figure 1). These findings led us to question whether the structural changes at the focal adhesion proteins were associated with protein level alterations. Thus, we analyzed talin and paxillin expressions during the course of infection by *T. cruzi*. After 24 hpi, the expression of both focal adhesion proteins remained unaffected, displaying protein levels comparable to control cells (Figures 1 and 2). The immunoblotting analysis revealed a significant decrease of both proteins at the later stage of infection (72 hpi). Reductions in protein content reached 32% and 34% in talin and paxillin expression, respectively (Figures

1F and 2K). Next, we assessed whether the disturbances observed in protein levels correlated with transcriptional gene regulation. Quantitative Real-Time PCRs performed for *talin* and *paxillin* transcripts revealed that *T. cruzi* infection affects the expression of these genes, leading to a 23% and 26% reduction at 72 hpi, respectively. (Figures 1G and 2L). We also evaluated whether other proteins associated with talin and paxillin organization, such as integrin, a transmembrane protein, and focal adhesion kinase (FAK), which regulates integrin-mediated mechanotransduction, also undergo alterations during structural talin/paxillin disruption. Integrin and pFAK, corresponding to FAK activation, displayed 38% and 50% upregulation, respectively, at the early stage of infection (24 hpi). Interestingly, a downregulation of 30% in pFAK expression was noticed at 72 hpi but no change was seen in β1-integrin and total FAK levels. (Figure 3). Both total and activated FAK (FAK-Tyr^{397}) were also revealed by immunofluorescence. Intense FAK and pFAK staining, detected as punctate dots, was distributed throughout the uninfected cardiomyocytes cytoplasm (Figures and 5). Infected cultures (24 and 72hpi) had similar immunoreactivity profile for total FAK as compared to controls. In contrast, pFAK signal was greatly reduced in highly parasitized cells at 72 hpi (Figure 5). Myofibrillar breakdown was consistently observed in highly infected cells at 72 hpi (Figures 2, 4 and 5).

DISCUSSION

Mechanosignaling has been highlighted as a key feature in cardiac homeostasis. Changes in mechanosensing proteins, responsible for balancing the mechanical force between cells and their microenvironment, seem to alter mechanotransduction in pathophysiological responses [14]. Clinical manifestations associated to biomechanical stress, such as hypertrophy, arrhythmia and heart failure, have been evidenced in cardiac diseases, including Chagas disease [15], which emphasizes the role of physical myocardium

properties in pathological process regulations. Thus, based on the fact that changes in integrin signaling, through adhesion-dependent adapter and signaling molecules, lead to abnormalities in cardiac performance, we investigated the effect of *T. cruzi* infection on mechanosensitive proteins in cardiomyocytes.

Talin, a non-channel type protein that acts as mechanosensor, is involved in contraction force transmission by modulating cytoskeleton-integrin-extracellular matrix interaction and also triggers downstream signaling by recruiting paxillin and others mechanosensing proteins [16]. As expected, our confocal microscopy analysis revealed talin in a striated pattern in cardiomyocytes. This finding is consistent with the presence of multiple binding sites for vinculin within the folded talin rod domain and its location in costameres as an integrin-talin-vinculin complex, allowing the transduction force from sarcomere to extracellular matrix. In fact, vinculin is essential in focal adhesion and its activation and nano-scale spatial localization depends on the association between talin and paxillin [17]. Additionally, paxillin location at the cell-substrate attachment site also corroborates its involvement in the regulation of focal adhesion dynamics. It has been demonstrated that paxillin phosphorylation modulates its interface with talin and triggers FAK signaling.

At the earliest infection time (24 hpi) talin and paxillin presented no changes in their expression and distribution pattern. However, the significant increase of FAK activity concomitant with β1-integrin expression may be related to the parasite invasion process, as reported previously [18]. The interaction of trypomastigotes, free in the culture supernatant, with integrin receptors may induce integrin clusters and trigger FAK signaling pathway to promote parasite entry.

A remarkable finding is the lack of talin and paxillin intracellular distribution and their downregulation in cardiomyocytes induced by *T. cruzi* infection (72h). Our data demonstrate

that both mechanosensitive proteins, talin and paxillin, exhibited reduced expression levels after 72 h of infection along with massive loss of immunoreactivity in highly infected cells. Surprisingly, intracellular parasites were also reactive with the anti-paxillin antibody. Although no match for paxillin or paxillin-like proteins was identified in the *T. cruzi* genome by a BLAST analysis (http://www.dbbm.fiocruz.br/TcruziDB/), the positive cross-reaction may be related to similarities (30%) with a mucin associated surface protein (MASP).

Several studies have pointed out the ability of many pathogens to modulate focal adhesion proteins. It has been recently demonstrated that *Leishmania amazonensis* infection disturbs macrophages migration by altering actin dynamics [(19)]. Inhibition of macrophage motility seems to be driven by downregulation of paxillin and FAK phosphorylation, suggesting that the reduction of migration is responsible for the retention of *L. amazonensis*-infected macrophages in the cutaneous lesion. Although the mechanisms involved in disorganization of focal adhesion are not completely elucidated, some are correlated with cleavage of focal adhesion proteins. The bacteria *Porphiromonas gingivales*, for example, causes paxillin proteolysis in epithelial cells [(20)]. Loss of focal adhesion by ExoU enzyme activity has also been reported in HeLa cells infected by *Pseudomonas aeruginosa*, releasing talin from cell periphery [(21)]. *T. cruzi* infection of cardiac myocytes leads to metalloprotease-2 and -9 activation and secretion, which results in the degradation of ECM proteins and changes in focal adhesion proteins, being correlated to the severity of chagasic cardiomyopathy [(22)]. Evidence also demonstrated that collagen reduction induces paxillin and talin cleavage in smooth muscle cells [(23)]. Interestingly, *in vitro* cardiomyocyte infection by *T. cruzi* greatly reduces ECM protein levels [(24)], thus suggesting a mechanism by which the infection alters focal adhesion.

We also questioned whether damage to focal adhesion proteins would be noted at the transcriptional levels. Both paxillin and talin transcripts, as well as protein levels, were

downregulated, suggesting that *T. cruzi* alters mRNA regulation and, therefore, protein synthesis. Alpha-cardiac actin mRNA as well as poly(A) mRNA have also been reported to be negatively regulated by *T. cruzi* infection [25]. Therefore, two distinct events may be proposed: (i) a deficiency in translation process due to decreased mRNA levels or (ii) protein degradation, since it has been reported that *T. cruzi* possesses a calpain-like protein [26] that may directly cleave both talin and paxillin. Cytokines present in the serum of infected individuals can also increase calpain activity in cardiomyocytes which, in turn, degrades structural host cell proteins [27]. Additionally, mechanical stimulation also induces increased m-calpain expression in C2C12 cells, leading to destruction of focal adhesion proteins identified as the enzyme substrate [28]. Considering this scenario, it is reasonable to suggest that *T. cruzi* infection leads to the proteolysis of focal adhesion components combined with transcriptional down-regulation.

Studies in the *Drosophila melanogaster* heart model, widely applied to cardiovascular system analyses, demonstrated that talin deletion results in reduced heart contractility and reported cardiac dilatation in the first instar [29]. Talin and paxillin decrease in cardiomyocytes may be the link between the changes previously reported in *T. cruzi* infection, including cytoskeleton component [30, 10], extracellular matrix [24] and junctional complex [12] disorganization. Alteration in mechanical transduction has been previously suggested due to disturbance in costamere organization and irregular alignment of intercalated disks in the cardiac fibers in *T. cruzi*-infected mice [11]. Herein, downregulation of FAK phosphorylation associated to alterations in a mechanosensor protein (talin) contributes to disturbances in mechanotransduction during *T. cruzi* infection. Total FAK expression remained unaltered even at the later stage of infection (72 hpi), demonstrating a selective downregulation of FAK activity. Thus, changes in mechanosignaling proteins, namely FAK and paxillin, may result in force transmission defects and heart failure. In fact, the data from this study

demonstrate that the change in mechanotransduction proteins in infected cardiac cells goes beyond the disorganization of the spatial distribution of mechanosensitive proteins, since highly infected cultures displayed a reduction of FAK activation. Furthermore, our results suggest absence of fluorescent signal of talin at the sarcolemmal focal adhesion complexes, i.e. costameres, in highly infected cells, but remains visible at sites of focal adhesion that keep the cells adhered to the substrate. Our previous data with an experimental murine model of acute infection have demonstrated disorganization of vinculin costameres in myocardium areas containing amastigote nests as observed in the *in vitro* model of infection [(11)]. However, at the end of the acute phase, when few amastigote nests are observed, areas of intense inflammatory infiltrate in the myocardium, characteristic of the chronic Chagas cardiomyopathy, also induced changes in the costameric distribution of vinculin, suggesting that disturbances in the vinculin-talin-integrin-ECM interface may be responsible for the change in mechanotransduction in the chronic phase of Chagas' disease [(11)].

In summary, our data demonstrated that *T. cruzi* infection downregulates talin and paxillin expression and loss of FAK activation, resulting in integrin-mediated mechanotransduction alterations. Defects in the mechanisms of force sensing and transduction may modulate the remodeling of myocardium in response to cardiac overload. Increased knowledge of the mechanisms that activate the cardiac gene expression program may highlight new targets for drug development. Thus, further studies on mechanosensitive microRNA expression may provide new insights into the molecular mechanism underlying Chagas cardiomyopathy.

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AUTHORS' CONTRIBUTION

TGM and MCSP conceived and designed the proposal. TGM, DA and MCSP performed lab experiments and processed the data; TGM and MCSP draft the manuscript; MNM and MCSP coordinated the resources.

COMPETING INTEREST

The authors declare no competing interest.

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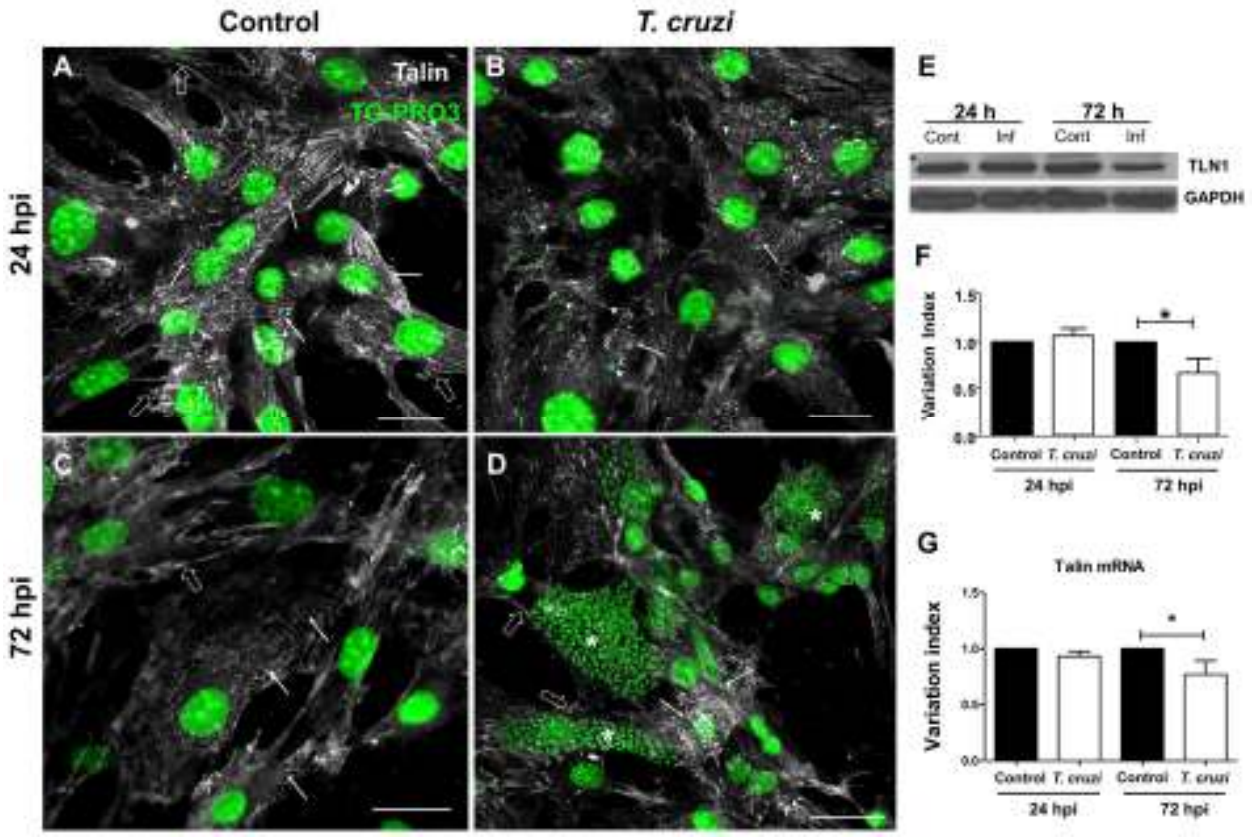

Figure 1: *Trypanosoma cruzi* infection disrupts talin in cardiomyocytes. Cardiac cells, cultivated for 24 h before infection with trypomastigote forms of *T. cruzi* (Y strain), were fixed at desired times and immunostained with anti-talin specific antibody (white). Nuclear chromatin was stained with To-PRO-3 Iodide (green), allowing the visualization of host cell nucleus and intracellular parasite nuclei and kinetoplasts (arrowheads in C). Uninfected cells displayed abundant talin immunoreactivity at 48h (A) and 96h (B) *in vitro* (left panels), and revealed a striated pattern in fully differentiated myocytes (arrows), as well as staining in focal adhesion sites (opened arrows). After 24 hours of infection, few intracellular parasites are seen in the host cell cytoplasm and no change is observed in talin spatial distribution (C). Talin location was drastically disturbed in highly infected cells (*), at 72 hpi, in which staining was only noticeable in focal adhesion sites. Uninfected cells in infected cultures maintained the striated talin staining pattern (D). Western blot analysis for talin (E) revealed that infected cultures had a significant decrease (32%) at 72 hpi (F). Quantitative RT-PCR showed a 23% reduction in the relative expression of talin transcripts at 72 hpi (G). *: $p<0.05$, Unpaired Student's T-test. Bars: 20 μm

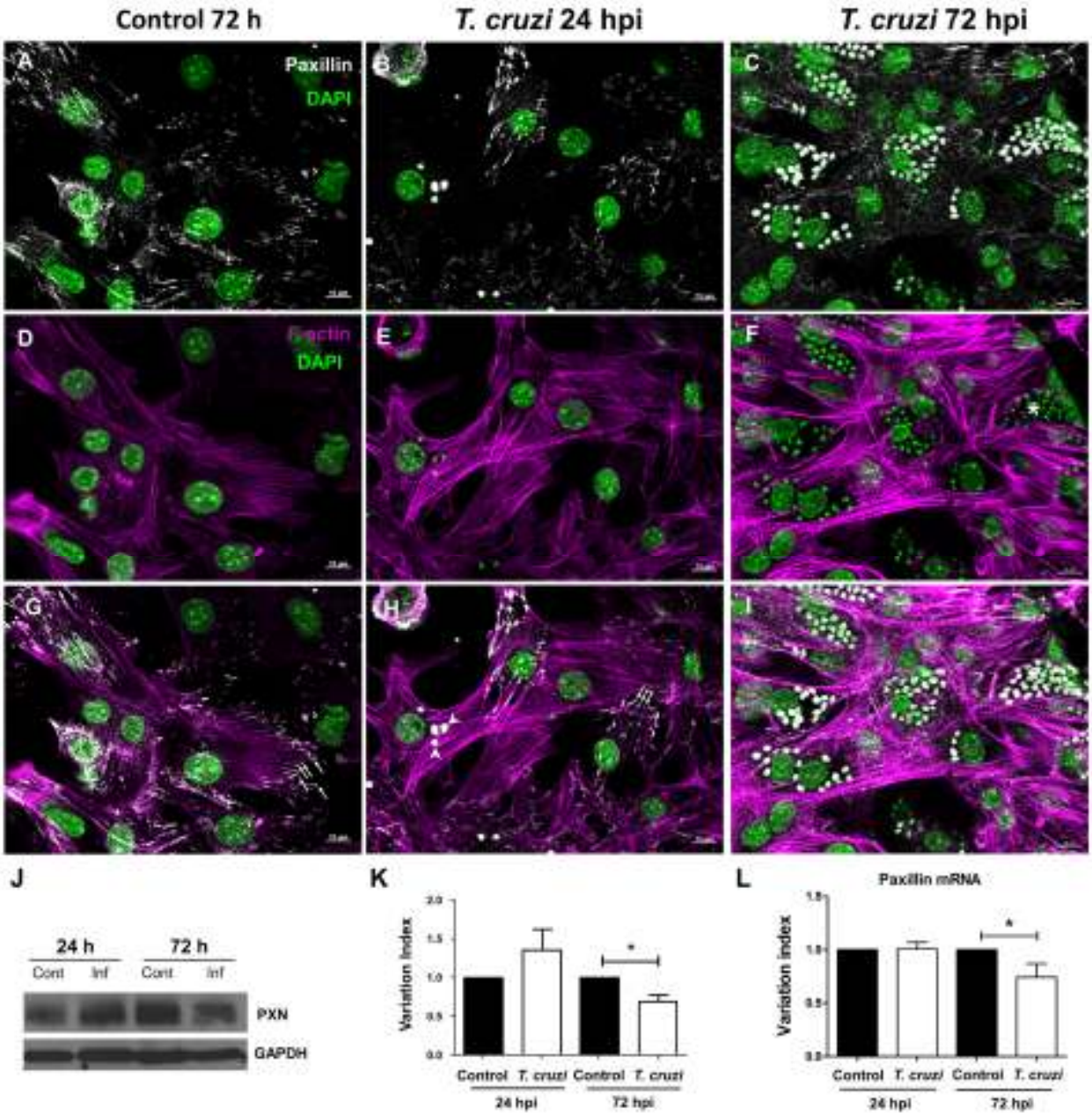

Figure 2: Changes in paxillin induced by *T. cruzi* infection. Double labeling of uninfected and *T. cruzi*-infected cardiomyocytes with anti-paxillin antibody (white) and To-PRO-3 iodide (green). Cardiac cells cultured for 48h (A) and 96h (B) displayed striations as shown by Phalloidin staining (magenta, in D-F) and paxillin staining (white in A, B and C) at focal adhesion sites (arrows in A and G). Paxillin location was unaltered after 24 hours of infection, even in the presence of intracellular amastigotes within the host cell cytoplasm (arrowheads in B and H). Loss of paxillin in focal adhesions was observed in highly infected cells (*), concomitant with myofibrillar breakdown as evidenced by Phalloidin staining (F). Parasites were also labeled by the paxillin antibody (B and C). Paxillin content

and expression were also affected at 72 hpi. Representative blots from three independent experiments are shown in J. Densitometric analyses revealed a 34% decrease in paxillin (K) protein content after 72 h of infection. Quantitative RT-PCR showed a 26% reduction in the relative expression of the paxillin transcripts (L). *: $p<0.05$, Unpaired Student's T-test. Bars: 10 μm.

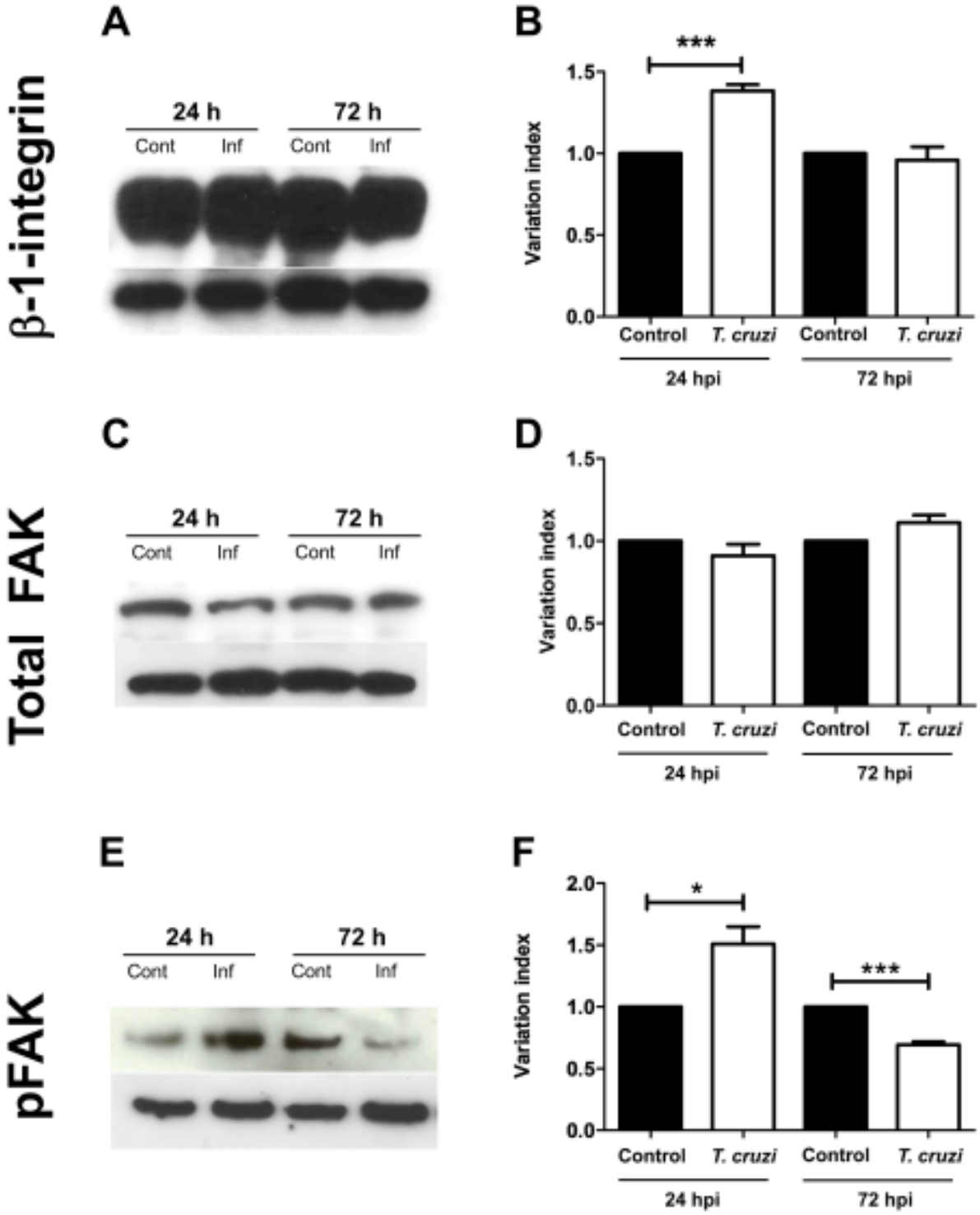

Figure 3: *T. cruzi* infection alters integrin and FAK-Tyr397. β-1-integrin (A), FAK (C) and FAK-Tyr397 (E) protein expression was analyzed by Western blot. Densitometric analyses revealed an upregulation of 38% and 50% of integrin (B) and FAK-Tyr397 (F) respectively, at the early stage of infection (24 hpi). A downregulation of 30% in FAK-Tyr397 expression was showed at 72 hpi (F) but no change was seen in β1-integrin (B) and total FAK (D) levels. GAPDH was used as housekeeping control. **: p<0.01; ***: p<0.0001, unpaired Student's T test.

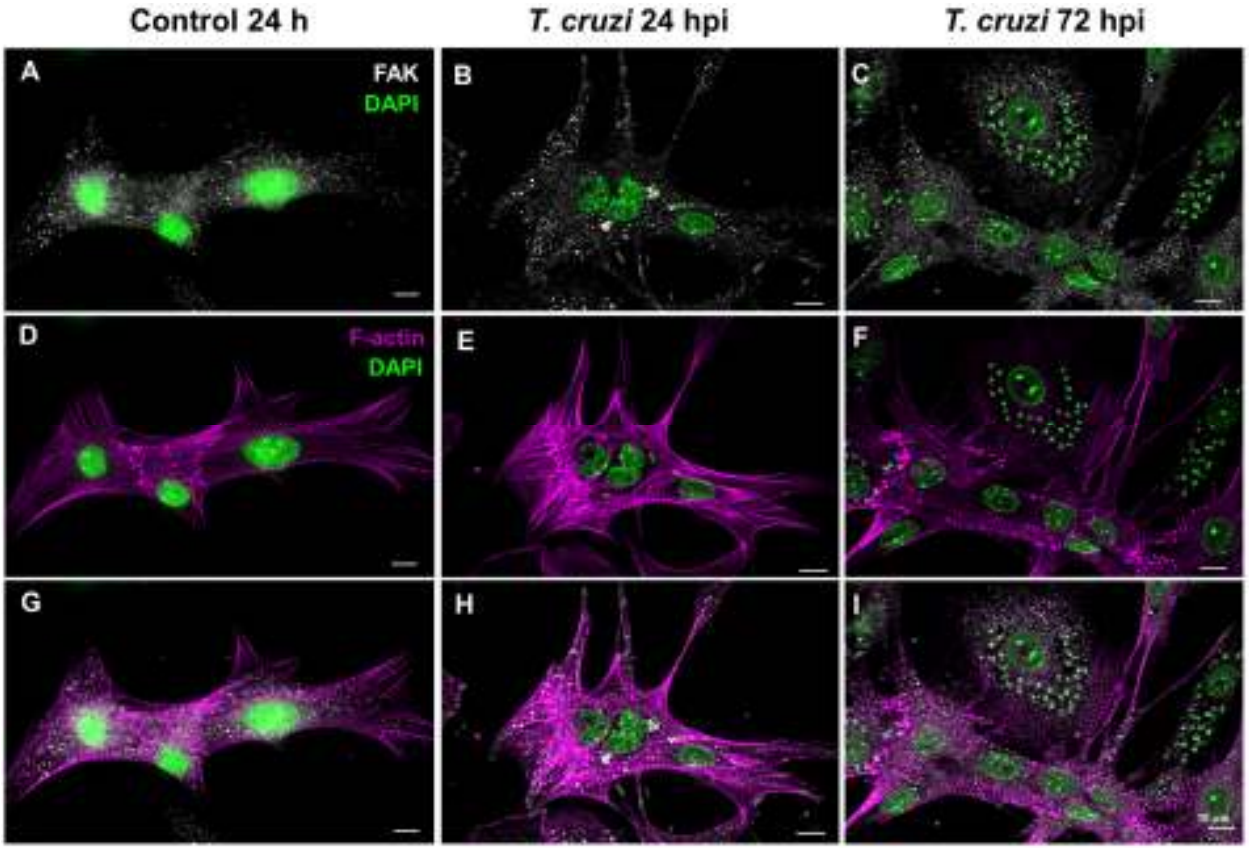

Figure 4: FAK immunolocalization in cardiomyocytes during *T. cruzi* infection *in vitro*. Primary cultures were stained for total FAK protein and co-labeled with Phalloidin-Alex488 and DAPI for F-actin and nuclear visualization, respectively. FAK immunolocalization was found as punctate dots throughout the cytoplasm of cardiomyocytes (white in A, B and C), showing well developed myofibrils (D, E and F). Infected cells showed no significant changes in FAK immunoreactivity at 24 or 72 hpi (H and I), whereas actin filaments are absent around intracellular amastigotes at later time of infection (I). Bars: 10 μm.

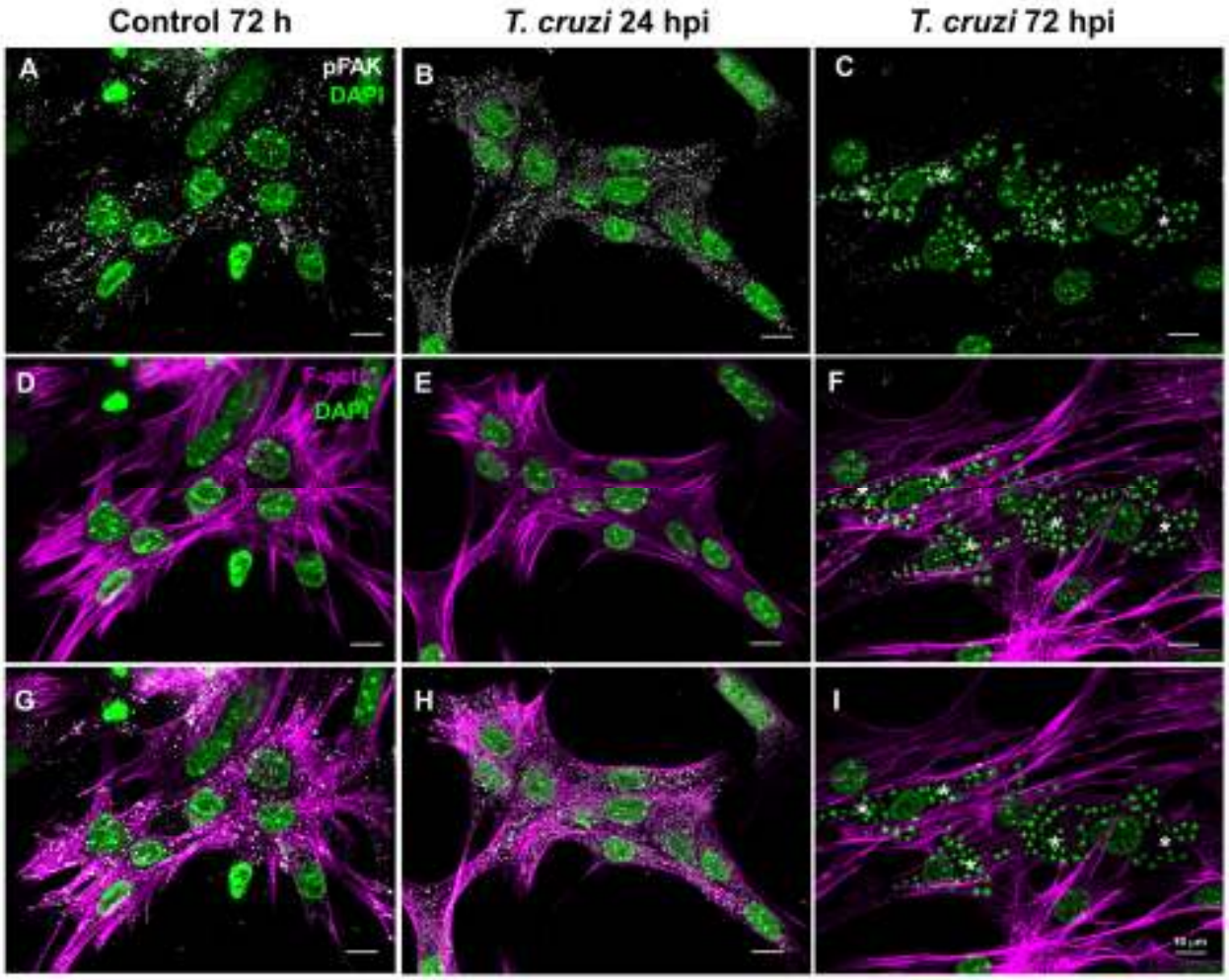

Figure 5: *T. cruzi* induces a biphasic effect on FAK phosphorylation in cardiac myocytes. Cultures were infected and immunostained for anti-phosphorylated FAK (white in A-C), F-actin (magenta in D-F) and DAPI for host cell and parasite chromatin (green). Intense pFAK staining was observed at 24 hpi (B and H), whereas highly infected cells at 72 hpi (* in C and I) showed a clear reduction of immunoreactivity for pFAK. F-actin staining evidenced the striations of the differentiated myocytes (magenta in D, E and F). Bars: 10 μm.