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## ***Trypanosoma cruzi* hijacks TrkC to enter cardiomyocytes and cardiac fibroblasts while exploiting TrkA for cardioprotection against oxidative stress**

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### **Abstract**

Chronic Chagas cardiomyopathy (CCC), caused by the obligate intracellular protozoan parasite *Trypanosoma cruzi*, is a major cause of morbidity and mortality in Latin America. CCC begins when *T. cruzi* enters cardiac cells for intracellular multiplication and differentiation, a process that starts with recognition of host-cell entry receptors. However, the nature of these surface molecules and corresponding parasite counterreceptor(s) is poorly understood. Here we show that antibodies against neurotrophin (NT) receptor TrkC, but not against family members TrkA and TrkB, prevent *T. cruzi* from invading primary cultures of cardiomyocytes and cardiac fibroblasts. Invasion is also selectively blocked by the TrkC ligand NT-3, and by antagonists of Trk autophosphorylation and downstream signaling. Therefore, these results indicate that *T. cruzi* gets inside cardiomyocytes and cardiac fibroblasts by activating TrkC preferentially over TrkA. Accordingly, short hairpin RNA interference of TrkC (shTrkC), but not TrkA, selectively prevents *T. cruzi* from entering cardiac cells. Additionally, *T. cruzi* parasite-derived neurotrophic factor (PDNF)/*trans*-sialidase, a TrkC-binding protein, but not family member gp85, blocks entry dose-dependently, underscoring the specificity of PDNF as TrkC counterreceptor in cardiac cell invasion. In contrast to invasion, competitive and shRNA inhibition studies demonstrate that *T. cruzi*-PDNF recognition of TrkA, but not TrkC on primary cardiomyocytes and the cardiomyocyte cell line H9c2 protects the cells against oxidative stress. Thus, this study shows that *T. cruzi* via PDNF favors neurotrophin receptor TrkC for cardiac cell entry and TrkA for cardiomyocyte protection against oxidative stress, and suggests a new therapeutic opportunity in PDNF and/or fragments thereof for CCC therapy as entry inhibitors and/or cardioprotection agonists.

### **Introduction**

Chagas disease, caused by infection with the protozoan parasite *Trypanosoma cruzi*, is a major source of disease burden in the Western Hemisphere, including developed countries (Bern *et al.*, 2009, Rassi *et al.*, 2010, Machado *et al.*, 2012). It starts with an acute phase where robust parasite growth throughout the body coincides with non-descript symptoms

such as fever, chills and nausea. Parasitemia typically peaks several weeks after infection, usually following the bite of reduviid insects, and is nearly undetectable after eight weeks, at which point blood and tissue parasitism wane under control from the immune response, giving rise to asymptomatic and pathology-free indeterminate phase that can last many years or a life-time. For reasons that remain unknown, around 30% of Chagas disease patients suffer or will suffer from chronic Chagas cardiomyopathy (CCC) whose symptoms include arrhythmias, heart failure and sudden death, and for which there are no cure except heart transplant (Fiorelli *et al.*, 2011). CCC occurs without a recrudescence of parasitism, and, in spite of the paucity of parasites in chronically infected heart, progression to CCC appears to be driven by the inflammatory response to both residual heart parasitism and to cardiac autoantigens, particularly myosin (Marin-Neto *et al.*, 2007, Machado *et al.*, 2012).

As *T cruzi* is an obligate intracellular parasite, it must gain access into the host cell cytoplasm for multiplication and differentiation and for avoiding killing by the immune system. Hence, efficient invasion of cardiac cells is critical for replication and differentiation of *T cruzi* and, thus, for the development of acute heart disease and for parasite persistence in chronic infection, including CCC. Still, mechanisms underlying *T cruzi* recognition of cardiac cell surface receptors required for the intracellular cycle is poorly understood. Although *T cruzi* invades both cardiomyocytes and cardiac fibroblasts *in vivo* (Tafari, 1970, Wong *et al.*, 1992, Huang *et al.*, 2003b), structural and molecular alterations and immune responses resulting from *T cruzi* infection of the heart are studied almost exclusively in the context of cardiomyocytes (Machado *et al.*, 2012, Calvet *et al.*, 2012, Araujo-Jorge *et al.*, 2012), even though cardiac fibroblasts are critical for proper cardiomyocyte function either by direct cell-cell interaction or paracrine signaling in physiological conditions and diseases such as myocardial infarction (Souders *et al.*, 2009, Kakkar *et al.*, 2010) and other types of myocarditis (van Nieuwenhoven *et al.*, 2012). Therefore, cardiac fibroblasts likely play an important role in Chagas heart disease and studies designed to understand *T cruzi*-cardiac fibroblast interactions are sorely needed to better understand Chagas disease progression.

Early findings suggested that *T cruzi* invasion of primary cultures of embryonic cardiomyocytes may involve mannosyl residues (Soeiro Mde *et al.*, 1999) and recent work indicates that cell entry depends on low-density lipoprotein (LDL) receptor in the cardiomyocyte cell line H9c2 (Nagajyothi *et al.*, 2012). However, the nature of the *T cruzi* ligand that recognizes mannosyl residues and LDL receptor has not been identified. Given that the protozoan parasite *Leishmania*, which infects a single cell type (macrophage), takes advantage of at least seven distinct surface receptors for invasion (Cunningham, 2002), and that the relatively simple organism HIV employs three entry receptors (CD4, CCR5 and CXCR4) to invade a single cell type (T-helper cell) (Wilen *et al.*, 2012), then *T cruzi* should exploit an array of entry receptors in the heart and elsewhere as it is able to enter a wide range of nucleated host cells (Brener, 1973).

We showed earlier that *T cruzi* binds receptor tyrosine kinases TrkA (Chuenkova *et al.*, 2004) and TrkC (Weinkauff *et al.*, 2009) and that the binding leads to invasion of neurons and glial cells (Weinkauff *et al.*, 2011, de Melo-Jorge *et al.*, 2007). *T cruzi* recognition of TrkA and TrkC is through its surface parasite derived neurotrophic factor (PDNF), a GPI-linked neuraminidase/transsialidase; however, PDNF interaction with TrkA and TrkC, and

with another protein kinase (Akt) (Chuenkova *et al.*, 2009) is independent of sialic acid-binding activity (Chuenkova *et al.*, 2011). TrkA and TrkC are activated primarily by the neurotrophins nerve growth factor (NGF) and neurotrophin-3 (NT-3), respectively, and the activation is essential for development and maintenance of the nervous systems (Huang *et al.*, 2003a). Recently, both TrkC and TrkA have been identified in cardiomyocytes (Kawaguchi-Manabe *et al.*, 2007, Meloni *et al.*, 2010), and very recently in our lab, in neonatal and adult cardiac fibroblasts (Aridgides, 2013), which led to the hypothesis that Trks are responsible, at least in part, for *T cruzi* homing into the heart.

Here, we report that *T cruzi* via PDNF invades both primary cardiomyocytes and cardiac fibroblasts preferentially through TrkC compared to TrkA, and that TrkA recognition on cardiomyocytes protects the host cells against oxidative stress. This study is, as far as we know, the first to identify a molecular mechanism underlying *T cruzi* invasion of cardiac fibroblasts and also the first to show that, prior to homing into the cytosol habitat, direct *T cruzi* recognition of TrkA on cardiomyocytes protects the cells against oxidative stress.

## Results

### Preferential use of TrkC for *T cruzi* entry into cardiomyocytes

To determine whether *T cruzi* exploits Trk receptors to invade cardiomyocytes, we first assessed the ability of *T cruzi* strains Colombian (cardiotropic), CL-Brener (skeletal muscle tropic) and Tulahuen (reticulotropic) (Brener, 1973) to invade primary cultures of cardiomyocytes. We find that all three strains readily invade cardiomyocytes dose-dependently, although the Colombian strain is slightly more efficient than the other two (Fig. 1a). We used these strains (as indicated) in subsequent experiments.

To determine whether *T cruzi* uses Trk receptors to invade cardiomyocytes, which express all members of the Trk family (TrkA, TrkB and TrkC) (Kawaguchi-Manabe *et al.*, 2007, Meloni *et al.*, 2010, Cai *et al.*, 2006), we pre-incubated primary cultures of cardiomyocytes with neutralizing antibodies against TrkA ( $\alpha$ -TrkA), TrkB ( $\alpha$ -TrkB), or TrkC ( $\alpha$ -TrkC) followed by *T cruzi* Colombian strain. We found that infection is inhibited by TrkC antibodies ( $62\% \pm 2.3$  inhibition at  $1 \mu\text{g/ml}$ ) but not by TrkA and TrkB antibodies (Fig. 1b), suggesting that *T cruzi* invades cardiomyocytes preferentially via TrkC. To further explore this point, we pre-incubated cardiomyocytes with NGF, brain derived neurotrophic factor (BDNF), or NT-3 to block their corresponding primary TrkA, TrkB and TrkC receptors, respectively, and then co-cultured the cardiomyocytes with *T cruzi*. Similar to the selective inhibition of infection by neutralizing TrkC antibody, the TrkC ligand NT-3 ( $10 \text{ ng/ml}$ ) potently blocks infection ( $80\% \pm 3.1$ ), whereas NGF is much less effective ( $30\% \pm 4.2$ ) and BDNF is without effect (Fig. 1c). Knocking down TrkC in primary cardiomyocytes (five cardiomyocyte clones infected each with distinct lentiviral construct encoding short hairpin against TrkC (shTrkC) prevents *T cruzi* from efficiently invading cardiomyocytes, whereas knocking down TrkA does not significantly affect *T cruzi* invasion of cardiomyocytes (Fig. 2a), further establishing TrkC as a preferred invasion receptor for *T cruzi* invasion of cardiac cells.

In addition, we find that invasion is blocked by the Trk autophosphorylation inhibitor AG879 (Ohmichi *et al.*, 1993) and by U0126, an inhibitor of Erk signaling that is activated downstream of Trks (Huang *et al.*, 2003a), but not by a pharmacological inhibitor of insulin-like growth factor-1 receptor (IGF-1R), which does not mediate *T cruzi* invasion of neuronal cells (de Melo-Jorge *et al.*, 2007) (Fig. 2b). Thus, *T cruzi* invasion of cardiomyocytes via TrkC requires TrkC signaling.

### Selective inhibition of cardiomyocyte invasion by the *T cruzi* surface protein parasite-derived neurotrophic factor (PDNF)/ trans-sialidase

Given that the *T cruzi* surface protein PDNF is the counterreceptor for TrkA (de Melo-Jorge *et al.*, 2007) and TrkC (Weinkauff *et al.*, 2011) in invasion of neuronal cells, PDNF may act likewise during *T cruzi* entry into cardiomyocytes. We find that a bacterially expressed short version of PDNF (sPDNF) (Chuenkova *et al.*, 1999) lacking C-terminal tandem repeat SAPA (Affranchino *et al.*, 1989) effectively blocks cardiomyocyte invasion by Colombian, CL-Brener and Tulahuen strains (Fig. 3a). PDNF has intrinsic neuraminidase activity (Pereira, 1983, Scudder *et al.*, 1993) but this activity may not be responsible for the ability of sPDNF to competitively inhibit cardiomyocyte invasion because pre-treatment of cardiomyocytes with *Vibrio cholera* neuraminidase (VCN) (same number of units as sPDNF) does not affect invasion (Fig. 3a). In addition, PDNF family member gp85 (Alves *et al.*, 2008) is also inactive in blocking invasion (Fig. 3b), underscoring the specificity of PDNF/TrkC interaction in triggering cardiomyocyte invasion.

### Preferential use of surface receptor TrkC for *T cruzi* via PDNF entry into cardiac fibroblasts

To determine whether *T cruzi* invades primary cardiac fibroblasts through Trk receptors (Aridgides, 2013) we performed invasion inhibition assays analogous to those described above for cardiomyocytes. As with cardiomyocytes, we find that antibodies against TrkC specifically inhibit *T cruzi* entry (Fig. 4a), as does the TrkC ligand NT-3 (Fig. 4b), knockdown of TrkC by shRNA as determined with nine distinct lentiviral vectors encoding shTrkC or shTrkA (Fig. 5a), and the Trk autophosphorylation inhibitor K252a (Tapley *et al.*, 1992) (Fig. 5b). These results indicate that *T cruzi* uses TrkC preferentially over TrkA to invade cardiac fibroblasts, that TrkC must be activated to drive cell entry, and that TrkC-mediated invasion depends on parasite-bound PDNF as judged by the effect of soluble recombinant sPDNF in inhibiting invasion dose-dependently (Fig. 5c).

### Recognition of cardiomyocyte-TrkA by *T cruzi* or recombinant PDNF triggers protection against oxidative stress

As TrkA activation is a *bona fide* trigger of cell survival and trophic events in neural cells (Huang *et al.*, 2003a) and cardiomyocytes (Caporali *et al.*, 2008), we sought to determine whether *T cruzi* exploits TrkA for cardiomyocytes protection.

First, to test whether *T cruzi* infection of cardiomyocytes elicits protective events via PDNF, we pre-incubated *T cruzi* with the extracellular domain of TrkA (TrkA<sup>ECD</sup>) or (TrkC<sup>ECD</sup>), which, through their neurotrophin-binding sites, bind to and block *T cruzi*-anchored PDNF (de Melo-Jorge *et al.*, 2007, Weinkauff *et al.*, 2009). *T cruzi*-bound Trk<sup>ECD</sup> complexes were then co-cultured with cardiomyocytes exposed to a lethal dose of H<sub>2</sub>O<sub>2</sub>. This way, and

consistently (five experiments, similar results), TrkA<sup>ECD</sup> or TrkC<sup>ECD</sup> prevents cardiomyocyte death as assessed by propidium iodide (PI)/Hoechst 33342 staining (Fig. 6a). In contrast, TrkB<sup>ECD</sup>, which does not bind to PDNF in solution or on the surface *T cruzi* (Weinkauff *et al.*, 2009), is inactive in preventing H<sub>2</sub>O<sub>2</sub>-mediated cardiomyocyte toxicity (Fig. 6a). This indicates that *T cruzi* infection of cardiomyocytes protects the host cells against oxidative stress through the parasite outer membrane PDNF.

Second, to directly test the possibility that PDNF triggers cardioprotective events, we sensitized primary cardiomyocytes with recombinant sPDNF (to activate prosurvival Trk signaling) and then challenged the cells with the H<sub>2</sub>O<sub>2</sub> insult. We find that sPDNF treatment rescues cardiomyocytes from H<sub>2</sub>O<sub>2</sub> cell toxicity (Fig. 6b, first three scatter plots) (three experiments, similar results, as it does on the cardiomyocyte cell line H9c2 (Fig. 6c) (two experiments, similar results). sPDNF protection is specific because it does not happen when cardiomyocytes are sensitized with PDNF family member gp85 before exposure to H<sub>2</sub>O<sub>2</sub> (Fig. 6b, first, second, and fourth scatter plots). These findings establish that *T cruzi* via PDNF protects cardiomyocytes against oxidative stress.

Third, to determine whether *T cruzi* protects cardiomyocytes against oxidative stress via TrkA, we reacted cardiomyocytes with anti-TrkA IgG or anti-TrkC IgG, and found that only anti-TrkA IgG effectively reverses the protective effect of sPDNF (Fig. 6b, first, second, fifth and sixth scatter plots). This underscores the requirement of cardiomyocyte TrkA for the beneficial effect of sPDNF to take place, the opposite of TrkA usage for cell entry (Fig. 1b). Furthermore, knockdown of TrkA gene expression in primary cardiomyocytes (four clones each infected with lentivirus encoding a distinct short hairpin TrkA construct) annuls sPDNF-induced neutralization of H<sub>2</sub>O<sub>2</sub> cardiotoxicity relative to control cardiomyocytes bearing lentivirus-encoding GFP but without mRNA short hairpins (Fig. 6d). Contrary to TrkA knockdown, interference with TrkC mRNA (five cardiomyocytes clones transfected with different TrkC shRNAs) is much less effective in reversing the protective effect of sPDNF (Fig. 6d).

## Discussion

TrkC/NT-3 signaling promotes cardiomyocyte proliferation and ventricular trabeculation in embryogenesis (Lin *et al.*, 2000) and TrkC deletion gives rise to defects in cardiac septation, valvulogenesis, and truncal formation (Tessarollo *et al.*, 1997). However, little is known about the role TrkC signaling plays in cardiomyocyte function in adulthood except that it increases the expression of cardiac hypertrophic markers (skeletal  $\alpha$ -actin and atrial natriuretic peptide), cardiomyocytes size, and phenylalanine uptake (Kawaguchi-Manabe *et al.*, 2007). Our results suggest a role for TrkC in Chagas myocarditis as they demonstrate that *T cruzi* binding to, and activating of, TrkC through its PDNF/*trans*-sialidase, preferentially over family members TrkA and TrkB, is a prelude to cardiomyocyte invasion, thereby implicating TrkC in *T cruzi* homing into the heart. Earlier studies showed that activation of transforming growth factor- $\beta$  (TGF- $\beta$ ) receptor enhances *T cruzi* entry into cardiomyocyte and other cell types (Ming *et al.*, 1995, Waghbi *et al.*, 2007, Waghbi *et al.*, 2005), as does binding to kinin receptors (Todorov *et al.*, 2003). Although our results suggest that PDNF family member gp85 does not initiate a cascade of events triggering

TrkC-dependent cardiomyocyte invasion, independent studies showed that gp85 is important in *T cruzi* adhesion to laminin and other ECM components (Alves *et al.*, 2008, Mattos *et al.*, 2012).

The idea that *T cruzi* recognition of TrkC contributes to cardiac homing is further supported by our findings that *T cruzi* entry into cardiac fibroblast is selectively driven by TrkC-PDNF interaction. Most studies on Chagas heart disease focus on *T cruzi* recognition of cardiomyocytes and mechanisms underlying *T cruzi*-cardiac fibroblast interaction are rare to find (Tafari, 1970, Wong *et al.*, 1992), even though cardiomyocytes require cardiac fibroblasts for proper function either through gap junction-based direct cell-cell interaction or by releasing growth factors and cytokines such as TGF- $\beta$ , fibroblast growth factor-2, interleukin-6 paracrine signaling (Souders *et al.*, 2009, Kakkar *et al.*, 2010). Cardiac fibroblasts express receptors for growth factors, several in common with cardiomyocytes and some more abundant in the fibroblasts, like angiotensin receptors, which link the renin-angiotensin-aldosterone system to myocardial and extracellular matrix remodeling and which convey paracrine signals to cardiomyocytes via angiotensin secretion (Gray *et al.*, 1998). Results presented here provide a molecular basis for *T cruzi* entry into cardiac fibroblasts as they show, for the first time, that TrkC is an entry receptor for *T cruzi* invasion of cardiac fibroblasts, analogous to invasion of cardiomyocytes.

Although TrkA is relatively unimportant for *T cruzi* entry into cardiomyocytes and cardiac fibroblasts, our results demonstrate that *T cruzi* interaction with TrkA on cardiomyocytes protects the cells against oxidative stress (Fig. 6). This is in concert with cardiomyocyte-TrkA activation by its natural ligand, NGF, which reduces myocardial infarction (Meloni *et al.*, 2010) and hypoxia/reoxygenation (Caporali *et al.*, 2008) injury, and with our recent findings that *T cruzi*-PDNF activation of TrkA in cardiac fibroblasts triggers the secretion of NGF that averts cardiomyocyte damage (Aridgides, 2013).

How might *T cruzi*-induced cardioprotective events fit in with Chagas heart disease progression? Patients with acute Chagas heart infection present several cardiac dysfunctions including alterations in ventricular repolarization and cardiomegaly due to pericardial effusion (Beltrao Hde *et al.*, 2009, Parada *et al.*, 1997, Pinto *et al.*, 2001, Valente *et al.*, 2009). Paradoxically, acute Chagas myocarditis wanes without sequelae and most patients remain asymptomatic and pathology-free for many years (indeterminate phase) until CCC emerges. But only approximately 30% chagasic patients progress to CCC (Marin-Neto *et al.*, 2007, Rassi *et al.*, 2010, Machado *et al.*, 2012). Thus it may be that the interaction of *T cruzi*, via PDNF, with cardiac cells in acute infection enhances mutually beneficial host survival mechanisms, as originally suggested in *T cruzi* recognition of neuronal cells (Chuenkova *et al.*, 2000) and in concert with other beneficial mammalian host/parasite interactions such as suppression of bacterial infection resulting from herpesvirus infection (Barton *et al.*, 2007) and of allergies by worms (Fallon *et al.*, 2007).

Cardiomyocytes are particularly vulnerable to reactive oxygen species due to their high metabolic rate, and oxidative stress is important in Chagas cardiomyopathy (Machado *et al.*, 2012). A *T cruzi*-driven cardioprotection against oxidative stress such as by direct activation of TrkA on cardiomyocytes (this study) and by paracrine signaling through the stimulation of

NGF secretion from cardiac fibroblasts (Aridgides, 2013) fits with the concept of acute heart infection waning without sequelae and with chronic pathology-free infection (indeterminate phase). Consistent with this idea, earlier studies showed that *T cruzi* can prevent apoptosis in cultured cardiomyocytes via activation of the transcription factor NF- $\kappa$ B (Petersen *et al.*, 2006), an activation that must be distinct from PDNF signaling because NF- $\kappa$ B is not activated by Trks (Huang *et al.*, 2003a).

Given that Maraviroc and other entry receptor antagonists of HIV infection of CD4 lymphocytes are currently used in the treatment of AIDS (Meanwell *et al.*, 2007), and given that sPDNF potently blocks cardiac cell entry of three biologically distinct *T cruzi* strains, PDNF and/or PDNF fragments may have an opportunity as therapeutics in CCC for which anti-infective therapy is unavailable (Matta Guedes *et al.*, 2012). Furthermore, TrkA-mediated cardioprotective effect should add another dimension for PDNF as therapeutics in CCC.

## Experimental procedures

### Ethics statement

All mouse work was approved by the Institutional Animal Care and Use Committee of Tufts University School of Medicine and Division of Laboratory Animal Medicine.

### Trypanosoma cruzi culture

Parasites were maintained in monolayers of Vero cells. Trypomastigotes, when released from infected cells, were harvested from supernatants by low speed centrifugation (250 xg, 5 min) in a table-top centrifuge to remove any contaminating Vero cells, then at high speed (2500 x g, 10 min) to pellet parasites, which were then resuspended in DMEM/0.1% FCS and counted in a hemacytometer before use.

### Cardiomyocyte and cardiac fibroblast preparation

Neonatal (1-3d old) C57BL/6 mice (Jackson Laboratories) were sacrificed by decapitation, hearts dissected, and cardiac cells were prepared as described previously (Aridgides, 2013) according to a procedure modified from Sreejit *et al.* (Sreejit *et al.*, 2008). Briefly, hearts cut into small cubes and digested with trypsin, separated by differential adhesion to a gelatin-coated substrate, then plated in 20% fetal calf serum and 5% horse serum. Purity of cardiac fibroblasts was assessed by immunofluorescence microscopy using antibodies to vimentin and of cardiomyocytes using antibodies to myosin heavy chain, and used when purity for each cell type was >90%; cardiomyocytes showed a beating phenotype.

### In vitro infection assay

Cardiac fibroblasts or cardiomyocytes in 20% FCS/5% horse serum were plated at  $\sim 2 \times 10^4$ /mL on 1% gelatin-coated 96-well plates and, after incubation for 14 h, medium was changed to DMEM/0.1% FCS. For inhibition of infection assays, cells were pretreated for 30 min with  $\alpha$ -Trk antibodies (Abcam), neurotrophins (R&D Systems), Trk chemical inhibitors (Sigma-Aldrich), sPDNF, gp85 (see below), or *Vibrio cholera* neuraminidase (VCN) (same number of units as sPDNF). Parasites were added at the concentrations

indicated in figure legends of figures, and allowed to infect for 3 h, after which non-invaded parasites were washed away twice with PBS and cells were left in DMEM/1% FCS. After two days, cells were fixed with methanol, stained with Diff Quik, and infected cells were counted in each well by microscopy. Inhibition of infection was calculated relative to vehicle-treated cells in each experiment.

### Purification of recombinant sPDNF and gp85

sPDNF was cloned into BL21 expression bacteria and purified by Ni-chromatography as previously described (Aridgides, 2013). gp85 (Giordano *et al.*, 1999, Alves *et al.*, 2008) was cloned from CLBrener *T. cruzi* DNA using a nested PCR approach to specifically amplify this single member of a highly conserved gene family (NCBI Ref Seq XM\_808586). Flanking primers (Forward: 5'GTG GCC TCA CAG TGA TTA AG 3', Reverse: 5'ACA GGA AGA GTG CGG AAG AA 3') generated a 3035 base pair product which was then used as a template for PCR to produce the gp85 cDNA product with additional BamHI and XhoI sites (underlined) (Forward: 5'GCA GGA TCC ATG CTC TCA CGT GTT G 3', Reverse: ATA CTC GAG CGC AGT CGC AAG G 3'). This PCR product was then cloned into the pET-23b vector (Novagen) and transformed into BL21 *E. coli* for expression. Bacteria were grown in LB medium to an OD<sub>600</sub> of 0.7, then induced for 3 h with 200 μM IPTG (isopropyl β-D-1-thiogalactopyranoside). Bacteria were then pelleted, lysed by sonication, and gp85 was purified over a Ni<sup>++</sup> column. Purity and concentration were assessed in SDS-PAGE gels after staining with Coomassie brilliant blue.

### Lentiviral transfection and shRNA knockdown

Lentiviral vectors (Open Biosystems) encoding shRNA constructs targeting TrkA, TrkC or GFP mRNA were generated from transfected HEK 293 cells according to manufacturer instructions, aliquoted, and frozen at -80°C until use. Lentiviral infection of primary cultures was performed after pretreatment with Polybrene (8 μg/mL) (Sigma-Aldrich) then adding lentiviral particles to each well. Optimal concentrations (8 μL vector/100 μL well on 96-well plates) were discerned by preliminary dose-response experiments on cardiac fibroblasts where cells were infected for 7 days, fixed and stained with antibodies specific for TrkA and TrkC (abcam) and assessed by fluorescence microscopy.

### Cardiomyocyte survival assay

Primary cardiomyocytes or H9c2 cardiomyocytes (ATCC) were plated on 96-well plates, pre-treated with indicated proteins for 30 min, and hydrogen peroxide or PBS vehicle was added to the cultures for four h. Cells were fixed with 1% paraformaldehyde overnight at 4°C. Cells were stained with Hoechst 33342 (20 μg/mL) and propidium iodide (PI) (10 μL) to visualize live and dead (apoptotic) cells, as we described earlier (Chuenkova *et al.*, 2004). After 5 min, the cells were visualized under ultraviolet irradiation at 340–380 nm with an inverted microscope. DAPI staining was performed as described earlier (Chuenkova *et al.*, 2000). More than 200 cells/well were counted and percent viable cells (Hoechst 33342 positive and PI negative) were calculated based on the percentage of viable cells



## Statistics

Statistical analyses were performed using GraphPad Prism software using Student's t-test or ANOVA with Tukey's post-test.

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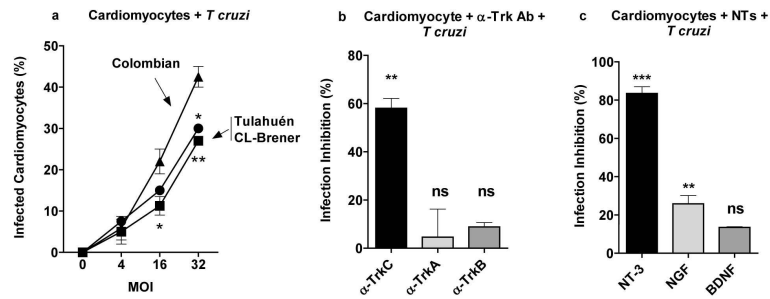
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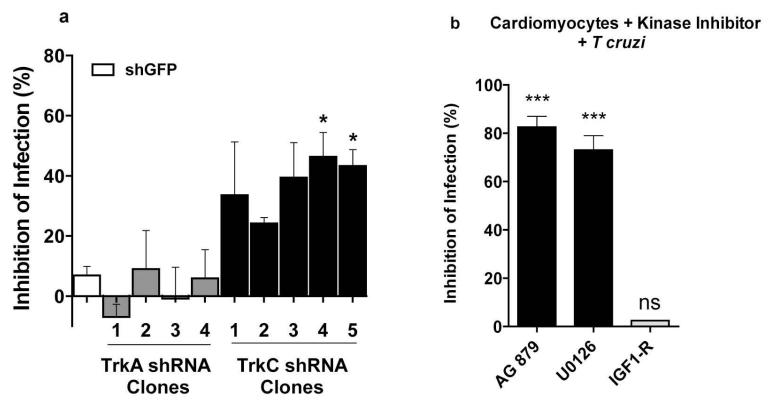


**Figure 1. Cardiomyocytes are preferentially invaded by *T. cruzi*'s Colombian strain and invasion is specifically inhibited by TrkC antibodies and the TrkC ligand NT-3**

**(a)** Primary cultures of mouse cardiomyocytes were plated in 96-well plates, and infected in triplicate with *T. cruzi* Colombian, CL-Brener and Tulahuén strains at various multiplicity of infection (MOI). After 2-3 h, parasites that did not invade were washed away and the ones that invaded were allowed to differentiate and multiply for 3 days. Infected cells were counted after Diff-Quik staining<sup>18</sup> (average of three experiments).

**(b)** Primary cardiomyocytes were plated in 96-well plates, pretreated with antibodies (1  $\mu$ g/ml) against TrkC (a-TrkC), (TrkA (a-TrkA) or TrkB (a-TrkB) followed by *T. cruzi* Colombian strain as in **(a)**; Standard deviation bars were calculated using Student t test, and the results shown here were similar in three other experiments;\*, P<0.05, \*\*\*, P<0.001, ns, not significant.

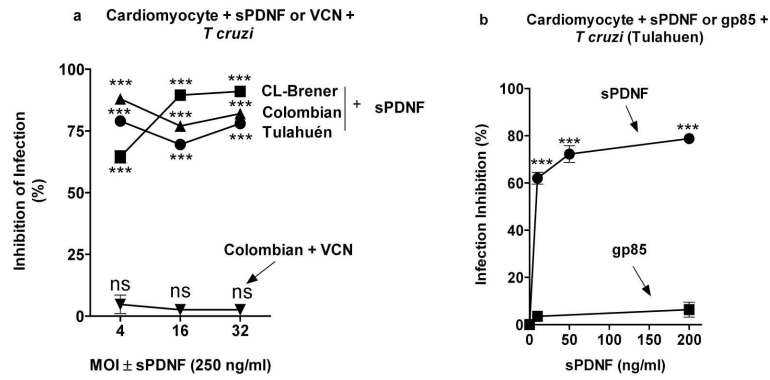
**(c)** Primary cardiomyocytes were pre-treated with the neurotrophins (NTs) NT-3 (TrkC ligand), NGF (TrkA ligand) and BDNF (TrkB ligand) (10 ng/ml, 30 min), infected with *T. cruzi* and the infection quantitated as in **(a)**. Standard deviation bars were calculated using Student t test, and the results shown here were similar in three other experiments;\*, P<0.05, \*\*\*, P<0.001.



**Figure 2. *T. cruzi* infection of primary cardiomyocytes is inhibited by TrkC knockdown preferentially over TrkA knockdown and by antagonists of Trk autophosphorylation and Trk downstream signaling**

**(a)** Primary cardiomyocytes (in quadruplicate wells per point) were plated on 96-well plates, and after one day, were infected with lentivirus expressing small hairpin knockdown plasmids for GFP (shGFP), TrkA (shTrkA, four distinct constructs), or TrkC (shTrkC, five distinct constructs). Six days after lentiviral infection, cells were infected with *T. cruzi* Tulahuen strain ( $2 \times 10^5$  /mL) for three hours, washed to remove unattached parasites, the infection allowed to proceed for two days at 37°C, fixed in 4% paraformaldehyde, and stained with DAPI to visualize host and parasite nuclei. Infected cells (>two parasites/cell) were counted in >200 cells/well, and inhibition of infection was calculated relative to control shGFP-infected cells; \*  $p < 0.05$  relative to control shGFP-infected cells.

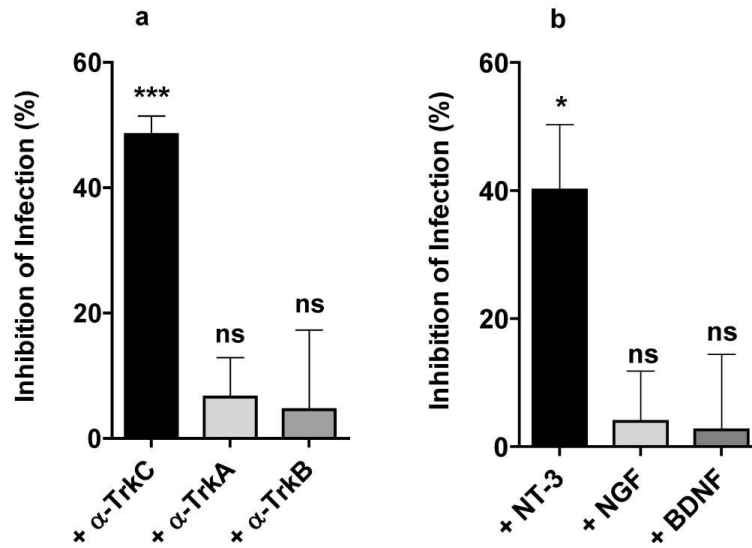
**(b)** Primary cardiomyocytes were pre-treated (30 min) with AG879 (Trk antagonist), U0126 (Erk signaling antagonist) or IGF1-R (insulin-like growth factor-1 receptor antagonist) followed by *T. cruzi* Tulahuen, as in **(a)**; two experiments, similar results.



**Figure 3. *T. cruzi* infection of cardiomyocytes is blocked by PDNF and not by *Vibrio cholerae* neuraminidase or PDNF family member gp85**

**(a)** Primary cardiomyocytes (triplicate) were pre-treated with sPDNF (250 ng/ml, 30 min) or *Vibrio cholerae* neuraminidase (VCN, same number of units as PDNF) followed by Colombian, CL-Brener and Tulahuén strains of *T. cruzi* at 4, 16 and 32 MOI for 3 h, washed to remove unattached parasites, and allowed infection to proceed for 2 days. Infection was quantified by light microscopy after Diff-Quik staining, two experiments, similar results.

**(b)** Primary cardiomyocytes were pre-treated with various doses of sPDNF or gp85 (30 min) followed by *T. cruzi* Tulahuén, as in **(a)**.

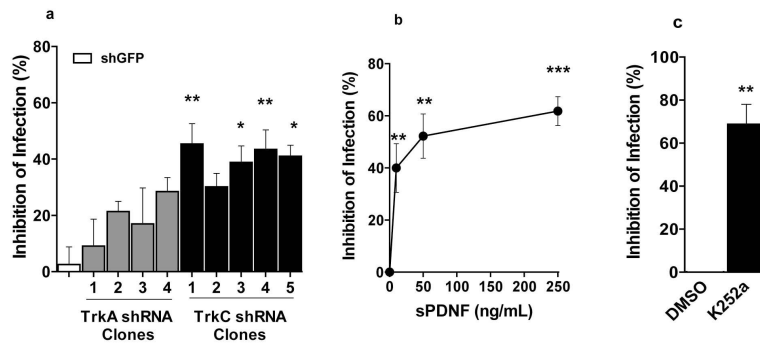


**Figure 4. *T. cruzi* entry into cardiac fibroblasts is specifically inhibited by antibodies against TrkC and by the TrkC ligand neurotrophin-3 (NT-3)**

**a)** Primary cultures of cardiac fibroblasts (in triplicate) were preincubated for 30 min with antibodies against TrkA ( $\alpha$ -TrkA), TrkB ( $\alpha$ -TrkB), or TrkC ( $\alpha$ -TrkC) (1  $\mu$ g/ml), infected with *T. cruzi*(Tulahuen) for 4 h, washed to remove unattached parasites, allowed to grow for 2 days, and stained with Diff-Quik. Infected cells (more than two parasites per cell) were counted for each well (>200 cells/well); inhibition of infection was computed relative to cells preincubated with medium vehicle; similar results were obtained in three other experiments; \*\*,  $p < 0.001$ , ns, not significant.

**b)** Primary cardiac fibroblasts were preincubated (30 min, 37°C) with NGF, BDNF, or NT-3 (50 ng/ml) prior to *T. cruzi* infection and quantification of infection ascertained as in **a)**; \*,  $p < 0.05$ , ns, not significant.



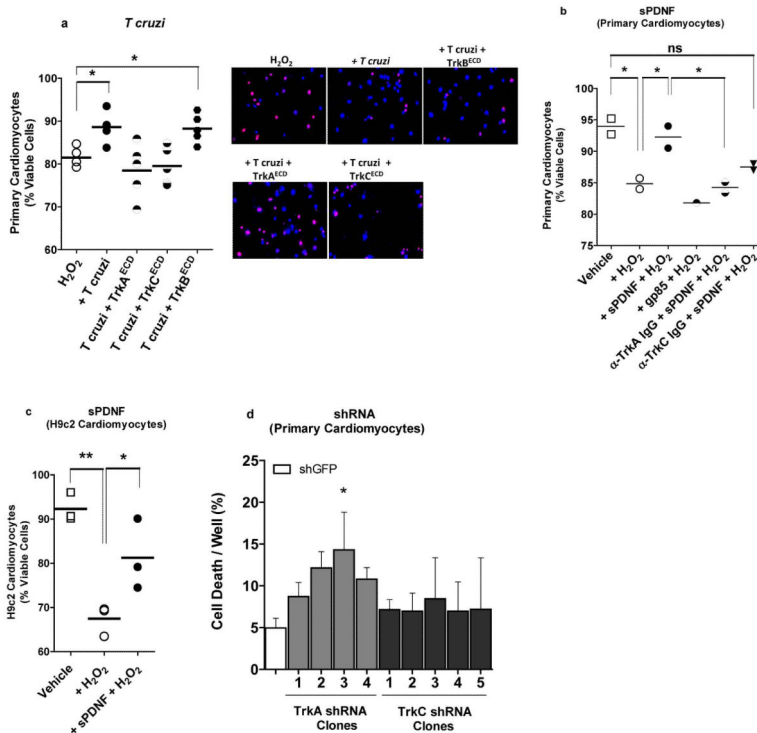


**Figure 5. *T. cruzi* entry into primary cardiac fibroblasts is inhibited by TrkC knockdown, sPDNF and pharmacological inhibitor of Trk autophosphorylation**

**(a)** Cardiac fibroblasts were infected with lentiviral knockdown vectors against GFP (shGFP), TrkA (shTrkA, four distinct vectors), or TrkC (shTrkC, five distinct vectors) for 7 d before infection, using a protocol otherwise identical to that described in Fig. 2a legend; \*,  $p < 0.05$ ; \*\*\*  $p < 0.001$ ; experiments repeated twice with similar results.

**(b)** Primary cultures of cardiac fibroblasts (in triplicate) were preincubated or not with the indicated concentrations of recombinant sPDNF for 30 min prior to *T. cruzi* infection (Tulahuen) for four h. Inhibition of infection was computed relative to cells preincubated with vehicle (PBS); similar results were obtained in three other experiments; \*\*\*,  $p < 0.001$ .

**(c)** Primary cardiac fibroblasts were preincubated (30 min, room temperature) with 250 nM K252a prior to infection; inhibition of infection was calculated relative to infection of fibroblasts pre-incubated with vehicle (DMSO); \*\*,  $p < 0.01$ .



**Figure 6. *T. cruzi* infection or sPDNF stimulation of cardiomyocytes confers protection against oxidative stress**

**a)** *T. cruzi* infection protects cardiomyocytes against H<sub>2</sub>O<sub>2</sub>-induced cell death and the protection is blocked by *T. cruzi* interaction with PDNF receptors TrkA<sup>ECD</sup> and TrkC<sup>ECD</sup> but not with PDNF non-receptor TrkB<sup>ECD</sup>. Primary cultures of cardiomyocytes were exposed to 150 μM H<sub>2</sub>O<sub>2</sub> for 4 h following preincubation with vehicle media (H<sub>2</sub>O<sub>2</sub>) or with *T. cruzi* (2 × 10<sup>5</sup>/mL) pretreated with vehicle (*T. cruzi*) or with the extracellular domains (ECDs) of TrkA (TrkA<sup>ECD</sup>), TrkC (TrkC<sup>ECD</sup>) or TrkB (TrkB<sup>ECD</sup>) (200 ng/mL, 30 min) to block (TrkA<sup>ECD</sup> and TrkC<sup>ECD</sup>) or not (TrkB<sup>ECD</sup>) GPI-anchored PDNF. Cell death was assessed by counting >200 cells stained with the propidium iodide (PI)/ Hoechst 33342 mixture. Data are combined from five separate experiments after normalization to media alone, each giving results similar to those shown here. Panel on the right shows representative staining from each condition; dead cardiomyocytes are seen as pink dots (the result of the merge of PI and Hoechst staining), while viable cardiomyocytes are stained blue by the vital dye Hoechst 33342 and no PI staining.

**b)** sPDNF specifically protects primary cardiomyocytes from H<sub>2</sub>O<sub>2</sub>-induced cell death. Primary cardiomyocytes were unexposed (Vehicle) or exposed to 150 μM H<sub>2</sub>O<sub>2</sub> for 4 h without (+ H<sub>2</sub>O<sub>2</sub>). Prior to H<sub>2</sub>O<sub>2</sub> exposure, cardiomyocytes were preincubated (30 min, room temperature) with sPDNF or with the PDNF/trans-sialidase family member gp85 (150 ng/ml). Prior to H<sub>2</sub>O<sub>2</sub> exposure and sPDNF treatment, cardiomyocytes were additionally preincubated with antibodies against TrkA (α-TrkA IgG) or TrkC (α-TrkC IgG) (1 μg/ mL); \*\*, p<0.001, \*, p<0.01; ns, not significant statistically; three experiments, similar results.

**c)** *T. cruzi* sPDNF protects H9c2 cardiomyocytes from H<sub>2</sub>O<sub>2</sub>-induced cell death. H9c2 cardiomyocytes were exposed to 150 μM H<sub>2</sub>O<sub>2</sub> for 4 h with or without preincubation with sPDNF (150 ng/mL); p<0.05, \*\*p<0.01, \*\*\*p<0.001; two experiments, similar results.

**d)** sPDNF protection of cardiomyocytes is selectively abrogated by TrkA knockdown. Primary cardiomyocytes were lentivirally transfected with shRNA to generate four shTrkA clones and five shTrkC clones, and after one week, clones were exposed to 150 ng/mL sPDNF and 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> for four hours in DMEM/0.1% FCS, and cell death quantified by PI/ Hoechst 33342; \*p<0.05; two experiments, similar results.