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Article

The Chagas' Disease Parasite *Trypanosoma cruzi* Exploits Nerve Growth Factor Receptor TrkA to Infect Mammalian Hosts

Milena de Melo-Jorge[1] and Mercio PereiraPerrin[1,*]
[1] Parasitology Research Center, Department of Pathology, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111, USA
*Correspondence: mercio.perrin@tufts.edu
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SUMMARY

Trypanosoma cruzi, the agent of Chagas' disease, is an obligate intracellular parasite that invades various organs including several cell types in the nervous system that express the Trk receptor tyrosine kinase. Activation of Trk is a major cell-survival and repair mechanism, and parasites could use Trks to invade cells as a strategy to protect their habitat and prolong parasitism of vertebrate hosts. We show that _T. cruzi_ binds to TrkA specifically and activates TrkA-dependent survival mechanisms. This interaction facilitates parasite adherence and promotes efficient invasion of neuronal, epithelial, and phagocytic cells via a process that requires TrkA kinase activity. Diffusible TrkA and TrkA-blocking agents neutralized infection in cellular and animal models of acute Chagas' disease, suggesting cellular receptors as therapeutic targets against parasitic diseases. Thus, TrkA, the nerve growth factor receptor commonly associated with neural survival and protection, may also underlie clinical progression of an important human parasitic disease.

INTRODUCTION

The receptor tyrosine kinases (RTK) TrkA, TrkB, and TrkC are widely expressed in peripheral (PNS) and central (CNS) nervous systems, where they primarily regulate survival and proliferation of cells (Bibel and Barde, 2000; Huang and Reichardt, 2003). Trks are also expressed on dendritic cells, lymphocytes, and other cells of the immune and inflammatory system (Vega et al., 2003), but their function in nonneural tissues is not as well understood, though it may also entail cell-survival promotion (Torcia et al., 1996). The Trk family shares a common structure: the extracellular domain (ECD) comprises leucine-rich motifs sandwiched between two cysteine clusters, followed by two immunoglobulin (Ig)-like domains, a single transmembrane domain, and an intracellular domain (ICD) that contains a conserved tyrosine kinase (see Figure 4D). The second Ig-like domain of TrkA, TrkB, and TrkC reacts selectively with the neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), respectively. Neurotrophin engagement initiates receptor dimerization, autophosphorylation, and activation of three main signaling pathways, one of which, phosphatidylinositol (PI) 3-kinase/Akt protein kinase, is a foremost mediator of Trk-induced cell survival (Huang and Reichardt, 2003; Kaplan and Miller, 2000). Thus, it stands to reason that microbial pathogens should greatly facilitate and prolong parasitism if, while invading host cells, they were to bind Trks and trigger survival and protection mechanisms normally associated with neurotrophin engagement.

Trypanosoma cruzi, the agent of a chronic and incurable illness (Chagas' disease) that afflicts millions of people in the Americas, is an obligate intracellular parasite that invades various organs throughout the body. In the nervous system, it inhabits Trk-expressing astrocytes, microglia, Schwann cells, enteric glial cells, and neurons (Da Mata et al., 2000; Rosenberg et al., 1991; Tafuri, 1970). Infection of the gastrointestinal tract can almost completely destroy the autonomous nervous system and cause megacolon and megaesophagus (megaviscera) (Brener, 1973; Köberle, 1968). *T. cruzi* also invades Trk-expressing cells in nonneural tissues such as keratinocytes and Langerhans cells (dendritic cells) in the skin (Nargis et al., 2001), a site where transmission of Chagas' disease starts after a reduviid insect bite (Brener, 1973).

Recent studies showed that a *T. cruzi* protein with neuraminidase (Pereira, 1983) and sialyl-transferase (transsialidase or TS) (Parodi et al., 1992; Schenkman et al., 1992; Scudder et al., 1993) activities mimics NGF by binding and activating TrkA; hence, TS is also known as parasite-derived neurotrophic factor (PDNF) (Chuenkova and Pereira, 2000; Chuenkova and PereiraPerrin, 2004, 2006). Given that PDNF/TS is readily shed into the extracellular milieu (Prioli et al., 1991), we originally thought that it operated as a diffusible exogenous molecule facilitating neuroregenerative events in Chagas' disease (Chuenkova and Pereira, 2000). However, PDNF/TS is also bound to the outer membrane of invasive *T. cruzi* trypomastigotes through a glycosylphosphatidylinositol (GPI) anchor (Prioli et al., 1991; Rosenberg et al., 1991)

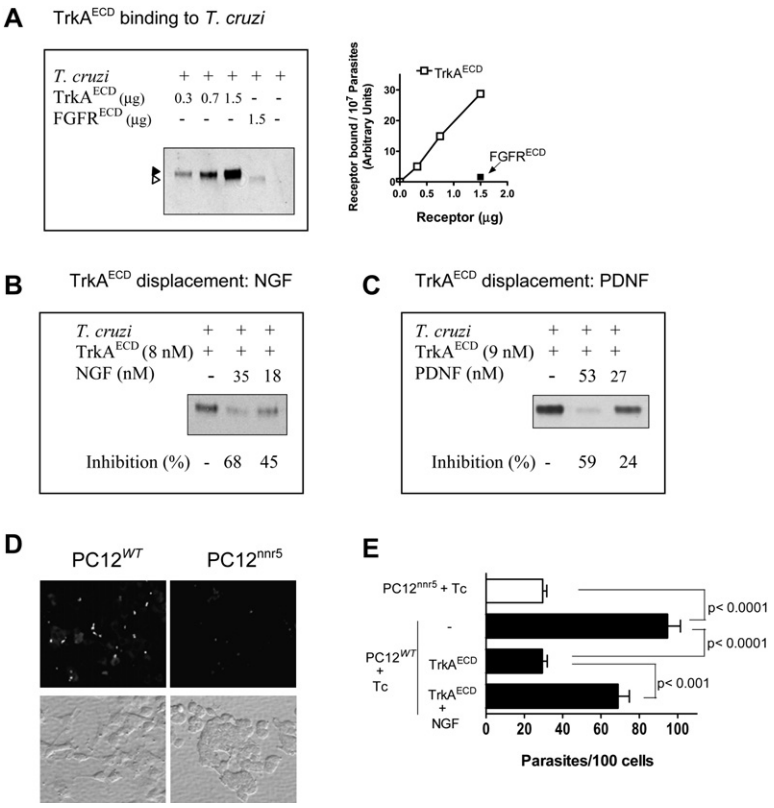

Figure 1. Specific Interaction of *T. cruzi* Trypomastigotes with Soluble TrkA^{ECD} and Transmembrane TrkA

(A) Binding of TrkA^{ECD} to *T. cruzi*. Trypomastigotes were incubated with the indicated amounts (per ml) of TrkA^{ECD} or FGFR^{ECD} for 1 hr at 4°C, centrifuged, resuspended in SDS, and analyzed by immunoblotting with anti-human IgG to identify *T. cruzi*-bound growth factor receptor. Arrowheads indicate bands corresponding to TrkA^{ECD} (closed arrowhead) and FGFR^{ECD} (open arrowhead). Amount of *T. cruzi*-bound receptors was estimated by scanning densitometry and plotted using the GraphPad software.

(B) Displacement of *T. cruzi*-bound TrkA^{ECD} by NGF. TrkA^{ECD} was pulled down by *T. cruzi* as described in (A) in the presence or absence of NGF. TrkA^{ECD} binding to *T. cruzi* in the absence of NGF was set as 100%.

(C) Displacement of *T. cruzi*-bound TrkA^{ECD} by PDNF. Same protocol as in (B), except PDNF and not NGF was included in the TrkA^{ECD}/*T. cruzi* mixtures. Results in (A)–(C) are representative data of three independent experiments.

(D) Visualization of *T. cruzi* adherence to PC12 cells. Trypomastigotes were labeled with CellTracker Red CMTPX and added to PC12^{WT} and PC12^{nnr5} cells for 30 min at 4°C, and the cell monolayers were gently washed to remove unattached parasites, fixed with 4% paraformaldehyde, and visualized by fluorescence (upper panels) and phase-contrast (lower panels) microscopy. Scale bar, 10μm.

(E) Quantitation of TrkA-mediated adherence of *T. cruzi* to neuronal cells. Adherence assay performed exactly as in (D) except parasites were incubated without (–) or with TrkA^{ECD} (1 μg/ml) in the absence or presence of NGF (3 μg/ml), 1 hr prior to addition to the cells. Parasites adhering to PC12 cells were estimated by fluorescence microscopy. Results represent the average of two independent experiments. Error bars indicate the standard error of the mean.

and thus it is strategically located to directly interact with host cell surface receptors such as TrkA. Here we report that *T. cruzi* adhered to PC12 cells in a TrkA-dependent manner and that it invaded neuronal and nonneuronal cells through functional, but not through kinase-deficient, TrkA. In synchrony with concepts of antiadhesion therapy in infectious diseases (Sharon, 2006), an inhibitor of TrkA kinase autophosphorylation specifically attenuated infection in a mouse model of acute Chagas' disease, as did passive administration of diffusible TrkA^{ECD}, presumably by competing with transmembrane TrkA for TrkA-dependent cellular invasion.

RESULTS

T. cruzi Adheres to PC12 Cells through the TrkA Receptor

The rat pheochromocytoma PC12 cell line is widely used in studies designed to understand mechanisms of NGF binding to TrkA and resultant intracellular signaling cascades. Therefore, we exploited the PC12 cell model to find out whether TrkA is a cellular receptor for *T. cruzi* to adhere to and invade host cells.

Intracellular microbial pathogens must first bind to surface receptors as a prelude to entry, differentiation, and multiplication inside host cells (Cossart and Sansonetti, 2004; Sibley, 2004; Smith and Helenius, 2004). To determine whether TrkA mediates *T. cruzi* adhesion to PC12 cells, we first ascertained whether trypomastigotes (~25 μm × 3 μm), the invasive stage of the parasite, interact specifically with the neurotrophin RTK. For this, we performed whole-cell pull-down assays in which parasites were incubated with various concentrations of TrkA^{ECD} fused to the Fc domain of human IgG at 4°C for 1 hr (to prevent receptor internalization). Figure 1A and the Figure 1A inset show that TrkA^{ECD}-Fc, but not fibroblast growth factor receptor (FGFR^{ECD}-Fc), bound to *T. cruzi* in a dose-dependent manner. The negative result with FGFR^{ECD}-Fc implied that the Fc fragment did not appreciably contribute to TrkA^{ECD}-Fc binding to *T. cruzi*. Preincubating TrkA^{ECD}-Fc with various amounts of endogenous (NGF) (Figure 1B) or exogenous (PDNF/TS) (Figure 1C) ligands effectively inhibited adsorption of the recombinant receptor to the parasites, reinforcing the conclusion that TrkA^{ECD}-Fc bound to *T. cruzi* specifically.

To find out if the parasites interact with transmembrane TrkA, trypomastigotes were labeled with the fluorescent dye CellTracker Red and added to wild-type PC12 cells (PC12^{WT}) and TrkA-deficient PC12 cells (PC12^{nnr5}) (Loeb and Greene, 1993) at 4°C for 30 min to allow adhesion to

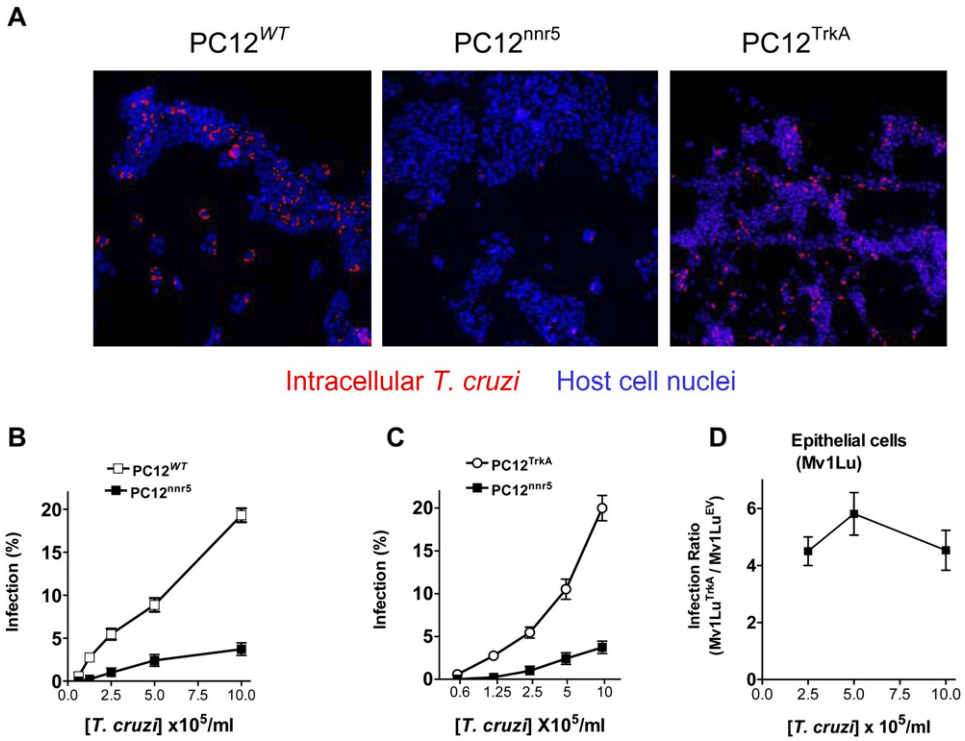

Figure 2. TrkA-Dependent *T. cruzi* Invasion of Neuronal and Epithelial Cells

(A) PC12^{WT} cells, TrkA null mutant PC12^{nnr5}cells, and PC12^{nnr5} cells stably expressing human TrkA (PC12^{TrkA}) were infected with *T. cruzi* and, after 2 days, fixed and stained with DAPI and with human chagasic serum followed by anti-human IgG-Alexa 594 to visualize host cell nuclei and intracellular parasite, respectively, by fluorescence microscopy.

(B and C) PC12^{WT}, PC12^{nnr5}, and PC12^{TrkA} cells were infected with the indicated concentration of *T. cruzi* for 2 hr, and the degree of infection was estimated, after 2 days, by counting infected and noninfected cells (>300 cells) by phase-contrast microscopy after staining with Diff-Quik.

(D) Infection ratio of mink lung epithelial cells (Mv1Lu) stably transfected with human TrkA gene (Mv1Lu^{TrkA}) over cells transfected with empty vector (Mv1Lu^{EV}) following a protocol analogous to the one for PC12 cells. Experiments were performed in triplicates and repeated at least three times with similar results.

Error bars indicate the standard error of the mean.

host cell plasma membrane with minimum cellular entry. Adherent *T. cruzi* was readily ascertained by fluorescent microscopy, which revealed a great preference ($p < 0.0001$) for PC12 cells expressing TrkA (PC12^{WT}) compared to the mutant cells (PC12^{nnr5}) (Figures 1D and 1E). Adsorption of TrkA^{ECD}-Fc (1 μg/ml) to trypomastigotes (10^7/ml) in a ratio that produced strong binding of chimeric receptor to the parasites (Figures 1A, 1B, and 1C) brought down adherence to PC12 cells to a level analogous to that of TrkA-minus nnr5 cells ($p < 0.0001$) (Figure 1E). Furthermore, TrkA^{ECD}-Fc interference in *T. cruzi*-PC12 cell adhesion was, in turn, reversed by NGF ($p < 0.001$) (Figure 1E). Therefore, these results supported the conclusion that transmembrane TrkA mediates *T. cruzi* adhesion.

T. cruzi Exploits the TrkA Receptor to Invade Neuronal and Engineered Epithelial Cells

Adhesion of microbes to surface receptors may or may not conduce invasion into cells (Sharon, 2006; Smith and Helenius, 2004), thus we sought to determine whether TrkA-mediated adhesion leads to TrkA-dependent invasion.

Monolayers of PC12^{WT} cells and PC12^{nnr5} cells infected with trypomastigotes (~30 parasites per cell, 37°C) revealed wild-type PC12 cells to be much more permissive to *T. cruzi* than mutant PC12 cells (Figures 2A and 2B); transfecting TrkA to the TrkA-deficient PC12^{nnr5} (Loeb and Greene, 1993) cells restored wild-type cell infection levels (Figures 2A and 2C).

Similarly, *T. cruzi* invaded mink lung epithelial Mv1Lu cells, which normally do not express TrkA, about 4-fold more efficiently in cells engineered to express wild-type human TrkA (Mv1Lu^{TrkA}) (Figure 2D), which additionally suggests TrkA to be a mediator of *T. cruzi* invasion. TrkA expressed in the epithelial cells was biologically active, as NGF activated phosphorylation of TrkA-dependent transcription factor CREB in Mv1Lu^{TrkA}, but not in empty-vector-transfected Mv1Lu (Figure S1 in the Supplemental Data available with this article online), consistent with earlier results by other investigators (Ip et al., 1993).

To determine whether the TrkA-binding site on *T. cruzi* mediates invasion, trypomastigotes were preincubated with TrkA^{ECD}-Fc to block trypanosome-bound PDNF/TS,

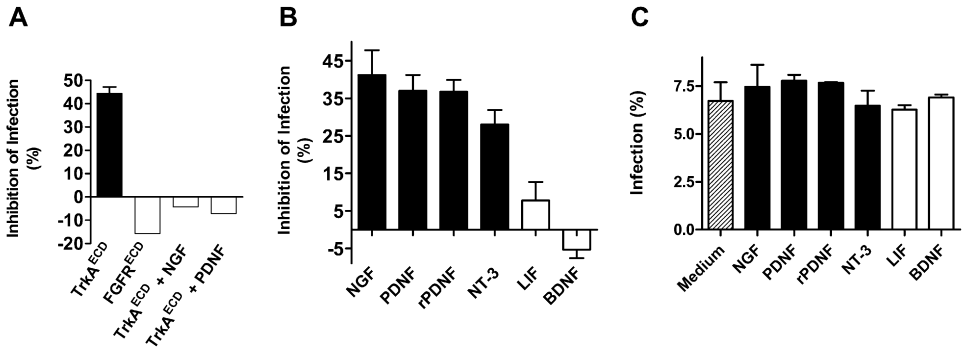

Figure 3. Specific Inhibition of *T. cruzi* Invasion by TrkA^{ECD} and TrkA Ligands

(A) TrkA^{ECD} inhibits *T. cruzi* invasion of PC12 cells. *T. cruzi* trypomastigotes were incubated in DME medium for 1 hr without (vehicle medium) or with control FGFR^{ECD} (1 μg), and TrkA^{ECD} (1 μg) without (TrkA^{ECD}) or with either 3 μg NGF (TrkA^{ECD} + NGF) or 12 μg PDNF (TrkA^{ECD} + PDNF). Parasites were washed by centrifugation to remove unbound TrkA^{ECD} and allowed to infect PC12 cells for 2 hr. Infection in vehicle medium was set at 0% inhibition.

(B) TrkA ligands (NGF, PDNF, rPDNF, and NT-3), but not non-TrkA ligands (LIF, BDNF), inhibit infection of PC12 cells. Cells were treated with DME medium without (vehicle) or with 100 ng/ml TrkA ligands and 100 ng/ml non-TrkA ligands for 30 min prior to the infection. Infection levels of vehicle-treated cells were used to calculate percentage of inhibition.

(C) TrkA ligands do not inhibit residual infection in PC12^{nnr5} cells, as determined by a protocol similar to that in (B). Experiments in (A)–(C) were repeated five times, and the results represent average of those five experiments.

Error bars indicate the standard error of the mean.

washed to remove parasite-unbound receptors, and used to infect PC12 cells. The results showed that TrkA^{ECD}-Fc, but not control FGFR^{ECD}-Fc, inhibited *T. cruzi* invasion and that the inhibition was reversed by loading the neurotrophin-binding site of TrkA with either endogenous (NGF) or exogenous (PDNF/TS) ligands (Figure 3A).

To verify whether *T. cruzi* invasion depends on the interaction of the parasite with the neurotrophin-binding site of TrkA, PC12 cells were preincubated for 30 min with TrkA-binding ligands (NGF, NT-3, and PDNF isolated from *T. cruzi* or from engineered bacteria) and with non-TrkA-binding ligands (leukemia inhibitory factor [LIF] and BDNF). The results showed that all TrkA ligands inhibited *T. cruzi* infection of PC12^{WT} cells (Figure 3B), but not of TrkA-deficient PC12^{nnr5} cells (Figure 3C), whereas non-TrkA ligands did not alter *T. cruzi* invasion of PC12 cells (Figure 3B). These results suggest that *T. cruzi* invasion of PC12 cells depends on TrkA NGF-binding site occupancy by the parasite PDNF/TS.

TrkA-Dependent *T. cruzi* Invasion Requires TrkA Kinase Activity

Microbes do not automatically activate the surface receptors that they use to invade cells, as exemplified by rabies virus, which enters host cells by binding, but without activating, the panneurotrophin receptor p75^{NTR} (Langevin et al., 2002). Three distinct approaches suggest that *T. cruzi* invasion through TrkA requires receptor tyrosine kinase activity.

First, we determined whether *T. cruzi* triggers phosphorylation of TrkA and the TrkA-dependent survival signaling pathways. The results showed that *T. cruzi* induced TrkA autophosphorylation and activated MAPK and PI3K/Akt kinase pathways in a TrkA-dependent manner (Figure S2), consistent with earlier results showing that soluble PDNF/TS induces neurite outgrowth and cell survival through TrkA activation (Chuenkova and PereiraPerrin, 2004).

Second, cells were treated with K252a (Berg et al., 1992) and AG879 (Ohmichi et al., 1993) to see if these specific inhibitors of TrkA kinase autophosphorylation affect *T. cruzi* invasion. The results showed that infecting PC12^{WT} cells sensitized with K252a (Figure 4A) and AG879 (Figure 4C) reduced permissiveness to the level of TrkA-deficient PC12^{nnr5} cells. Neither K252a nor AG879 affected *T. cruzi* invasion of the TrkA-deficient cells (Figures 4B and 4C), strongly suggesting that the two compounds reduced infection by specifically inactivating the TrkA kinase pathway of *T. cruzi* invasion. Furthermore, screening various cell lines with TrkA kinase inhibitors showed that only TrkA-expressing cells (PC12 cells and dendritic cells) became less permissive to invasion upon treatment with the TrkA kinase inhibitors (Table 1). K252a-dependent inhibition of infection was not due to toxicity on *T. cruzi* because preincubating parasites, but not host cells, with the inhibitor did not affect invasion (Figure S3). Treatment of PC12 cells with a specific tyrosine kinase inhibitor of insulin-like growth factor receptor (IGF-1R) did not reduce *T. cruzi* infection (Figure 4D) (Girnita et al., 2004; Zheng and Quirion, 2006), further underscoring specific action of K252 and AG879.

And third, a TrkA deletion mutant was expressed in neuronal and epithelial cells to additionally find out if the kinase domain of the receptor is required for TrkA-dependent invasion. A TrkA derivative (TrkA$^{\Delta ICD}$) that contained intact ECD and transmembrane domains but lacked most of the intracellular kinase domain, including all residues that become phosphorylated upon TrkA dimerization (Figure 4E), was generated. Transfecting TrkA-deficient PC12^{nnr5} cells with TrkA$^{\Delta ICD}$ produced cells (PC12-nnr5$^{TrkA\text{-}\Delta ICD}$) that were poorly permissive, comparable to cells transfected with empty vector (PC12-nnr5^{EV}),

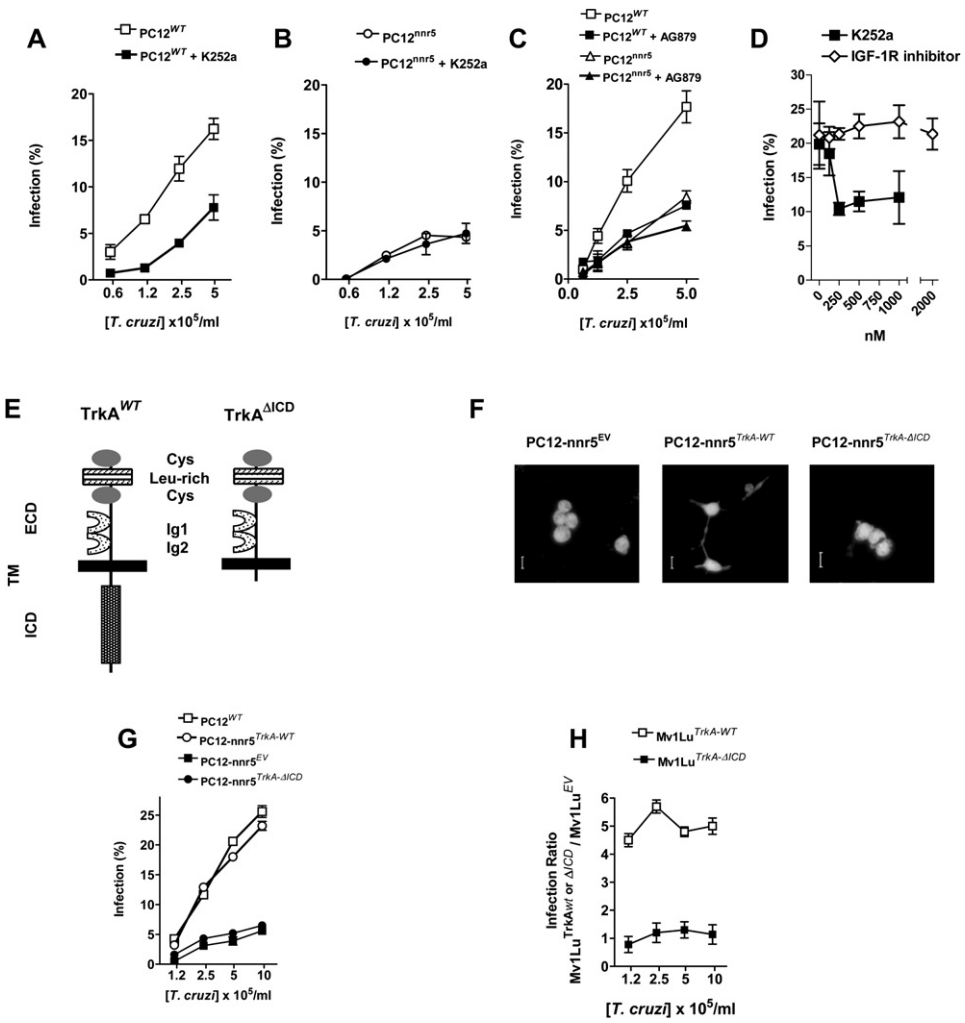

Figure 4. TrkA Kinase Inhibitors and Deletion of TrkA Intracellular Domain Block *T. cruzi* Invasion

(A) K252a inhibits *T. cruzi* infection of PC12 cells. PC12^{WT} cells were pretreated with 500 nM K252a for 1 hr prior to parasite addition. Infection assay is similar to that described in the Figure 2 legend.

(B) K252a does not inhibit *T. cruzi* infection of PC12^{nnr5} cells; protocol is similar to that in (A).

(C) AG879 inhibits *T. cruzi* infection of PC12^{WT} but not of PC12^{nnr5} cells. Protocol is similar to that in (A) and (B) except for the pretreatment with 50 μM AG879.

(C) IGF1-R inhibitor does not block *T. cruzi* infection of PC12^{WT} cells. Protocol is similar to that in (A) except that cells were pretreated with several concentration of IGF-1R inhibitor and K252a.

(E) Diagram of wild-type TrkA (TrkA^{WT}) and TrkA lacking intracellular domain (TrkA$^{\Delta ICD}$). Tyrosine residues that become phosphorylated upon TrkA dimerization are missing in TrkA$^{\Delta ICD}$.

(F) NGF (10 ng/ml, 48 hr) does not stimulate neurite extension in PC12^{nnr5} cells transfected with TkA$^{\Delta ICD}$ (PC12-nnr5$^{TrkA-\Delta ICD}$) or empty vector (PC12-nnr5^{EV}), in contrast to the response of PC12^{nnr5} cells transfected with TrkA^{WT}. After treatment, cells were fixed and stained with anti-neurofilament-200 antibody and Alexa-conjugated secondary antibody. Scale bar, 10μm.

(G) TrkA^{WT}, but not TrkA$^{\Delta ICD}$, rescues *T. cruzi* invasiveness in TrkA null PC12^{nnr5} cells. Infection assays are similar to the protocol in (A)–(C), which show PC12-nnr5$^{TrkA-WT}$ cells supporting *T. cruzi* infection as well as PC12^{WT} cells, in contrast to equally poorly permissive PC12-nnr5$^{TrkA-\Delta ICD}$ cells and PC12$^{nnr5-EV}$ cells.

(H) TrkA^{WT}, but not TrkA$^{\Delta ICD}$, enhances *T. cruzi* invasion of mink lung epithelial cells (Mv1Lu). Mv1Lu$^{TrkA-WT}$, Mv1Lu$^{TrkA-\Delta ICD}$, and Mv1Lu^{EV} cells were infected with the indicated concentrations of *T. cruzi*. Results were plotted as the ratio of Mv1Lu$^{TrkA-WT}$ and Mv1Lu$^{TrkA-\Delta ICD}$ over Mv1Lu^{EV}.

The experiments in (A)–(D), (G), and (H) were repeated three times. The results are representative of one experiment of triplicate samples. Error bars indicate the standard error of the mean.

both in contrast to the rescue produced by TrkA^{WT} in PC12-nnr5$^{TrkA-WT}$ cells (Figure 4G). The rescue in invasion produced by TrkA^{WT}, but not TrkA$^{\Delta ICD}$, could not be explained by low expression of the kinase-deficient constructs because TrkA$^{\Delta ICD}$ and TrkA^{WT} gene products were expressed at similar levels (Figure S4). In agreement with the expectation that TrkA$^{\Delta ICD}$ is devoid of kinase activity, PC12-nnr5$^{TrkA-\Delta ICD}$ cells did not extend neurites in

Table 1. Selective Inhibition of *T. cruzi* Invasion by TrkA Kinase Inhibitors

Cell Type	TrkA Expression	Infection[a]		p Value[b]	Inhibition of Infection (%)
		Vehicle Medium (% ± SD)	TrkA Kinase Inhibitor (% ± SD)		
Dendritic cells[†] (primary culture)	+	11.9 ± 3.2	5.6 ± 1.7	0.008*	52.94
$PC12^{WT}$ (neuronal cell line)	+	14.6 ± 0.9	7.8 ± 2.3	0.02*	46.70
Smooth muscle cells (primary culture)	−	57.0 ± 1.6	53.9 ± 13.4	ns	5.57
L_6E_9 (skeletal muscle cell line)	−	39.4 ± 8.0	39.8 ± 4.1	ns	−0.96
Schwann (glial cell line)	−	47.0 ± 12.6	44.7 ± 7.5	ns	4.99
$PC12^{nnr5}$ (neuronal cell line)	−	4.3 ± 0.1	4.7 ± 1.4	ns	−10

[a] Cell monolayers were treated with vehicle medium (DMEM + 0.001% DMSO) or TrkA inhibitor (500 nM K252a for neuronal cells, smooth and skeletal muscle cells, and glial cells, and 10 μM TrkA inhibitor for dendritic cells [†]) for 1 hr prior to infection with 5×10^6 *T.cruzi*/ml. After 2 days, cell were fixed and stained with Diff-Quick to visualize intracellular parasites. + and − denote expression and absence of TrkA receptor, respectively.
[b] p value was calculated based on paired t test. *Statistically significant infection inhibition; ns, not statistically significant.

response to NGF, in contrast to PC12-nnr5$^{TrkA\text{-}WT}$ cells (Figure 4F). Furthermore, TrkA^{WT}-transfected Mv1Lu (Mv1Lu$^{TrkA\text{-}WT}$) became 4- to 5-fold more permissive to *T. cruzi* than TrkA$^{\Delta ICD}$-transfected Mv1Lu (Mv1Lu$^{TrkA\text{-}\Delta ICD}$) (Figure 4H), further supporting the conclusion that the kinase domain of TrkA is important for *T. cruzi* invasion. Preliminary experiments showed that Mv1Lu transfectants expressed TrkA^{WT} and TrkA$^{\Delta ICD}$ proteins at similar levels (data not shown), excluding the possibility that Mv1Lu$^{TrkA\text{-}\Delta ICD}$ cells were poor *T. cruzi* hosts due to low expression of TrkA$^{\Delta ICD}$ compared to TrkA^{WT}.

Competitive Inhibitors of NGF-TrkA Interaction Attenuate *T. cruzi* Infection in Experimental Chagas' Disease

Chagas' disease is mostly transmitted to man after the bite of reduviid insects on the skin, where resident nerves and TrkA-expressing cells (such as mast cells, keratinocytes, and dendritic cells) upregulate TrkA expression after injury (Pincelli, 2000; Shu and Mendell, 1999). While some parasites may immediately leave the bite site, many remain at the inoculation site and invade local host cells (Schuster and Schaub, 2000). To find out whether *T. cruzi* exploits the TrkA pathway to infect cells in subcutaneous tissues in vivo, we inoculated TrkA^{ECD}-Fc/*T. cruzi* complex into the footpads of mice in a protocol meant to mimic the inhibition of adhesion and invasion brought by the chimeric receptor-parasite complex in vitro (Figures 1E and 3A, respectively). On days 8, 11, and 14 postinoculation, mice infected with TrkA^{ECD}-Fc-trypomastigotes exhibited parasitemia 81%, 80%, and 53% lower than mice inoculated with control *T. cruzi* adsorbed with vehicle medium or control receptor FGFR^{ECD}-Fc (Figure 5A and inset).

One likely explanation for these in vivo results is that binding of TrkA^{ECD}-Fc to trypomastigotes blocked parasite recognition of transmembrane TrkA. A corollary to this hypothesis is that addition of NGF to TrkA^{ECD}-Fc should inhibit receptor binding to the parasites and reverse the blockade of parasite recognition of transmembrane TrkA in vivo, which was observed experimentally (Figure 5B and inset).

Given that TrkA-dependent *T. cruzi* invasion of cells in vitro requires tyrosine kinase activity of the receptor, we also sought to determine whether this would be the case for infection in vivo. First we sensitized footpads of mice with K252a (11.7 μg/kg body weight) to inhibit TrkA tyrosine kinase of permissive host cells in situ, followed by inoculation of *T. cruzi* in the same site 1 hr later. Priming with K252a at 11.7 μg/kg body weight was based on preliminary dose-response experiments that showed this dosage to optimally inhibit parasitemia (data not shown). Measurement of tissue parasitism by real-time PCR (Cummings and Tarleton, 2003) in the inoculation site 3 and 10 days later, and parasitemia at multiple days thereafter, showed that K252a significantly reduced parasite load in the inoculation site (Figures 6A and 6B) and in the blood (parasitemia) (Figure 6C). Such reduced parasite load was reflected in the decreased inflammatory response in histological slices of the inoculation site 10 days after infection (Figures 6E and 6F). That K252a attenuated infection in mice by inhibiting TrkA kinase activity of susceptible cells in the inoculation site was validated by the results of two other experiments. Here, we inoculated parasites in a footpad distinct from the one primed with K252a or in the same footpad sensitized with a tyrosine kinase inhibitor specific for a growth factor receptor unrelated to TrkA (IGF-1R). K252a did not reduce parasitemia when injected into a site far away from the *T. cruzi* inoculation site (Figure S5), nor did the IGF-1R kinase inhibitor administered in the same site as the trypanosomes (Figure 6D).

DISCUSSION

During prenatal development and adulthood, NGF mediates proliferation, differentiation, and survival of neuronal (Bibel and Barde, 2000; Huang and Reichardt, 2003) and nonneuronal (muscle, astrocytes, monocytes, and

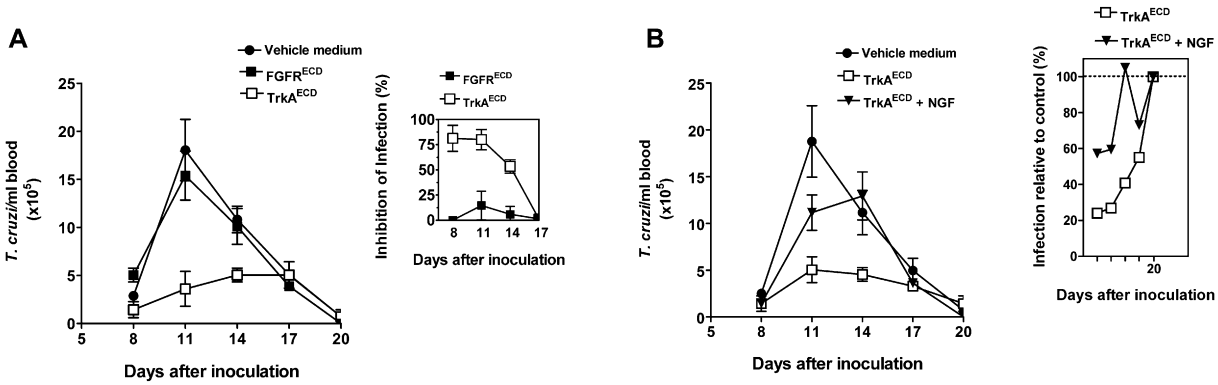

Figure 5. Specific Inhibition of *T. cruzi* Infection In Vivo by Soluble TrkA^{ECD}

(A) TrkA^{ECD}, but not FGFR^{ECD}, reduces parasitemia in a murine model of Chagas' disease. Trypomastigotes (2 × 10^6/ml) were incubated with DME medium without (vehicle medium) or with TrkA^{ECD}, FGFR^{ECD} (1 μg/ml, 1 hr), washed by centrifugation to remove receptor unbound to parasites, and inoculated (3 × 10^3/mouse) subcutaneously in the mice footpads. Parasitemia was monitored every 3 days for the indicated times. Inset shows the degree of inhibition by TrkA^{ECD} (but not FGFR^{ECD}).

(B) NGF reverses the infection-inhibition action of TrkA^{ECD}. Protocol is similar to that in (A) except parasites were incubated for 1 hr with vehicle medium without or with TrkA^{ECD} (1 μg/ml), TrkA^{ECD} (1 μg/ml) + NGF (3 μg/ml), washed by centrifugation, and inoculated into mice footpads. Inset shows the degree of inhibition of parasitemia produced by TrkA^{ECD} and TrkA^{ECD} + NGF relative to control vehicle medium (dotted line). Experiments were repeated twice with similar results.

Error bars indicate the standard error of the mean.

dendritic) cells through the activation of tyrosine kinase receptor TrkA (Hutton and Perez-Polo, 1995; Rende et al., 2000; Vega et al., 2003). NGF binding to TrkA also induces receptor endocytosis and retrograde transport of signaling endosomes (Huang and Reichardt, 2003; Kaplan and Miller, 2000). Thus, if pathogenic microbes were to exploit a neurotrophic survival receptor such as TrkA to access the host cell intracellular milieu, one would expect such a type of invasion to stimulate survival and metabolism of the infected cells.

The results presented here show that the intracellular protozoa parasite *T. cruzi* adheres to and invades PC12 cells through the recognition of TrkA, and that such recognition triggers TrkA-dependent MAPK and PI3K/Akt kinase signaling. Blocking TrkA activation in neuronal (PC12) and nonneuronal (dendritic) cells by pharmacological and genetic means greatly reduced *T. cruzi* invasion. Conversely, forced expression of wild-type TrkA, but not kinase-deficient TrkA, in a cell line naturally resistant to *T. cruzi* (mink lung epithelial Mv1Lu cells) increased invasion by 4-fold. Most interesting, interfering with the *T. cruzi*/TrkA crosstalk either with a TrkA autophosphorylation inhibitor—K252a—or an NGF antagonist—soluble ectodomain of TrkA—profoundly attenuated tissue parasitism, parasitemia, and inflammation in an animal model of Chagas' disease. Therefore, our findings support the novel concept that a survival receptor—TrkA—traditionally associated with development and repair of the nervous system, functions as a vehicle for invasion by the intracellular pathogen *T. cruzi*. Activated TrkA-dependent survival pathways, whether by extracellular *T. cruzi*, *T. cruzi* product PDNF/TS, or NGF, can last at least 2–3 days (Chuenkova and Pereira, 2000; Chuenkova et al., 2001; Chuenkova and PereiraPerrin, 2004). Such durable response is consistent with the idea of invasion receptor TrkA prolonging the life span of infected cells, which could be further extended and/or augmented by intracellular *T. cruzi*, as they, through mechanisms that have yet to be elucidated, seem to inhibit apoptosis in Schwann cells (Chuenkova et al., 2001) and cardiomyocytes (Aoki et al., 2004; Petersen et al., 2006). And these cell-survival-promoting actions would be particularly valuable in nondividing postmitotic cells such as neurons and heart muscle cells.

The ligand that mediates TrkA-dependent *T. cruzi* invasion is PNDF/TS, a protein widely studied for its ability to catalyze the transfer of sialic acid from glycoconjugates into the aqueous environment (neuraminidase activity) (Pereira, 1983) and to β-galactosyl substrates (trans-sialidase activity) (Parodi et al., 1992; Schenkman et al., 1992; Scudder et al., 1993). Although earlier work suggested that these enzymatic activities may mediate *T. cruzi* interaction with epithelial and fibroblast cell lines (Ming et al., 1993; Schenkman et al., 1993), subsequent findings did not support such idea because a monoclonal antibody against PDNF/TS, which completely inhibit trans-sialidase activity, did not alter infection in vivo (Risso et al., 2006). Similarly, TrkA-dependent entry of *T. cruzi* into cells may not require the sialic-acid-binding site of PDNF/TS because a deletion mutant of the enzyme that inactivate both neuraminidase and sialyl-transferase activities did, nevertheless, promote neurite extension and survival of PC12 cells to the same extent as enzymatically active enzyme (Chuenkova and Pereira, 2000). Furthermore, a PDNF/TS-based synthetic linear peptide of 21 amino acids (Y21) reproduced neurotrophic properties of the intact enzyme (Chuenkova and PereiraPerrin, 2005). That trans-sialidase activity is dispensable for TrkA-dependent

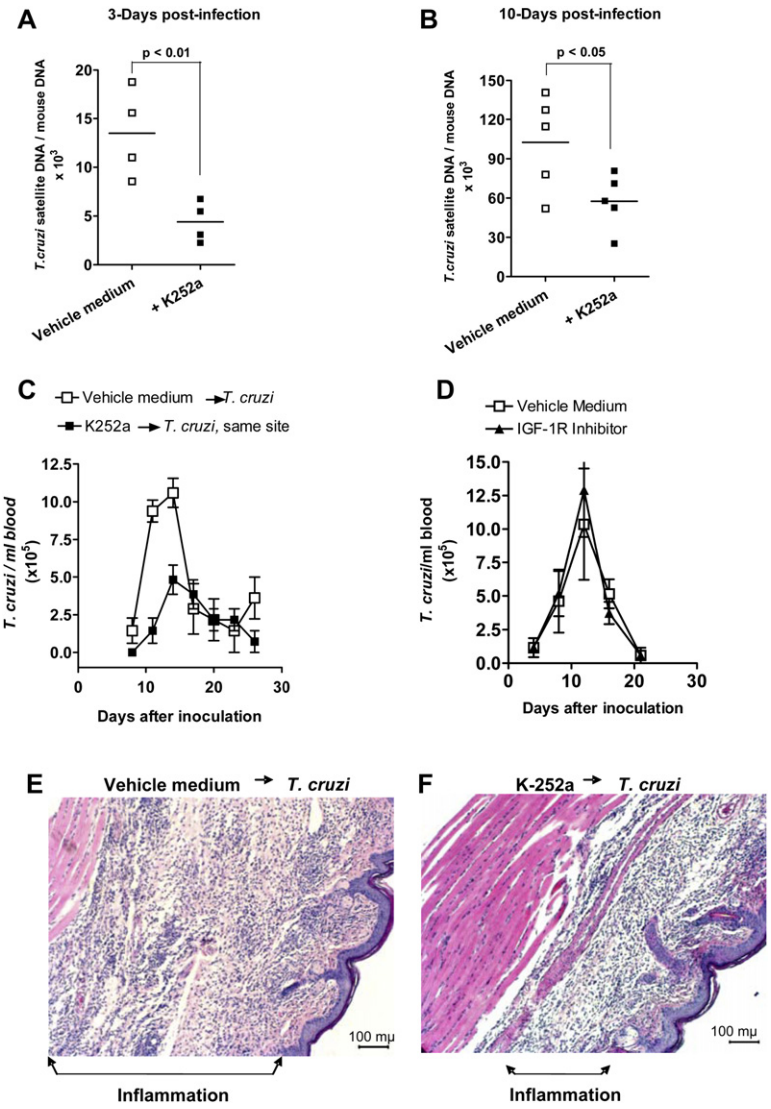

Figure 6. TrkA Kinase Inhibitor K252a Reduces Parasite Tissue Load, Parasitemia, and Inflammation in Experimental Chagas' Disease

(A and B) K252a reduces tissue parasitism in the inoculation site. Vehicle medium without and with K252a (11.7 μg/kg of body weight) was injected in the right footpad of C57BL/6 mice followed by inoculation of *T. cruzi* (3 × 10^3/ml) in the same site 1 hr later. Parasite load at the inoculation site was quantified after 3 (A) and 10 days (B) by real-time PCR. Each symbol represents a single infected mouse. Note that the magnitude on the ordinate in (B) is 10× greater than that in (A), reflecting parasite growth with disease progression in early acute disease.

(C) Kinetics of parasitemia were measured every third day in groups of five mice infected with *T. cruzi* using a protocol similar to that in (A) and (B). Experiments were repeated three times with similar results.

(D) Kinetics of parasitemia were measured every 4 days in mice infected with *T. cruzi* using protocol similar to that in (A) and (B) except that mice were primed with IGF-1R inhibitor (11 μg/kg of body weight).

(E and F) H&E-stained section of inoculation site (right footpad) 10 days postinfection, revealing reduced inflammation in the subcutaneous tissue infected with *T. cruzi* after priming with K252a (F) compared with analogous site sensitized with vehicle medium (G). Inflammatory cells in both (E) and (F) consist of neutrophils, macrophages, and mononuclear cells. Analysis is from two experiments with similar results.

Error bars indicate the standard error of the mean.

invasion was further validated by recently identified PDNF/TS-modeled synthetic peptides that bind TrkA and potently inhibit *T. cruzi* invasion of PC12 cells (M.d.M.-J., M. Chuenkova, and M.P., unpublished data). Thus, PDNF/TS moonlights as a carbohydrate-binding enzyme and as a neurotrophic factor, analogous to other moonlighting proteins like phosphoglucose isomerase, which can function as carbohydrate-binding enzyme and as a growth factor for neurons independently of carbohydrate-binding activity (Petsko and Ringe, 2004).

Schneider and Schweiger proposed more than a decade ago that Trks mediate cell-cell interactions (Schneider and Schweiger, 1991) based on the role played by leucine-rich/cysteine-rich cassette of other receptors such as microbial recognition of Toll-like receptors (Kedzierski et al., 2004) and *Leishmania infantum* binding to macrophages (Kedzierski et al., 2004). The results presented here provide experimental support, perhaps for the first time, for TrkA-dependent cell-cell adhesion in a heterophilic (mammalian host cell and unicellular trypomanosome) system (Figure 1). Such TrkA-dependent adhesion, in turn, serves as a prelude for *T. cruzi* invasion of cells of the nervous system and other tissues that express the NGF receptor. It highlights one invasion mechanism among various others that *T. cruzi* most certainly exploits to infect a diverse range of distinct host cell types in mammals.

Indeed, two other receptors, transforming growth factor β receptor II (TGFβ-RII) and bradykinin receptor, have been proposed to mediate *T. cruzi* infection of cultured mink lung epithelial Mv1Lu cells and human umbilical vein endothelial cells, respectively (Ming et al., 1995; Scharfstein et al., 2000). *T. cruzi* is not alone in taking advantage of distinct receptors in the quest to invade mammalian cells, for even a protozoan parasite—*Leishmania*—that inhabits a single cell type—macrophages—uses at least three surface receptors (complement receptors 1 and 3, and mannose/fucose receptor) (Handman

and Bullen, 2002). Simpler organisms such as viruses may also penetrate cells through multiple receptors as best exemplified by HIV-1, which exploits at least four distinct molecules (CD4, mannose binding C-type lectin, and chemokine receptors CXCR4 and CCR5) to infect cells (Smith and Helenius, 2004). Nevertheless, TrkA is the first among the Trk receptors and the other two major families of receptors for neurotrophic factors (neurokines and the glial-cell-line-derived family of ligands) to be identified as a mediator for the intracellular invasion of a microbe. Given that neurotrophins can bind to more than one Trk receptor, as in the case of NT-3, which can activate TrkA and TrkC (Huang and Reichardt, 2003), it may be that PNDF binds and mediates invasion not only through TrkA, but also via TrkB and/or TrkC as well.

T. cruzi is believed to persist for many years inside myocytes in the heart and gastrointestinal tract of chronic chagasic patients (Brener, 1973). Adipocyte is also a likely important *T. cruzi* reservoir, as parasite DNA can be detected in adipose tissue as long as 300 days postinfection (Combs et al., 2005). Given that most patients with acute Chagas' disease have *T. cruzi* in their cerebrospinal fluid (Hoff et al., 1978) and that chronic chagasic patients immunosuppressed with HIV, drugs, or irradiation can reactivate infection predominantly in their brains, including striatum and other parts of the basal ganglia (Antunes et al., 2002; Kohl et al., 1982; Rosemberg et al., 1992), it may be that *T. cruzi* also remains dormant in cholinergic basal forebrain and striatum, the only CNS site that contains TrkA-expressing neurons (Steininger et al., 1993).

EXPERIMENTAL PROCEDURES

Parasites and Mammalian Cell Lines

The Silvio X-10/4 and Tulahuen strains of *T. cruzi* were propagated in a human Schwann cell line as described previously (Chuenkova and Pereira, 1995) and used for in vitro and in vivo assays, respectively. Tulahuen strain was chosen for in vivo experiments because it produces readily detectable parasitemia and mortality, while Silvio strain was used in the in vitro experiments because it grows robustly in cell cultures. $PC12^{WT}$ and $PC12^{nnr5}$ cells were gifts from Dr. Lloyd Green (College of Physicians and Surgeons, Columbia University, NY), as were $PC12^{nnr5}$ cells stably expressing human $TrkA^{WT}$ used in Figure 2A (Green et al., 1986; Loeb and Greene, 1993). The permanent human Schwann cells cell line was used in a previous study (Chuenkova et al., 2001). Primary cultures of human smooth muscle cells were a gift from Herbert Tanowitz (Albert Einstein College of Medicine, Bronx, NY), and bone marrow-derived dendritic cells were a gift from Kristin Stephan (Tufts Medical Center, Boston, MA). Mink lung epithelial cells (Mv1Lu) and the myoblast cell line L_6E_9 were purchased from ATCC.

Binding of $TrkA^{ECD}$ to *T. cruzi*

Trypomastigotes (10^7/ml) were incubated with $TrkA^{ECD}$-Fc and $FGFR^{ECD}$-Fc (R&D systems) in binding buffer (DMEM, 0.1% BSA) for 1 hr at 4°C without or with putative competitors, washed four times with binding buffer by centrifugation (1,500 × g, 10 min) to remove unbound receptor, and processed to quantitate bound receptor and measure cell adhesion and invasion in vitro and in vivo. To ascertain bound $TrkA^{ECD}$, parasites were resuspended in SDS sample buffer, run on reducing SDS-PAGE (7.5%), transferred to nitrocellulose, and probed with anti-human IgG peroxidase-labeled antibody (Promega) and scanning densitometry (Bio-Rad laboratories). For cell adhesion, trypomastigotes were labeled with fluorescent CellTracker Red CMTPX (Molecular Probes) as recommended by the manufacturer and added to $PC12^{WT}$ and $PC12^{nnr5}$ cells for 30 min at 4°C. Cell monolayers were washed four times in the cold, fixed in 4% paraformaldehyde for 15 min, washed in PBS three times, and analyzed by fluorescent microscopy.

T. cruzi Infection In Vitro

Cells were infected with *T. cruzi* (Silvio), and infection was visualized by fluorescence or phase-contrast microscopy as previously described (Chuenkova and PereiraPerrin, 2004). For inhibition experiments, cells were preincubated for 30 min with PDNF or rPDNF purified by affinity chromatography (Chuenkova and Pereira, 2000; Scudder et al., 1993), NGF (Sigma-Aldrich), NT-3 (R&D Systems), LIF (Sigma-Aldrich), BDNF (R&D systems), washed three times with DMEM, and infected with various concentration of *T. cruzi*. To test the effect of TrkA kinase inhibitors (Calbiochem) on *T. cruzi* invasion, cells were preincubated for 1 hr with TrkA kinase inhibitor (10 μM, IC_{50} = 6 nM), K252a (500 nM, IC_{50} = 3 nM), and AG879 (50 μM, IC_{50} = 10 μM), and IGF-1R inhibitor (125 nM to 2 mM, IC_{50} = 1 nM) followed (without washing) by various concentration of parasites for 2–3 hr.

Murine Model of Acute Chagas' Disease and Real-Time Quantitative PCR

Groups of five female C57BL/6 mice (Jackson Laboratory), 6–8 weeks old, were injected subcutaneously into footpad with 3×10^3 trypomastigotes (Tulahuen) pretreated with $FGFR^{ECD}$-Fc or $TrkA^{ECD}$-Fc with or without NGF. In some experiments, footpads were primed with vehicle medium containing, or not, K252a (11.7 μg/kg of body weight) or IGF-1R inhibitor (11 μg/kg of body weight) followed by *T. cruzi* inoculation in the same site 1 hr later. Parasitemia was monitored every 3 days (Chuenkova and Pereira, 1995). In some cases, footpads were collected 3 and 10 days postinfection and tissue processed for hematoxylin and eosin staining or for quantification of parasite load by real-time PCR (Cummings and Tarleton, 2003). Parasite load (relative amounts of *T. cruzi* DNA per mouse DNA) was calculated by considering DNA from uninfected mice as baseline and TNF (one-copy mouse gene) as internal control. These experiments were approved by the Institutional Animal Care and Use Committees (IACUC) of Tufts University-New England Medical Center.

TrkA Constructs and Transfection Assays

Primers were designed to amplify TrkA cDNA encoding 796 amino acids (full-length $TrkA^{WT}$) (TrkA-1: 5′-CCGCTCGAGATGCTGAG AGGCCA-3′ and TrkA-2: 5′-CCGGAATTCCGCCCAGAACGTC 3′) or 489 amino acids (truncated form that lacks kinase domain, or $TrkA^{\Delta icd}$) (TrkA-1 and TrkA-4: 5′-CCGGAATTCCCTGTGGGTTCTC-3′), for which XhoI and EcoRI restriction sites were added to the 5′ and 3′ ends, respectively. DNA was digested and ligated into the vector pEYFP-N1 (Clontech). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. TrkA function was tested by neurite outgrowth assay. Transfected $PC12^{nnr5}$ cells were treated with 10 ng/ml NGF in 0.5% FCS DMEM for 48 hr. Cells were fixed in 4% paraformaldehyde, blocked in 5% BSA/PBS, and probed with anti-neurofilament 200 (Sigma-Aldrich) followed by Alexa 594-conjugated anti-rabbit IgG (Molecular Probes). Cells were analyzed in a fluorescent microscope.

Supplemental Data

The Supplemental Data include five supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/1/4/251/DC1/.

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