Role of the neurotrophic receptor TrkC in the pathogenesis of *Trypanosoma cruzi* infection

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ABSTRACT

Chagas' disease is caused by infection with the obligate intracellular parasite *Trypanosoma cruzi*. Gastrointestinal failure, which results from nerve tissue destruction, is a major complication of Chagas' disease. The neurotrophic receptor TrkA mediates *T. cruzi* cellular invasion of some neural and non-neural cells, but several infected cell types such as cardiomyocytes and nerve tissue cells in the colon and heart lack TrkA expression. Using *in vitro* assays we report that TrkC, which shares a common ligand (NT-3) and sequence homology with TrkA, mediates *T. cruzi* cellular invasion. Under the same conditions, the third Trk family receptor, TrkB, does not mediate cellular invasion. The parasite molecule responsible for binding TrkC is PDNF/TS, as it co-immunoprecipitates with TrkC and also inhibits invasion in TrkC expressing cells.

Trk receptors and their neuroprotective signaling underlie the development and maintenance of mammalian nervous tissue. We show that *T. cruzi* can promote TrkC-dependent cell survival, as it does with TrkA, and that invasion in TrkC- or TrkA-expressing cells results in more robust intracellular parasite division. As such, and even though *in vitro* cellular invasion leads to cell death due to intracellular parasite overgrowth during the course of 4-7 days, TrkC and TrkA may serve a dual beneficial role for *T. cruzi* by mediating cellular invasion and simultaneously providing trophic signals that help maintain the parasite's environment.

To determine the significance of *T. cruzi*-Trk interactions in Chagas' disease, we focused on a mouse model of *T. cruzi* infection. Acute infection in mice was specifically blocked using α-TrkA and α-TrkC antibodies, which suggests both receptors play an important role in mammalian infection. Continuing with this model,

as parasites spread from the initial inoculation site, parasitemia peaks 11 days post inoculation (dpi) and colon parasitism peaks at 18 dpi. Focusing on the nerve tissue cells of the colon, which express TrkB and TrkC but not TrkA, we found a roughly 60% reduction of TrkC-expressing cells in infected mice versus uninfected mice, whereas TrkB mRNA remains constant, which suggests TrkB cells are spared. These data raise the possibility that *T. cruzi* preferential invasion of TrkC-expressing cells results in the destruction of a specific subpopulation of nerve tissue cells in the colon. In addition, TrkC cell death correlates with increased colon size in infected mice. Although megacolon (an enlarged non-functional colon) can occur in Chagas' disease patients, this is the first such evidence showing mice infected with *T. cruzi* develop enlarged colons.

Our data support a novel model of *T. cruzi* colon pathology such that *T. cruzi* preferential invasion of TrkC-expressing cells leads to specific destruction of TrkC-expressing cells in the colon, which results in pathological enlargement of the colon. Given the broad expression of TrkC in the PNS and in other highly relevant tissues in Chagas' disease, such as muscle cells of the heart, our data raise the further possibility that TrkC plays a prominent role in mediating invasion and tissue damage in organs other than the colon.

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LIST OF ABBREVIATIONS

BDNF Brain-derived neurotrophic factor

CNS Central nervous system

DMEM Dulbecco's modified eagle's medium

EBA Erythrocyte-binding-like family

ECD Extracellular domain

ENS Enteric nervous system

Erk Extracellular-signal regulated kinase

FBS Fetal Bovine Serum

GI Gastrointestinal

HPRT Hypoxanthine-guanine phosphoribosyltransferase

Ig Immunoglobulin

Ig-2 Membrane proximal immunoglobulin-like domain of Trk receptors

LRR Leucin-rich region

mAb Monoclonal antibody

MAPK Mitogen-activated protein kinase

NGF Nerve growth factor

NT-3 Neurotrophin-3

PDNF Parasite-derived mimic of neurotrophic factor

PI-3K Phosphoinositide-3 kinase

PLC Phospholipase C

PNS Peripheral nervous system

rPDNF Recombinant parasite-derived mimic of neurotrophic factor

RPMI Roswell Park Memorial Institute Media

RT Room temperature

SD Standard deviation

TGF-β Transforming growth factor- β

TGF-βR Transforming growth factor-β receptor

Trk Tropomyosin-related receptor kinase

TS *trans*-Sialidase

CHAPTER 1. INTRODUCTION

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1.1 The Discovery of Chagas' Disease

Infectious diseases have decimated human populations for millennia and have also played prominent roles in the more recent history of the Americas. Studying, understanding and controlling these diseases have been important steps in human health and economic growth. For example, yellow fever and malaria played a key role in both the Louisiana purchase (1803) and the construction of the Panama Canal (1914): in both situations, the French, after losing thousands of lives to the diseases in the new world, sold their properties to the United States. In large part, the American success with the Panama Canal can be credited to the recognition, finally, that Yellow Fever and Malaria are vector-borne. This started with the Cuban scientist Dr. Carlos Finlay first postulating in the later decades of the 19th century that the mosquito was the vector for these diseases. Dr. Walter Reed, an American physician working for the US Army, confirmed this hypothesis at the turn of the century and implemented a successful vector eradication strategy to limit the diseases, giving protection to the workers in Panama to complete the canal. As such, immense health and economic gains were being realized at the turn of the 19th century when physician-scientists were combating these terrifying diseases with new understanding of their origin.

Brazil was no exception. In 1908, the prominent physician Oswaldo Cruz—who helped eradicate Small Pox and Yellow Fever from Brazilian ports—sent his younger colleague and protégé of sorts, Carlos Chagas, to head an anti-malarial campaign that was ravaging the effort of railroad construction in the Minas Gerais region. By 1909 Chagas published a preliminary paper describing a new trypanosome that he named *cruzi* in honor of his mentor. More definitive papers followed, describing the parasite and the disease it causes in humans in greater detail, as well did controversy regarding the

diverse number of symptoms that Chagas associated with *Trypanosoma cruzi* infection. The diversity and chronicity of the disease course have greatly complicated the understanding and study of Chagas' disease. A good example of this is the first confirmed *T. cruzi* infected patient, Berenice, who, infected as a young child, was entirely healthy with no known clinical problems until she died in her 70's. She had no neurological problems (a prominent finding of Dr. Chagas, although less so today), no heart disease and no gastrointestinal disease, yet parasites could still be cultured from her blood when she was 53 years old (Lewinsohn, 1981).

In a way, this example is linked to where my project in lab started: Although *T. cruzi* parasitizes mammalian nervous and muscle tissue where pathology can be seen in some Chagas' disease patients, has it evolved mechanisms to limit host damage? What molecular mechanisms has *T. cruzi* evolved to interact with mammalian nervous tissue?

1.2 The Causative Agent of Chagas' Disease

It is now clear that Chagas disease is a vector-born infectious disease caused by the obligate intracellular parasite *Trypanosoma cruzi*. *T. cruzi* is a unicellular eukaryote that exists in three life forms: Epimastigote (5μM X 10uM, vector form), Trypomastigote (2μM x 15-20μM, flagellate form that invades mammalian cells) and Amastigote (2μM x 2μM, intracellular replicating form). After the initial infection of mammalian hosts, the parasite invades cells and divides locally; it then spreads systemically through the blood where nervous and muscle tissues develop a high parasite burden. Cellular invasion is a key component of these processes.

1.3 *T. cruzi* Evolution and Vector

T. cruzi is one of the many members of the order Kinetoplastida and family Trypanosomatidae that contribute significantly to disease in the world. *Trypanosoma cruzi* (Chagas' disease), *Trypanosoma brucei* (African Sleeping Sickness) and *Leishmania* species (Leishmaniasis) are the most common. Trypanosomes are thought to have evolved roughly 570 million years ago in aquatic organisms (living in both invertebrates and vertebrates) (Hamilton et al., 2005). Roughly 100 million years ago, the current *T. cruzi* life cycle became possible with the evolution of the hematophagous insect vectors of the subfamily Triatominae (Reduviidae Family). Reduviid beetles that support the *T. cruzi* life-cycle live in the Americas from parallel 42° North to parallel 42° South (Carcavallo, 1999).

This large area that stretches roughly from the northern United States to southern Argentina/Chile has likely promoted the further differentiation of *T. cruzi* parasites to fill specific niches. Isoenzyme profiles analyzed by gel electrophoresis led to the categorization of *T. cruzi* into specific zymodemes (Z1, Z2 and Z3); genetic analysis of polymorphisms of ribosomal RNA genes has led to two *T. cruzi* groups, lineage 1 and lineage 2 (TCI and TCII) (Yeo et al., 2005). TCI and TCII are further categorized, but the significance of these *T. cruzi* divisions is not always clear. Evidence exists to the contrary, but in general, TCI parasites predominate north of the Amazon basin and TCII parasites to the south; in addition, this geographic separation roughly correlates with more severe pathology in the Southern region compared to the North (Miles et al., 1981).

1.4 *T. cruzi* Life Cycle

T. cruzi lives in the midgut of a large number of reduviid beetles as dividing epimastigotes (and amastigotes, to a lesser extent). As parasites move through the gastrointestinal (GI) tract, the parasites eventually attach to mucosal surfaces in the

beetle hindgut where they differentiate into long slender flagellate forms called metacyclic trypomastigotes. Metacyclic trypomastigotes are then released in the feces of the beetle and gain access to mammalian hosts through mucosal surfaces (gut and eye) or through lesions in the skin (the bug bite). Parasites subsequently invade cells where they differentiate into amastigotes and divide. Intracellular reproduction occurs predominantly through asexual binary fission, although evidence suggests a form of sexual reproduction (genetic exchange and recombination with non-Mendelian gene distribution) can occur (Carrasco et al., 1996; Gaunt et al., 2003). Amastigotes differentiate again into trypomastigotes, burst from the infected cell, infect additional cells and eventually enter into the blood, at which point a bug taking blood meal may become infected. Differentiation into different life forms is in large part regulated by glucose availability and cAMP signaling (Tyler and Engman, 2001). The image in Diagram 1 below summarizes the infection cycle of *T. cruzi* through during both the insect vector and its mammalian hosts.

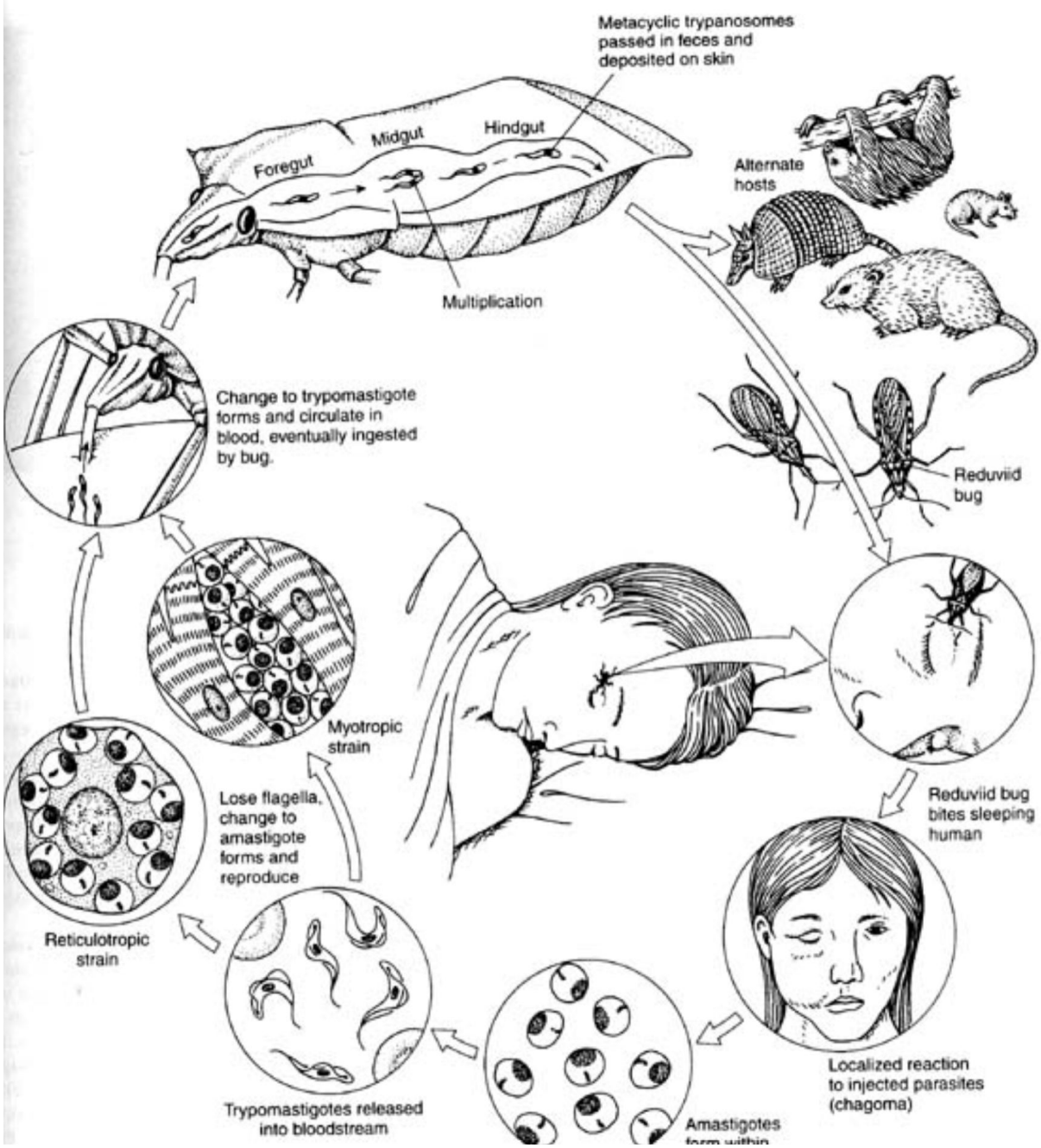

Diagram 1. *T. cruzi* Life Cycle in Insect Vector and Mammalian Hosts

\1.5 Infection and Clinic

http://jpkc.sysu.edu.cn/jscx/Textbook/six-5_clip_image008.jpg

The vast majority of contact between Reduviid beetles and human hosts occurs when humans live in poor, rural housing conditions (mud walls, thatched roofs) where beetles are often co-habitants. The most common story associated with infection is that the beetle lands on someone when they are sleeping, taking a blood meal and depositing feces. The parasites, which are in the feces, are then rubbed into the bite site or into the eyes. The large and very conspicuous size of the beetles (often 1" or greater) generally precludes the possibility of being infected during the day (see Diagram 2, below). Because the face is often the most exposed region of the body when one is sleeping, the beetle usually bites the face and a welt is left behind; hence, the beetle is called a Kissing Bug in English and Barbeiro (barber) in Portuguese. Other modes of infection include maternal-fetus transmission, blood transfusions, organ transplant and parasite ingestion.

www.foodsafetynetwork.ca/userfiles/image/Animals/Insects_Pests/Reduviid bug.jpg

www.inhs.uiuc.edu/~sjtaylor/reduviidae/Reduviid3.jpg

Diagram 2. *Reduviid* Beetle on Face (left) and Leaf (right).

After the initial infection the disease can be examined in three sequential phases: acute, indeterminate and chronic phase. The classic sign of the initial infection is unilateral periorbital edema (swelling around one eye, called Romaña's sign) or a raised welt on the skin (chagoma) when infection occurs in the eye or bite site, respectively. Lymph node swelling, muscle pain, a mild fever or no symptoms at all are the norm for acute infection, but the very young or immunosuppressed can develop fatal acute central nervous system (CNS) or cardiac disease. Carlos Chagas originally estimated the acute mortality of Chagas' disease at about 50% (Koberle, 1968), but currently that number seems closer to 5% when untreated (Prata, 2001). The indeterminate phase, when the infected person remains asymptomatic, can last a lifetime. However, roughly 30% of indeterminate patients develop the chronic form of the disease where patients, usually within 10-30 years, develop heart disease and/or GI tract disease (de Oliveira et al., 1998; Koberle, 1968; Punukollu et al., 2007). Below I discuss the most prominent complications of Chagas' disease, and in section 1.6 I examine the leading hypotheses for the pathophysiological mechanisms that may be responsible for the disease complications.

1.5a Cardiac Disease

Chagas' disease is the leading cause of heart failure in South America. During acute infection as many as 90% of infected individuals have cardiac involvement, although symptoms are mild enough that diagnosis is established less than 10% of the time (Parada et al., 1997; Punukollu et al., 2007). During the indeterminate phase, cardiac problems are subclinical, although pathology can be detected by Holter monitor and echocardiogram (Barretto and Ianni, 1995). Signs and symptoms vary widely in

patients who develop chronic Chagas' cardiac disease. Syncope, palpitations, shortness of breath and lethargy are common symptoms. A variety of heart murmurs and arrhythmias, left ventricular apical aneurysm, right bundle branch block and abnormal Q waves and low QRS voltage are common signs in Chagas' disease patients. In the most advanced stages, mitral and tricuspid regurgitation occurs and there is a global cardiac enlargement, which is a classic sign of Chagasic heart disease known as cardiomegaly (Punukollu et al., 2007).

1.5b Gastrointestinal Disease

Heart disease is the most common problem of Chagas' disease, while GI findings are the second most common. Complications with the GI tract develop progressively over several decades. Patients may complain of problems swallowing, vomiting, bowel discomfort, bloating and severe constipation. Peristalsis (the rhythmic, autonomic movement of the bowels) becomes progressively worse and the GI sphincters (most commonly of the esophagus and large intestine) can begin to lose function. As gut motility and sphincter function decrease, the areas directly proximal to the GI sphincters balloon because the contents cannot move. This is referred to as mega-esophagus and mega-colon and can be easily visualized with barium radiographs (de Oliveira et al., 1998). There are rare occurrences of gall bladder, biliary tree, stomach and small intestine disease in Chagas' disease patients (Koberle, 1968; Matsuda et al., 2009).

1.5c Additional, Rare Complications of Chagas' Disease

Encephalitis accounts for a small percentage of death during acute Chagas' disease, and when infected asymptomatic individuals become immunosupressed (HIV or cancer/transplant therapy), fulminant CNS Chagas' disease often occurs (Pittella, 2009). There are accounts of the ballooning of the ureter and bladder, destruction of salivary

glands, peripheral neuropathies and spinal cord lesions that result in impaired movements (Koberle, 1968; Matsuda et al., 2009).

It should be apparent at this point that *T. cruzi* infection can result in a diverse number of clinical complications. A common factor among these findings, and sometimes the underlying etiology of disease, is the is the destruction of nervous tissue in the PNS that occurs in Chagas' disease patients.

1.6 Pathophysiological Mechanisms of Chagas Disease

As I have briefly discussed, the great majority of Chagas' disease patients are infected as children and remain asymptomatic throughout their life, often only developing symptoms—if ever—decades after infection. Destruction of nervous tissue, particularly of the autonomic branch of the peripheral nervous system (PNS), and direct destruction of cardiac muscle are thought to explain the majority of pathologies seen in Chagas' disease. However, it has been a point of mystery and contention to imagine how an infected patient who acutely controls the parasite infection without symptoms can then die of heart failure or other complications 30 years later. The main theories to explain these findings include host-driven mechanisms (immunopathology) and parasite-driven mechanisms. Before discussing how the pathology may occur, I will review the general organization of the mammalian nervous system and the pathology found therein in Chagas disease patients.

1.6a Organization of the Nervous System

The CNS, which includes the brain and spinal cord, communicates with the rest of the body via the PNS. The PNS is comprised of somatic nervous system (SNS) and the autonomic nervous system (ANS). The SNS controls sensory and motor functions, generally considered to be voluntary actions. The ANS helps control respiration, heart

rate, gut motility and other functions that are generally considered involuntary. The ANS is comprised of the sympathetic branch, parasympathetic branch and enteric branch.

As with the rest of the PNS, nerves of the ANS relay messages to and from the CNS. For example, when I give my thesis defense seminar my brain will send signals to the rest of my body via sympathetic nerves: a “pre-ganglionic” sympathetic nerve originating in my spinal cord will make a synapse with a “post-ganglionic” nerve at an area called a sympathetic ganglia (a group of neuronal bodies) just outside the spinal cord. The post-ganglionic nerve will course a path to organs and blood vessels to increase my heart rate and respiratory rate, shut off blood to the GI tract and prepare me to run away. Hopefully my parasympathetic nerves, which generally counter the actions of the sympathetic nervous system, will make me relax and breath. Parasympathetic nerves are organized in a similar fashion except that pre-ganglionic nerves are long and postganglionic nerves are short; thus, parasympathetic ganglia are close to the organ of innervation (whereas the sympathetic ganglia are close to the spinal cord.)

The third branch of the autonomic nervous system, the enteric nervous system, controls the movement of food through the gut. The ENS has some input from sympathetic and parasympathetic nerves, but is often considered a separate branch of the nervous system because it functions in relative autonomy (below the upper esophagus) with fully enclosed neural circuits. The ENS is composed of a vast system of neurons and glial cells that migrate from the neural crest under the control of GDNF (glial-derived neurotrophic factor) and NT-3 (Chalazonitis, 2004; Costa et al., 2000; Ruhl, 2005).

The ENS is sandwiched between the layers of the GI, organized in a vast network of interconnected ganglia. The GI tract is a long tube of concentric layers of tissues. The main layers, starting from the lumen are: 1) the epithelial mucosa, 2) circular muscle and 3) longitudinal muscle. There are two layers of nerve tissue: the

submucosal plexus that is between the mucosa and circular muscle and the myenteric plexus that is between the circular muscle and the longitudinal muscle. There are over 20 types of neurons (Costa et al., 2000) and 2 types of glial cells (non-myelinating Schwann cells and astrocyte-like enteric glial cells) (Ruhl, 2005) that coordinate the synchronous involuntary movement of the gut, called peristalsis. As neurons die (or glial cells that support the neurons) peristalsis breaks down and the gut ceases to function properly.

1.6b Nerve Tissue Pathology in Chagas' Disease

Ganglia of the PNS are commonly destroyed in Chagas' disease. In ganglia of the gut and those that control the heart, 50-100% of the neurons are missing in Chagasic patients compared to age-matched controls based on autopsy reports (da Silveira et al., 2005; Koberle, 1968). The majority of human analyses and animal studies related to Chagas' disease focus on the nervous system of the gut and heart, but many original studies quantified neuron loss throughout the body, finding neuron loss in most areas of the PNS including those that control the lungs, gland secretion, the endocrine system and the muscular system (somatic nervous system). The parasympathetic and enteric system appears to be destroyed with more frequency. It is not clear what controls this selectivity, but these points will be discussed further in the Discussion.

Neuronal death is an accepted etiology of megacolon and megaesophagus: when specific neuronal populations die the sphincters and peristalsis they control no longer function and the organs balloon because waste cannot be cleared (Matsuda et al., 2009). Cardiomyopathy, however, is more complicated. Nerve damage and accompanying heart arrhythmias and ECG abnormalities appear early Chagas' disease (Ribeiro et al., 2005), but there is also often extensive inflammatory infiltrates and focal damage to cardiomyocytes and the conduction systems (non-neural tissue) (Morris et

al., 1990). This means that although nerve damage may likely be a contributing factor to the varied complications of Chagas' heart disease, even without nerve damage patients would likely still develop cardiomyopathy. Thus, GI complications are closely linked to nerve tissue damage and cardiac complications are likely a combination of cardiac tissue and nerve tissue damage.

The mechanisms that underlie this damage in the mammalian host are not clear. Hypotheses include 1) autoimmunity, 2) over-reactive immune response to few parasites, 3) a secreted parasite neurotoxin and 4) damage through cellular invasion and parasite release that leads to cell death. The neurotoxin hypothesis was suggested decades ago (Koberle, 1968), and there is not any evidence to support or refute it. The other possibilities will be discussed below.

1.6c Autoimmunity Hypothesis

The relative paucity of parasites in indeterminate Chagas' disease patients and the gradual onset of disease (many years after initial infection) led to the hypothesis that autoimmunity plays an important role in disease pathogenesis. There are over 50 review articles that are available through Pubmed if you search for "Autoimmunity in Chagas' Disease," and whether autoimmunity contributes to the pathogenesis of disease has been debated for nearly three decades. This is such an important question in the field because it greatly shapes therapeutic strategies for the disease. The resounding initial belief in the autoimmune hypothesis led to a severe lack of initiative and funds to develop vaccines and conventional drug therapy. The logic being that vaccines may promote autoimmunity (by presenting parasite antigens) and anti-parasitic drug therapy may not be beneficial because it is not the parasite causing the disease.

T. cruzi-induced autoimmunity could occur through molecular mimicry or through a cryptic antigen mechanism. Molecular mimicry, much like the mechanism involved with

rheumatic fever, is that antibodies raised against parasite epitopes cross-react with host antigens. A cryptic antigen is one that is not normally exposed, but infection or tissue damage allows for immune presentation of that previously cryptic self-antigen and autoimmunity can ensue. In general, it seems that molecular mimicry has received more attention in the Chagas' disease field.

Much of the evidence for the possibility of autoimmunity rests on several groups describing a number of autoreactive antibodies and T cells that are specific to humans or animals infected with *T. cruzi* (Cunha-Neto and Kalil, 2001; Fae et al., 2008; Khoury et al., 1979; Kierszenbaum, 2005; Leon et al., 2004) and have functional relevance *in vitro* (de Oliveira et al., 1997; Goin et al., 1997; Masuda et al., 1998). But does the autoreactivity translate to mammalian disease? To consider the hypothesis in more depth, I've summarized two key animal model studies from some of the leading groups in this field.

A recent paper from a prominent group that has been pursuing this hypothesis for decades (Leon et. al., 2004) showed convincing results that immunization of A/J mice using *T. cruzi* protein extracts and complete Freund's adjuvant lead to increased IgG specific for cardiac myosin in immunized versus non-immunized mice (Leon et al., 2004). One issue is that they could not detect any cardiac damage in the immunized mice. Furthermore, no group has ever demonstrated that the passive transfer of antibodies from *T. cruzi* infected animals recapitulates disease (Kierszenbaum, 2005). Another key example of work supporting the autoimmune hypothesis was a Journal of Experimental Medicine paper published in 1992. Santos et. al. showed that transfer of whole hearts into syngeneic mice led to rejection in mice that had been previously infected with *T. cruzi* whereas the hearts were not rejected from uninfected mice; they showed this to be dependent on CD4 T-cells. Additionally, the CD4 T cells expanded *in vitro* when incubated with myocardial antigens. These experiments are convincing and

clearly define an auto-reactive T cell population in infected mice. However, 4 years later using a similar system, Tarleton et al showed that the heart rejection was dependent upon parasites (from the infected mouse that received the graft) remaining and penetrating the transplanted heart (Tarleton et al., 1997). These data refute the most convincing experiments to demonstrate autoimmunity in a mouse model.

The last bit of information that seems relevant is that it has been known for decades that patients—even those with the indeterminate, asymptomatic form of the disease—quickly develop Chagasic symptoms if they become immunosupressed. Because autoimmune patients benefit from immunosuppression, this further suggests that autoimmunity is unlikely to play a prominent role in Chagas' disease pathogenesis.

1.6d Immunopathology Hypothesis

As an alternative to autoimmunity, where the immune system recognizes self as foreign, immunopathological mechanisms would entail an immune response that is directed against *T. cruzi*, but is out of proportion to what is needed to control the infection and therefore results in damage. Immunity regulated by Th1 and CD8+ T cells is key in the control of acute *T. cruzi* infection (Gutierrez et al., 2009), which would result in CTL-mediated killing of infected cells. In addition, in the chronic stage of the disease (in patients with or without symptoms) inflammatory foci with mononuclear infiltrates are found (da Silveira et al., 2005). Two important questions need to be answered about this finding: 1) Are parasites present in the sites of inflammation? 2) Is the level of inflammation appropriate?

Parasites are found even in chronic Chagasic patients (da Silveira et al., 2005), which would suggest an immune response of some sort is necessary and likely helpful. It is difficult to say, however, what is an appropriate degree of response. One way to address this might be to eliminate regulatory T cells (T regs), which play an important

role in limiting inflammation, and ask if the pathology is worsened. In mice, inactivation of CD4+CD25+ T regs was found to confer slight resistance to infection with similar levels of inflammation and antibodies found (Sales et al., 2008). This suggests there is not an over-reactive immune response, at least in that model of Chagas' disease.

Other reports find diffuse inflammatory infiltrates consisting of eosinophils, macrophages and plasma cells in heart lesions often accompanied by extensive fibrosis (Gutierrez et al., 2009; Morris et al., 1990; Parada et al., 1997). Because these cells are not classic responders to an intracellular infection, it may be that their presence (especially during chronic infection) is not beneficial to controlling infection and is damaging host tissue. However, those questions have not been addressed experimentally. Such experiments that could address these questions would be to selectively limit (possibly using deleting antibodies) specific parts of the immune system during the chronic phase of the disease and evaluate various parameters such as parasite reactivation, fibrosis and sites of inflammation.

In summary, it is likely that some host cell death could be secondary to immune-mediated killing, and it is at least possible that some of this tissue destruction may be out of proportion to what is needed.

1.6e Cellular Infection-mediated Cell Death

The hypothesis that cell death comes from the stress of cellular infection is supported by the fact that when cells are infected in culture they die once parasites have replicate and exit the cell. However, this mechanism alone seems inadequate because the number of infected cells observed *in vivo* is likely far less than the amount of cell death that occurs (see Discussion 4.5).

1.6f Disease Mechanism Summary

The etiology of Chagas' disease remains a major question that is unlikely to be explained by any single mechanism. Why is major pathology seen in specific tissues although the parasite invades nearly all tissues? Although technology has afforded us the ability to see early physiological signs of *T. cruzi* infection, why do symptoms often only appear 10-30 years after infection? These questions are key in understanding Chagas' disease and they are questions that I have tried to address, in however limited a fashion, in my thesis work, and I will revisit them in the Discussion section.

1.7 Current Epidemiology and Treatment

Roughly 10 million people are infected with *T. cruzi*, the majority of whom are in South and Central America (http://www.cdc.gov/chagas/epi.html). The best strategy to combat disease has been to control the vector and thereby limit infections. For example, the Southern Cone Initiative was launched in 1991 to control vector populations with insecticides and improved living conditions in rural areas of Brazil, Argentina, Uruguay and Chile. This campaign had enormous initial success, but efforts and effects waned when government interest (and therefore money) shifted (Dias et al., 2002). Reinfestation of cleared areas have hindered progress (Cecere et al., 2006), and many areas in Central America and Mexico have seen an increased prevalence of Chagas' disease (although this may be confounded by increased reporting) (Dumonteil, 1999; Dumonteil and Gourbiere, 2004). In recent years, Chagas' disease has received increased interest due to its emerging prevalence in developed countries.

In the United States, sylvatic transmission is the major reservoir for *T. cruzi*. For example, a 3-year study determined 33% of raccoons in Fairfax County, Virginia (a

suburban area of Washington D.C.) are infected with *T. cruzi* (Hancock et al., 2005). It is therefore not surprising that autochthonous infections in the United States do occur (Dorn et al., 2007), but because our living conditions are so much higher than in the rural areas where T. cruzi infection is common, infections that occur within the US are rare. The majority of cases are from immigrants and patients who traveled to endemic areas or those infected through contaminated blood (Young et al., 2007). The first widespread screening for *T. cruzi* in US blood supplies started in January of 2007. In the first 15 months, more than 500 cases of contaminated blood were confirmed (Bern et al., 2008). Given the number of contaminated samples in just over a year, one may think there would be more transfusion-acquired cases. One explanation for this may be that most infections are initially silent and unlikely to be diagnosed.

Studies in Switzerland found13% of immigrants from countries where *T. cruzi* infection is endemic tested positive for *T. cruzi* infection (Jackson et al.). Additionally, at the first Chagas clinic in the United States, run at the UCLA county hospital in Sylmar, CA, they find a similar level of infection (10-15%) in Latin Americans who present to the emergency department with cardiac symptoms (Unpublished data, personal correspondence with Dr. Sheba Meymadi, UCLA). This number is strikingly high given the disease is largely unrecognized by American physicians and there are no drugs approved by the FDA for treatment.

Treatment for *T. cruzi* infection is most effective during the acute stage of the disease, when relatively few know they are infected[1]. The two drugs that are commonly used are Benznidazole and Nifurtimox, both of which require special permits from the CDC on a case-by-case basis to obtain and use to treat patients in the United States,

[1] This is one reason for suggesting that it is the pathology that occurs during acute infection that eventually leads to the chronic complications. Some studies have shown better outcomes even when treated at later stages.

although they are widely used in Latin America. One possible reason for this is that both drugs have a long list of relatively severe side effects (Castro et al., 2006). However, all of the side effects are reversible (Rassi et al.). Current guidelines suggest that all patients 18 years or younger and patients 19-50 without advanced heart disease should be treated (Bern et al., 2007). Treatment is optional for those 50 years or older (Bern et al., 2007). Treatment for cardiac disease is symptomatic and treatment for megacolon is symptomatic or surgical. Due to the large number of people at risk for developing disease and the limited treatment options, Doctors Without Borders recently highlighted Chagas' disease as a key neglected disease in the world. As I previously discussed, considerations of autoimmunity have greatly hindered vaccine and drug development. There are currently novel therapeutics and vaccines being pursued, although most are at the first stages in laboratory settings (Cazorla et al., 2009; Doyle et al., 2007; Haolla et al., 2009).

1.8 *T. cruzi* Cellular Invasion

T. cruzi is capable of invading the majority of mammalian host cells, although nervous and muscle tissue is especially targeted. Cellular invasion is a key step that allows for parasite division and the maintenance of the parasite life cycle. Cellular invasion is a process wherein the trypomastigate that is roughly 20μm in length invades a host cell and within 24 hours has changed into an amastigote with a diameter of only 10% its original length and completed its first division, an impressive feat that can be further analyzed in several specific steps. After cellular adhesion, the trypomastigote can be found within the host cell in a parasitaphorous vacuole (host membrane derived vacuole) in roughly 10 minutes (Rodriguez et al., 1996; Tardieux et al., 1992). Vacuole acidification allows the parasite's escape to the cell cytoplasm and begins its transformation into the replicating amastigote form (Andrade and Andrews, 2004; Ley et

al., 1990; Tomlinson et al., 1995). This process involves a complicated and diverse orchestration of both parasite and host cell signaling events.

There are two defined pathways of parasite invasion that I describe below: 1) lysosomal exocytosis/wound repair pathway and 2) actin-dependent cellular membrane pathway.

1.8a Lysosomal Exocytosis/Wound Repair Pathway

Ca^{2+}-regulated lysosome exocytosis is a possible mechanism to repair damage to the extracellular membrane (Reddy et al., 2001). In a mechanism that is inhibited by wortmannin (a PI 3-kinase inhibitor), Ca^{2+}-dependent and actin-polymerization-independent, *T. cruzi* recruits lysomsomes to the cell membrane where it has attached and becomes enveloped by membrane that is saturated with lysosome markers (Rodriguez et al., 1997; Tardieux et al., 1992). The actual internalization process that occurs here is not entirely clear; nor is it defined how *T. cruzi* recruits lysosomes to the correct location at the cell membrane.

1.8b Actin-dependent Cell Membrane Pathway

A decade after the lysosome exocytosis was proposed, it was shown that roughly 80% of vesicles containing the invasive parasite did not express lysosomal markers (Woolsey et al., 2003). Instead the parasite was within invaginations of the host cell plasma membrane enriched for class I PI3-kinases. Host cell actin polymerization, after the first minutes of the initial invasion step, is required for lysosome fusion with the parasite vacuole (Woolsey and Burleigh, 2004). This also helps explain why actin reorganization had been shown for years to be a key process in parasite invasion (Rodriguez et al., 1995), although it did not fit the lysosomal invasion hypothesis. Each pathway quickly merges because the parasite is enclosed in a membranous vacuole

enriched in lysosomal markers (and a low pH) within 45 minutes after cell entry (Woolsey et al., 2003).

1.8c *T. cruzi*/Host Cell Molecular Interactions

In the early 1990's, it began to be appreciated that the invasion process is likely caused by specific receptor-mediated events (Schenkman et al., 1991a; Schenkman et al., 1991b). One important family of *T. cruzi* molecules is the *trans*-sialidase superfamily, which includes gp85, gp82 and TS, which is also called PDNF (parasite derived neurotrophic factor). Most of *trans*-sialidase superfamily molecules have *trans*-sialidase (alpha (2-3)-linked sialic acid transferase) and neuraminidase activity and share at least ~50% homology (Colli and Alves, 1999; Schenkman et al., 1991b; Uemura et al., 1992). Although these molecules have all been implicated in cellular invasion, it can be difficult to differentiate between a *T. cruzi* molecule that allows for increased cytoadhesion and a molecule that binds a specific host receptor that is capable of triggering the required signaling events that mediate invasion (Ca^{2+}, PI3Ks, actin rearrangement). A molecule that leads to increased cellular adhesion may lead to an increase in the frequency of contact between parasite and host molecules that mediate invasion. Put another way, an adhesion molecule may play an important role in tethering the parasite to the host cell surface, but not actually mediate the invasion process. For example, gp82 binding of mucin is likely important for cellular adhesion in the gastric mucosa, although not for the cellular signaling required for the invasion process itself (Cortez et al., 2003).

Another TS family member, PDNF, renamed for its ability to promote the survival and differentiation of neurons in culture, can mediate cellular invasion through the neurotrophic receptor TrkA (de Melo-Jorge and PereiraPerrin, 2007). PDNF, also known as *trans*-sialidase (TS), was the original molecule discovered within the *trans*-sialidase superfamily. TS was first identified for its ability to cleave α-2,3-linked sialic acid from

glycoconjugates (neuraminidase activity) and transfer them onto β-galactosyl substrates (*trans*-sialidase activity) (Pereira, 1983; Schenkman et al., 1991b). TS is a gpi-linked surface molecule expressed during the invasive life stage of *T. cruzi* (Pereira, 1983), and has been known since 1991 to be involved with invasion of mammalian cells (Schenkman et al., 1991b). TS was renamed PDNF due to its ability to mimic the effect of neurotrophic factors by promoting the differentiation and maintenance of neurons in culture through activation of classic neurotrophic signaling pathways (see Introduction 1.10) (Chuenkova et al., 2001; Chuenkova and Pereira, 2000, 2001, 2003). PDNF neurotrophic activity is independent of its enzymatic activity and mediated through the receptor tyrosine kinase TrkA (Chuenkova and Pereira, 2000; Chuenkova and PereiraPerrin, 2004, 2005). As such, *T. cruzi*/TrkA-mediated cellular invasion represents a unique situation wherein *T. cruzi* invades a cell and simultaneously activates survival signaling pathways that could help promote survival of the parasite's environment and intracellular division. This was the first invasion mechanism where both the parasite molecule and host receptor was defined. TrkA-*T. cruzi* interactions may have limited effects, however, because TrkA is expressed on only a small subset of cells in mammals.

We might expect that the invasion mechanisms evolved by a complex eukaryotic pathogen that has a near limitless number of hosts (all mammals and several insect species) and can invade and replicate in nearly every nucleated mammalian cell would be diverse. Our knowledge of *T. cruzi* cellular entry is developing, but it appears that indeed there are multiple intracellular signaling pathways (both of the host cell and the parasite) and multiple extracellular *T. cruzi*-host interactions that contribute to this process. Defining both the parasite molecule and the host receptor binding pair is important to understanding the *T. cruzi* invasion process, and as more are discovered, it is becoming important to determine what role the specific interactions have in models of

Chagas' disease. Do specific receptors mediate tissue tropism? Are specific receptors important at the various sites or steps of host colonization?

1.9 Animal Models of *T. cruzi* Infection

There are many potential animal models of Chagas' disease—even polar bears can succumb to *T. cruzi* infection (Jaime-Andrade et al., 1997). Canines, primates and rabbits recapitulate many aspects of Chagas' disease, but inbred mice are by far the most common models used. Murine susceptibility to infection varies greatly, dependent on which inbred mouse strain and which *T. cruzi* strain is used. Parasites are in general inoculated subcutaneously (to mimic skin infection routes) or intraperitoneally. Parasites invade local tissues then spread through the blood throughout the body (Guarner et al., 2001). Peak parasitemia usually occurs 10-20 days post inoculation (dpi) and peak tissue parasitism follows. Although no consensus exists, acute infection in mice is generally considered up to 30 dpi and chronic infection is beyond 60 dpi (Maifrino et al., 2005; Rodrigues et al., 2002). Mice are used to answer various physiological questions about Chagas' disease, but whether mice develop pathology analogous to what in humans is called cardiomegaly and megacolon is questionable. Nervous and muscle tissue pathology, including both inflammation and neuron death, in the heart and the colon is a common finding in mice infected with *T. cruzi* (Maifrino et al., 2005; Ribeiro et al., 2002; Rodrigues et al., 2002). These findings are in agreement with what is seen in humans (Adad et al., 2001; Cunha-Neto and Kalil, 2001; da Silveira et al., 2008; Koberle, 1968; Parada et al., 1997). Less studied pathology in the nervous system including sciatic nerve and spinal cord lesions can also be found in both humans and mice (Koberle, 1968; Molina et al., 1987). In addition, some data suggest that both cardiac dilation (de Souza et al., 2005) and intestinal dysmotility (de Oliveira et al., 2008) observed in Chagas' disease patients is also present in mice. No studies have shown megacolon in mice.

1.10 Trk Receptors

As discussed, *T. cruzi* can invade neurons through TrkA, a member of the Trk family of tyrosine kinase receptors. Although TrkA has a more limited expression in tissues and many cell types (Schwann cells, cardiomyocytes) that are highly parasitized by *T. cruzi*, TrkB and TrkC, which share significant homology with TrkA, have a broader distribution in several tissues.

Tropomyosin-related kinase (Trk) receptors have unique characteristics that may be beneficial for *T. cruzi* to exploit: 1) Ligand binding to Trk receptors activates signaling cascades that lead to cell membrane invagination and the internalization of the receptor/ligand complex within a "signaling endosome." This process is not unlike what occurs in the membrane-dependant pathway of *T. cruzi* invasion. 2) Signaling pathways that are important for *T. cruzi* cellular invasion are activated by Trk receptors. 3) The neurotrophic effects of Trk receptor signaling might help mitigate stress caused by invasion and parasite division, promoting the health of the parasite's environment.

1.10a Trk Receptors and their Neurotrophin Ligands and Signaling

In 1991, an oncogene containing 8 exons of nonmuscle tropomyosin fused to the cytoplasmic domain of a novel tyrosine kinase was cloned from a carcinoma cancer (Barbacid et al., 1991). It was named tropomyosin related kinase (Trk), now called TrkA, and the two other family members TrkB and TrkC were discovered based on their high homology to TrkA. Very quickly thereafter it was published that NGF (nerve growth factor) is the ligand that activates TrkA (Kaplan et al., 1991; Klein et al., 1991). NGF, discovered in the 1950's, was integral in the field of neurobiology and helped define mechanisms of innervation, survival, intercellular communication and continues to be

widely studied for its role in the Alzheimer's disease and many other areas (Levi-Montalcini, 1987). The Nobel Prize in Physiology or Medicine in 1986 was awarded to Dr. Stanley Cohen and Dr. Rita Levi-Montalcini for their discovery and work on NGF.

NGF binds TrkA; BDNF (brain-derived neurotrophic factor) and NT-4 (neurtrophin-4) bind TrkB; and NT-3 (neurotrophin-3) binds TrkC and also TrkA and TrkB with lower affinity. *Trk* receptor genes encode proteins of roughly 800 amino acids in length that have an extracellular domain, transmembrane domain and intracellular domain. The extracellular portion has several sites of sialylation and is comprised of multiple regions that include two cysteine clusters, leucine-rich repeat and two immunoglobulin-like domains, of which the Ig2 domain is responsible for ligand binding. After ligand binding, Trk receptor dimerization and auto-phosphorylation leads to the activation of several small G proteins (Ras, Rap and Rac-Rho family) and the MAP kinase, PI 3-kinase and PLC-γ signaling pathways (see below, Diagram 3). This signaling helps regulate a diverse number of functions including cell survival and proliferation, cell migration and differentiation, axon and dendrite growth and synaptic formation and maintenance (Huang and Reichardt, 2003). NGF-TrkA preeminence has promoted their study as the prototype of Trk receptor actions.

Much evidence supports the similarities between TrkA, TrkB and TrkC, but more recent studies have also started to reveal some differences between the receptors. For example, different levels of PLC-γ activation, Shc interaction and Ca^{2+} signaling have been demonstrated using BDNF, NGF and NT-3 in transfected cell lines (of appropriate receptors) even though the initial Trk receptor phosphorylation induced by the neurotrophins was the same (Yamada et al., 2002). Signaling differences are in agreement with common findings of morphological differences between TrkA versus TrkB versus TrkC-expressing neurons, but the majority of differences between the Trk

receptors has been attributed to cell type and cell location where each Trk receptor is expressed.

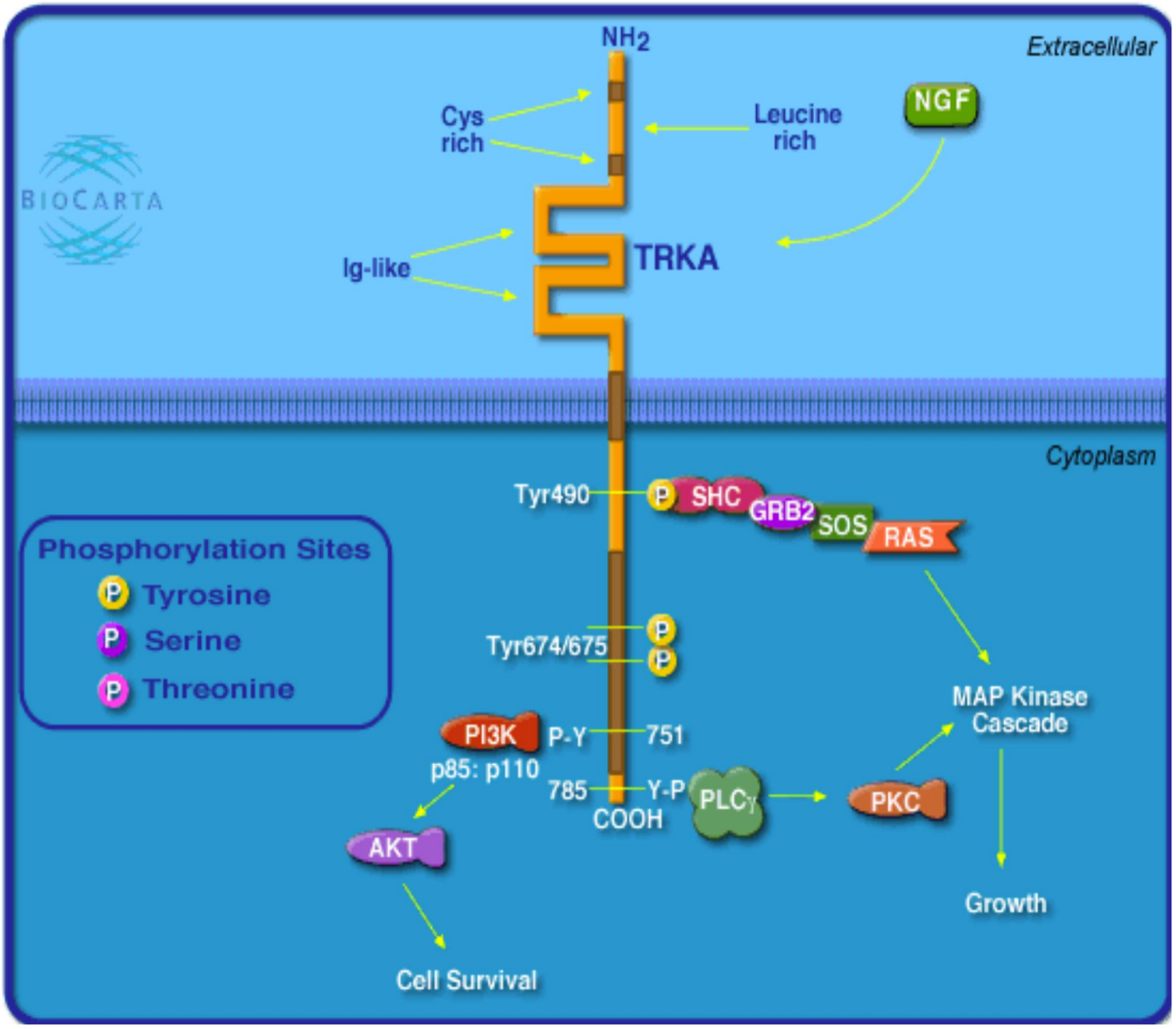

Diagram 3 Trk Signaling

Diagram 3 shows TrkA/NGF signaling, but TrkB/BDNF and TrkC/NT-3 would be very similar.

1.10b Trk Expression and Function

Although I discussed data addressing signaling differences between specific Trk receptors, the majority of knowledge about the diversity between TrkA, TrkB and TrkC relates to which cell type and in which tissues the receptors are expressed. Below I have tried to summarize the expression patterns of the individual Trk receptors in the CNS and PNS, but there are growing reports of Trk expression in many other non-neuronal organs, endothelial and epithelial tissues, salivary glands, pancreas, bone marrow, adrenal glands, prostate, ovary, uterus, and muscle (Shibayama and Koizumi, 1996). Trk receptors are also expressed by many cells of the immune system, including in both myeloid and lymphocyte lineage cells (Vega et al., 2003). Although Trk function in non-neuronal cells is poorly understood, it is assumed that, like their major functions in neuronal cells, Trk receptors help regulate survival and differentiation in non-neuronal cells as well.

TrkA

TrkA has a more limited expression compared to the other Trk receptors in the adult mammal nervous system, and is predominantly found on neurons and not glial cells. TrkA is expressed in sensory (Merkel fibers) and sympathetic neurons in the dorsal root ganglia and sympathetic ganglia of the PNS with fewer reports of TrkA expression in parasympathetic neurons, such as those in the vagus nerve (Cronk et al., 2002; Helke et al., 1998; Yamamoto et al., 1996). Within the brain, TrkA is expressed by cholinergic and non-cholinergic neurons located within the basal forebrain and brainstem (Holtzman et al., 1995). TrkA is expressed in neurons throughout ENS in humans, yet

only in specific segments, such as the small intestine, or not at all in other mammalian species (Esteban et al., 1998; Sternini et al., 1996). *TrkA*$^{-/-}$ mice, in agreement with adult expression patterns of TrkA, have major sensory deficits and a decreased number of neurons in their dorsal root and trigeminal ganglia. Most *TrkA*$^{-/-}$ mice die after weaning, but those that live to sexual maturity are infertile. In humans, some patients with mutations in their *TrkA* genes suffer from congenital insensitivity to pain with anhydrosis (CIPA), a disease characterized by fever, insensitivity to pain, self-mutilation, mental retardation, and absence of sweating (Indo, 2001).

TrkB

TrkB is expressed throughout the brain cortex, spinal cord and dorsal root ganglia in both neuronal and glial populations (Klein et al., 1990; Yamamoto et al., 1996). TrkB is expressed by neurons and glial in the enteric nervous system, although more evidence supports glial expression of TrkB (Hoehner et al., 1996; Levanti et al., 2009).

TrkC

TrkC, with high levels of expression throughout the CNS and PNS, has a broader distribution than TrkA or TrkB (Lamballe et al., 1991; Shelton et al., 1995) in embryonic and adult mammals. TrkC is crucial for normal heart development as well as for the development of several neuron populations in the PNS, including the dorsal root ganglia and enteric ganglia; *TrkC*$^{-/-}$ mice do however have normal brain development (Klein, 1994; Tessarollo et al., 1997). All *TrkC*$^{-/-}$ mice die within 25 days after birth from heart abnormalities that include septation defects, pulmonary stenosis and valvular defects (Tessarollo et al., 1997). TrkC expression wanes in adult mammals, but is still expressed in various glial, neuron, muscle and endothelial cell types. Of particular

interest with regards to Chagas' disease, in adult mammals TrkC is expressed in cardiomyocytes (Kawaguchi-Manabe et al., 2007), glial Schwann cells (Yamauchi et al., 2003), skeletal muscle (Shelton et al., 1995) and in glia and neurons in the ENS (Saffrey et al., 2000; Sternini et al., 1996).

1.10c Trk Receptor Endocytosis

Upon ligand binding, the Trk receptor/ligand complex is endocytosed and transported sometimes as far as 1 meter to the body of the cell. Trk receptor internalization is of interest in regards to *T. cruzi* cellular invasion because both processes share common signaling pathways and the outcome is the same: internalization. Trk receptor internalization occurs through three mechanisms: actin-mediated macroendocytosis, clathrin-mediated endocytosis and lipid-raft mediated endocytosis (Campenot and MacInnis, 2004). All pathways lead to endosome formation, as is seen in *T. cruzi* invasion. Additionally, PI3-K/Akt signaling is important in Trk receptor endocytosis (Clague, 1998; Kuruvilla et al., 2000) and *T. cruzi* invasion alike. Such common features suggest that *T. cruzi* may exploit normal Trk receptor function to promote cellular invasion, as was shown with TrkA.

CHAPTER 2. MATERIALS AND METHODS

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2.1 Mice

Female C57BL/6, aged between 6 to 8 weeks, were purchased from Jackson Laboratory. All murine experiments were approved by the Institutional Animal Care and Use Committees (IACUC) of Tufts University-New England Medical Center; protocol number 14-07.

2.2 Cell Lines

PC12-NNR5 cells were gifts from Lloyd Green (College of Physicians and Surgeons, Columbia University, NY). Trk receptor-deficient PC12 cell mutant NNR5 (Green et al., 1986) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS (Gemini Bio Products), 100 U/ml penicillin-streptomycin (Gibco), 2 mM L-glutamine (Gibco), 1x nonessential amino acids (Gibco), and 1 mM sodium pyruvate (Gibco). Human Schwann cells (permanent cell line) (Chuenkova et al., 2001) were maintained in DMEM supplemented with 10% FBS (Gemini Bio Products) and 100 U/ml penicillin-streptomycin (Gibco).

2.3 Parasites

All *in vitro* and *in vivo* studies were performed with Silvio X-10/4 (Postan et al., 1983) and Tulahuen (Silva et al., 1991) strains, respectively. Both strains of trypomastigotes were grown in Vero cells. The parasites were harvested by centrifugation at 500 x g for 5 min to remove host cells and cell debris and washed two times with DME medium at 1200 x g for 10 min.

2.4 Purification of PDNF and rPDNF

2.4a Purification of PDNF

PDNF was isolated by immunoaffinity chromatography, according to Scudder et al (Scudder et al., 1993). In brief, trypomastigotes (Silvio X-10/4 strain) were grown in Vero cell monolayers for 3-5 days in 2.5% FCS/DMEM. After removing cells and parasites by centrifugation, the conditioned media was filtered through a nitrocellulose membrane (0.22 μm). The filtered media was then added to a column containing 1 ml of Protein G Sepharose 4 Fast Flow (GE Healthcare) previously coupled with TCN-2 monoclonal antibody (mAb) and equilibrated with 50 mM PBS, pH 7.8. TCN-2 mAb is specific for a 12-amino acid (DSSAHGTPSTPA, peptide TR) that is repeated in tandem at the C terminus of the enzyme. The column was first washed with PBS and PDNF was eluted with PBS containing 10 mg/ml peptide TR in 0.1% N-octyl-βD-gluco-pyranoside (Sigma). Residual TCN-2 mAb was removed from PDNF through a 0.5 ml Protein G Sepharose column. The purified enzyme was concentrated by ultrafiltration (Amino Ultra-15 microconcentrator, Amicon) and washed extensively (> three times in similar Amicon devices) with PBS pH 7.2 to remove the synthetic peptide and detergent. *trans*-Sialidase activity was measured by sialylation of the acceptor 14C-labeled N-acetyllactosamine as described below (Section 4.c). To determine protein concentration of purified protein, samples of PDNF and BSA (used as a standard) were subjected to SDS-PAGE gels (7.5%), stained with Commassie blue (BioRad), and analyzed by scanning densitometry (GS-800 Calibrated Densitometer, BioRad).

2.4b Purification of rPDNF

Recombinant PDNF (rPDNF) was expressed in BL21(DE3) bacterial cells at the GRASP center (Tufts University Medical School/New England Medical Center). The protein was purified using a metal chelate column as described by Chuenkova and colleagues (Chuenkova et al., 1999). Bacterial lysates were prepared in 50 mM PBS pH 7.4, 0.5 M NaCl, 0.1% Triton X-100, 5 mM imidazol, and 1 mM PMSF by osmotic shock and subsequent sonification (2 cycles of 60 s, pulsating waves, at level 8) using the 550 Sonic Dismembrator (Fisher Scientific). The lysates were then centrifuged at 44,000 x g for 20 min at 4ºC, filtered through a nitrocellulose membrane (0.44μm), and added to chelating Sepharose Fast Flow column (Amersham Biosciences) pre-charged with Ni2+ ions. rPDNF was eluted with 20 mM Tris/HCl, 0.5 M NaCl, 100 mM imidazol. After desalting, the purified protein was used to determine *trans*-sialidase activity . The protein concentration was determined by Commassie blue staining, as detailed for PDNF (Section 4a).

2.4c *trans*-Sialidase Assay

trans-Sialidase activity was determined as previously described (Scudder et al., 1993). Purified TS/PDNF enzyme was added at various concentrations to a reaction at the final concentration of 10 mM Tris/HCl pH 7.2, 0.25 *μ*mol sialic acid acceptor [14C]Nacetyllactosamine (0.0759 *μ*Ci) (Sigma-Aldrich) and 17% FBS sialic acid donor in a total volume of 60 *μ*l. The reaction was incubated at 25ºC to allow for sialic acid transfer, and was inactivated with 1 ml water. A column containing 0.5 ml of QAESephadex A-50 Fast Flow (Sigma Aldrich) was equilibrated with water and used to adsorb the 14C-labeled sialyl-N-acetyl-lactosamine. Free [14C]N-acetyllactosamine was removed by washing the column with 5 ml water, and the sialylated [14C]Nacetyllactosamine was eluted with 1 ml of 1M NaCl. The sialylated product was quantitated by liquid scintillation counting.

2.5 The Binding of TrkC-Fc to *T. cruzi*

The extracellular domain of TrkA, TrkB, TrkC, and fibroblast growth factor receptor (FGFR) bound to the immunoglobulin Fc domain were purchased from R&D systems, as was TrkC without the Fc domain. For binding experiments 5 x 10^6 trypomastigotes/ml were incubated with each receptor in binding buffer (DMEM, 0.1% bovine serum albumin [BSA]) for 45 min at 4℃ and wa shed four times with binding buffer by centrifugation (6,000 x g for 5 min) to remove unbound receptor. Parasite pellets were resuspended in reducing (2% β-mercaptoethanol) sodium dodecyl sulfate (SDS)-Laemmli sample buffer, run on an SDS-polyacrylamide gel electrophoresis (PAGE) gel (7.5%), transferred onto nitrocellulose, and probed with anti-human immunoglobulin G (IgG) horseradish peroxidase (HRP)-labeled antibody (Promega); blots were quantified in a scanning densitometer (Bio-Rad Laboratories). Blots were stripped and reprobed using human chagasic serum or TCN-2 monoclonal antibody to evaluate loaded parasite and the effect of washing of the parasites. Similar procedures were performed for *T. cruzi*-TrkC competitive binding experiments except that NT-3, BDNF, NGF (Chemicon), or PDNF was coincubated with T. cruzi and TrkC.

2.6 Coimmunoprecipitation assay

A coimmunoprecipitation assay was performed by a slight modification of a previously reported coimmunoprecipitation procedure (de Melo-Jorge and PereiraPerrin, 2007): PDNF (1 μg) was incubated overnight at 4℃ wit h Fc receptors (1 μg) in binding buffer (DMEM, 0.1% BSA). Fc-receptors were immunoprecipitated on protein G-Sepharose (GE Healthsciences) and washed three times in binding buffer. The pellet was resuspended in SDS-sample buffer, run on reducing (2% β-mercaptoethanol) SDS-

PAGE (7.5%) gels, and transferred onto nitrocellulose. PDNF coimmunoprecipitated by the Fc receptors was identified with PDNF-specific monoclonal antibody TCN-2 and anti-mouse-HRP secondary antibodies. Receptors were evaluated using anti-human IgG-HRP antibodies.

2.7 Cloning: Constructs and Transfection Assay

TrkB and TrkC were directionally cloned from human RNA (RNA was a gift from Tugba Bagci, Neuroscience Department, Tufts Medical School, Tufts University) via reverse transcription-PCR into the pIRES-dsRed mammalian expression vector (Clontech). TrkC was amplified using primers designed to place and XhoI site at the 5' end of the product (see 2.14, Primer Table). The PCR product was ligated into the Topo vector (Invitrogen), excised, and religated into the pIRES vector using XhoI and EcoRI sites. A similar cloning strategy was employed for TrkB (see 2.14, Primer Table). TrkA was directionally cloned from plasmid 15002 (Addgene) that contained human TrkA cDNA with the same strategy (see 2.14, Primer Table). PC12-NNR5 cells were transfected with TrkB, TrkC, or empty vector (EV) clones using Fugene HD (Roche) according to the manufacturer's protocol. Transfected cells were selected for in NNR5 medium (see 2.1, Cell Lines) supplemented with 500 μg/ml Geneticin (Gibco) for 1 week and then sorted by use of a fluorescence-activated cell sorter (FACS) for dsRed expression at 1 week and 1 month posttransfection. Cells were regrown in selective medium and sorted a third time (by FACS) to obtain cell populations with homogenous expression levels of transfected receptors. Cultures were maintained without G418 for experiments and analyzed regularly for expression.

2.8 Trk Signaling Evaluation

2.8a Neurite Extension

Transfected NNR5 cells were plated in 96-well plates (10^4 cells/well) and treated with NT-3 or BDNF (100 ng/ml) or PDNF (250 ng/ml) in 10% fetal calf serum (FCS)-DMEM for 48 to 72 h. Cells were fixed in 4% paraformaldehyde, blocked in 5% BSA-phosphate-buffered saline, and probed with anti-neurofilament 200 (Sigma-Aldrich) followed by Alexa 488-conjugated anti-rabbit IgG (Molecular Probes). Cells were imaged (magnification of x20) by fluorescence microscopy (Olympus IX70). Using SPOT Advanced software, a minimum of 50 cells for each condition were analyzed for neurite length. All cells with at least one neurite greater in length than 100% of its cell body width were counted as being a cell with a neurite.

2.8b Erk Phosphorylation

Subconfluent transfected NNR5 cells were plated in regular medium for 1 day and then cultured in serum-free DMEM overnight. Cultures were then treated with NT-3 or BDNF (100 ng/ml) or PDNF (25 to 200 ng/ml) for 12 min. When indicated, cells were pretreated with the Trk-specific inhibitor K252a (1 μM) (Sigma-Aldrich) for 60 min. Cells were immediately washed with cold phosphate-buffered saline and lysed on ice with 1% NP-40 lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min. Lysates were cleared by centrifugation at 10,000 x g for 10 min, and equal amounts of total protein (20 to 60 μg) in SDS-loading buffer were run under reducing conditions on an SDS-PAGE gel (12.5%), transferred onto nitrocellulose, and probed with antibodies specific for phospho-Erk (P-Erk) (Cell Signaling) and Erk1/2 kinase (Cell Signaling). To determine whether T. cruzi activates Erk1/2, NNR5 transfectants were infected with 107 trypomastigotes/ml for 15 min, and P-Erk was ascertained as described above for PDNF cell activation.

2.9 NNR5 Survival Assay

Transfected NNR5 cells were plated in 96-well plates (104 cells/well), attached overnight in 10% FCS-DMEM, and grown in 1% FCS-DMEM, serum-free DMEM, or serum-free DMEM supplemented with 250 ng/ml PDNF for 72 h. Cultures were then treated with Hoechst 33342 stain (10 μg/ml) and propidium iodide (PI) (2 μg/ml) in prewarmed (37℃) DMEM for 10 min at 37℃ and visuali zed under UV light (340 to 380 nm). Hoechst 33342 stains live and apoptotic nuclei blue, and PI stains only dead cells (pink).

2.10 Schwann Cell Survival Assay

Schwann cells were plated in 96-well plates (5 x 103 cells/well), allowed to attach overnight to the substratum in 10% FCS-DMEM, and then washed twice in serum-free DMEM. Cells were then maintained in serum-free DMEM for 3 days with daily 1-h treatments of FCS (1%) or serum-free DMEM with or without TrkC-specific antibodies (1 mg/ml) (Upstate Biotech), TrkB-specific antibodies (1 mg/ml) (Upstate Biotech), or PDNF (250 ng/ml) with or without Trk antibodies. Cells were then stained with Hoechst 33342 dye-PI and imaged by fluorescence microscopy as described above.

2.11 *T. cruzi* Infection *In Vitro*

2.11a Infection

Cells (3,000/well) were plated in 96-well plates (Falcon), incubated for 1-2 days in growth media, and then incubated with the indicated concentrations of trypomastigotes in 0.1% BSA/DMEM (2 h, 37℃). After infection, cells were washed 5X with pre-warmed

DMEM, and were grown in 1% FCS/DMEM for 2 days. Cell monolayers were analyzed by phase-contrast microscopy, as follows: Cells were fixed with methanol for 2 min, stained with acid-base staining reagents (Diff Quick, Baxter) following the manufacturer's instructions. Infected and non-infected cells (>200 cells/well) were counted in triplicates.

2.11b Inhibition of Infection

To determine if TrkC ligands inhibit infection of TrkC+ cells, the specified cell lines were pre-incubated for 30 min at 37ºC with the indicated concentration of ligands [PDNF or rPDNF purified by affinity chromatography, NGF (Sigma-Aldrich), NT-3 (R&D Systems), BDNF (R&D systems)]. After treatment with ligands, cell monolayers were washed 1X with DMEM, and infected as described in Section 11a.

2.11c Kinase Inhibitors

To test whether invasion is dependent on TrkC signaling, specified cells were pre-incubated for 1 h at 37ºC with K252a (500 nM, IC50 = 3 nM) (Calbiochem), a Trk kinase inhibitor, AG879 (50 É M, IC50 = 10 É M), and IGF-1R inhibitor (125 nM to 2 mM, IC50 = 1nM). Cells were then incubated with parasites as described in Section 11a in the presence of the inibitors.

2.11d Soluble TrkC-Fc

To block the TrkC-binding site on the parasite surface, trypomastigotes were pre-treated with TrkC-Fc. Treated parasites were incubated with cell monolayers as described in Section 11a.

2.12 Murine Model of Acute Chagas' Disease

2.12a Infection

Trypomastigotes (Tulahuen strain) were harvested from Vero cell cultures, washed 1X in DMEM and 2X in PBS. Parasites (5000 in 30μL of PBS) were injected subcutaneously into the mouse footpad.

2.12b Footpad Parasitism

When noted, specific antibodies or BSA were injected into the footpad simultaneously with the parasites. Mice were sacrificed three days later and the footpad where parasites were injected was removed without the digits. The footpad was cut into small pieces and Footpad DNA was isolated using DNeasy Tissue kit (Qiagen) according to manufacturer's protocol. Total was then used to perform Real-Time PCR, as previously described (Cummings and Tarleton, 2003). Each reaction contained 50 ng of total DNA, and samples were analyzed with primers specific for *T. cruzi* DNA and a one-copy mouse gene, *tnf*, was used as internal control for each sample (see Primer Table). To determine the number of parasites, a standard curve was calculated using uninfected tissue spiked with a known quantities of parasites; the standard curve was run with each plate.

2.12c Parasitemia

Mice were tail bleeding at specified time points after parasite inoculation, roughly ever 2 days. The number of parasites were counted with a hemocytometer, as previously described (Chuenkova and Pereira, 1995).

2.12d Colon Weight

Mice were left undisturbed in their original cages until sacrificing by CO_2 and mice were sacrificed at roughly the same time of day (2pm) in all experiments. After the

mice were sacrificed the colon (just distal the cecum to the anus) was removed with all contents and weighed before they were processed for other experiments.

2.12e Colon Cell Quantification (qPCR)

After weighing the colon, the distal half of the colon is retained and washed with cold PBS. Sections are removed to isolate total RNA and the remaining sections were used for cryosectioning and immunohistochemistry. To obtain cDNA, RNA was isolated using Trizol (Invitrogen protocol) and reverse transcribed using Quantitect Reverse Transcription Kit (Qiagen). Total cDNA was anaylyzed by qPCR (see Materials and Methods, 14) using HPRT for an internal control. All tissues analyzed for each gene was run on the same plates or with overlapping samples to control for plate variability, as needed. Relative levels of expression were analyzed using the standard comparative CT method Livak (Livak and Schmittgen, 2001). The average gene level for uninfected control mice were standardized to 100% for graphing purposes.

2.12f Colon Immunohistochemistry

After weighing and washing the colons, segments of the distal colon were immersed in OTC baths and frozen in dry ice/acetone mixture. Samples were then frozen in the -80ºC freezer and later sectioned by Tufts Univeristy Animal and Tissue Processing Facility. Frozen sections (10μm thick) on slides were fixed in acetone at -20ºC for 20 minutes, washed in 2X PBS and blocked for 30 minutes at RT in blocking medium (10% goat serum, 5% milk and 4% BSA in PBS). Sections were incubated with primary antibodies overnight at 4ºC, washed 3X in PBS, incubated in secondary antibodies for 4 hours at RT, washed 3X and then coverslipped using Slowfade Gold (Molecular probes). Sections were analyzed by fluorescence microscopy. For TUNEL staining to detect cell death, the Roche kit and protocol were followed.

2.13 Quantitative PCR

For all qPCR experiments, reactions contained roughly 50ng of total DNA or cDNA, 0.4 μl of forward and reverse primers (10mM), 10 μl Qiagen QuantiTect Sybr Green PCR Master Mix (Qiagen), and PCR grade water to a final total volume of 20 μl. All primers used were ensured to have similar primer efficiencies of about 85% to 90% and have a single peak for a melting curve. Reactions were analyzed using the BioRad iQ5 qPCR machine with the following cycle parameters: 1X [10m, 95ºC] and 50X [15s, 94ºC; 30s, 54ºC; 30s, 72ºC].

2.14 Antibody Table

Antibody	Catalog #	Source	Company	Use	Dilution
TrkC	MAB3731	mouse	RnD	WB	500
TrkC	07-226	goat	Upstate	WB, CC, MM	
TrkC	SC-117	rabbit	Santa Cruz	IHC	100
TrkB	07-225	rabbit	Upstate	WB	1000
TrkB	sc-8316	rabbit	Santa Cruz	CC, MM	
TrkA	06-574	rabbit	Upstate	WB, MM	
SMA	Ab 5694	rabbit	Abcam	IHC	300
TuJ	MMS-435P	mouse	Covance	IHC	200
GFAP	Z 0311	rabbit	Dako	IHC	250
P-Erk 1/2	9101	rabbit	Cell Sig.	WB	1000
Erk 1/2	9102	rabbit	Cell Sig.	WB	1000

WB = Western blot; **CC** = Cell culture infection blocking;
MM = Mouse model injections; **IHC** = Immunohistochemistry

2.15 Primer Table

Primer Name	Sequence
Cloning	
hTrkC-F	CCGCTCGAGATGGATGTCTCTCTT
hTrkC-R	CTAGCCAAGAATGTCCAGGTAGATTG
hTrkB-F	CCGCTCGAGATGTCGTCCTGGATA
hTrkB-R	CTAGCCTAGAATGTCCAGGTAGACCG
hTrkA-F	ACTCGAGATGCTGCGAGGCGGAC
hTrkA-R	CCTAGCCCAGGACATCCAGGTAGA
qPCR	
mTrkC-F2	TACCTGGCTTCCCAGCACTTT
mTrkC-R2	GTGTCCTCCCACCCTGTAGTAATC
mTrkB-F1	AGCCCTGGTATCAGCTAT
mTrkB-R1	GGGTGTGGATGCTCTTGATG
mTrkA-F2	CGCTGAGTGCTACAACCTTC
mTrkA-R2	GAAAGTCCTGCCGAGCATTC
ms100b-F2	GCGAGAGGGTGACAAGCACAA
ms100b-R2	CTTCGTCCAGCGTCTCCATC
mHuD-F2	GGTGCTACAGAACCGATTAC
mHuD-R2	GTCCAGCCTGAATCTTTGAG
mGFAP-F1	AGCTCCAAGATGAAACCAACCTG
mGFAP-R1	GCAAACTTAGACCGATACCACTC
HPRT-F1	CAGCGTCGTGATTAGCGATGATG
HPRT-R1	CGAGCAAGTCTTTCAGTCCTGTC
b-Actin-F1	AGGTATCCTGACCCTGAA
b-Actin-R2	CACACGCAGCTCATTGTA
T. cruzi	
TCZ-F	GCTCTTGCCCACAMGGGTGC
TCZ-R	CCAAGCAGCGGATAGTTCAGG

mTNF-F	TCCCTCTCATCAGTTCTATGGCCCA
mTNF-R	CAGCAAGCATCTATGCACTTAGACCCC

CHAPTER 3. RESULTS

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The neurotrophic receptor TrkA mediates *T. cruzi* induced neuron protection in cultured neurons, and, more recently it was shown to mediate *T. cruzi* cellular invasion. TrkA shares homology with the related Trk family receptors, TrkB and TrkC. In addition to shared homology, the Trk family of receptors share NT-3 as a common ligand, although it binds TrkC with greater affinity. The three receptors are variably expressed on cells in the CNS, PNS, ENS and on cardiomyocytes, all tissues that are highly relevant in Chagas' disease. Thus, my project aimed to determine whether 1) *T. cruzi* interacts with TrkB and/or TrkC in addition to TrkA and 2) *T. cruzi*-Trk interactions help mediate pathology (or protection via trophic actions) in mice infected with *T. cruzi*.

3.1 *T. cruzi* Binds to TrkC

To determine whether *T. cruzi* binds TrkB and/or TrkC we used a pull-down assay with live parasites. Parasites were incubated with Fc chimera of the extracellular domain (ECD) of TrkB and TrkC receptors, hereafter called $TrkB^{Fc}$ and $TrkC^{Fc}$ unless stated otherwise. Second, parasite-receptor mixtures were spun down, washed to remove unbound receptors, and lysed with SDS-Laemmli sample buffer. Third, the lysates were analyzed by Western blotting to evaluate *T. cruzi*-bound receptor using an antibody specific for the human IgG Fc domain. An Fc chimera of FGFR1, widely expressed in the nervous system (Huang and Reichardt, 2003), was included as a negative control, and an Fc chimera of TrkA was included as a positive control. The results showed that *T. cruzi* binds $TrkC^{Fc}$ and not $TrkB^{Fc}$ (Fig. 1A) and that the binding is dose-dependent (Fig. 1B). Stripping and redeveloping the blots with a *T. cruzi*-specific

antibody showed that the motile parasites were not lost during washing (Fig. 1A). *T. cruzi* also binds recombinant TrkC-ECD not linked to Fc (Fig. 1C), suggesting that *T. cruzi*-TrkC binding is independent of the Fc domain.

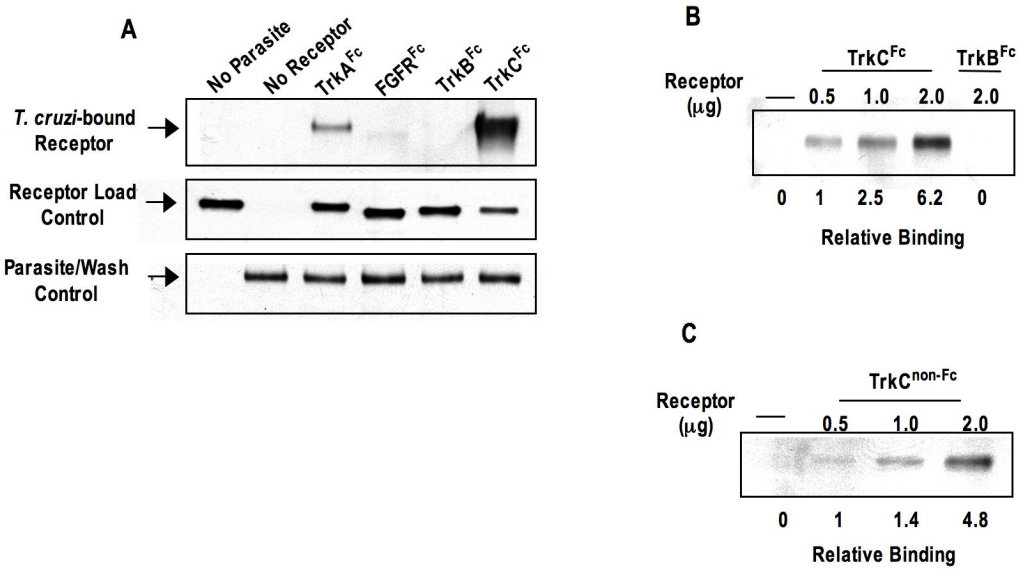


Fig. 1 *T. cruzi* binds TrkC.

(A) Live parasites (5 x 10^6 parasites) were incubated with equal amounts of the indicated soluble receptors (1 μg), spun down, washed to remove unbound receptors, and lysed to reveal bound receptor by Western blotting using anti-human IgG-HRP. The receptor load control represents an equal volume of supernatant from each sample after the first spin to evaluate unbound receptor by Western blotting. Note that $TrkC^{Fc}$ is visibly reduced in the supernatant obtained by incubating parasites with TrkC. The parasite/wash control represents the total number of parasites after washes revealed by Western blotting with *T. cruzi*-specific antibody TCN-2. Note that parasite load was constant for every lane. (B) Dose response *of T. cruzi*/$TrkC^{Fc}$ binding. (C) Dose response of *T. cruzi*/TrkC-non-Fc binding. Bound receptor was revealed using α-TrkC antibody. (B and C) Relative binding was calculated using the parasite/wash control as a standard. Experiments in B and C were done three times, and those in A were done five times, all with similar results.

3.2 Binding of *T. cruzi* to TrkC is Specifically Inhibited by NT-3.

We used competition assays to further define the specificity of *T. cruzi*/TrkC molecular interactions. For this, we incubated live parasites with $TrkC^{Fc}$, with or without the neurotrophins NGF, NT-3, and BDNF, to determine whether TrkC binding to *T. cruzi* is specifically blocked by NT-3. Indeed, NT-3, but not BDNF and NGF, inhibits $TrkC^{Fc}$ binding to *T. cruzi* in a dose-dependent manner (Fig. 2A and B). This result suggests that *T. cruzi* binds TrkC in a region that overlaps with the binding site of NT-3 and may also be able to activate TrkC signaling pathways, a possibility that was later tested (Fig. 6).

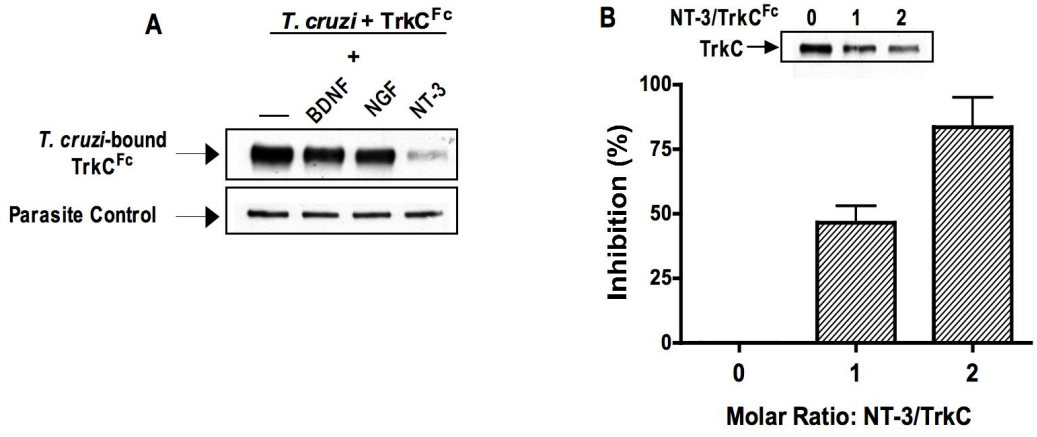


Fig. 2 NT-3 specifically inhibits *T. cruzi*-TrkC binding.

(A) A total of 2x molar amounts of recombinant NT-3, BDNF, or NGF (0.6 μg) to $TrkC^{Fc}$ were incubated with 5 x 10^6 *T. cruzi* parasites and $TrkC^{Fc}$ (1 μg). Parasites were then spun down and washed, and bound $TrkC^{Fc}$ was revealed by Western blotting using α-human IgG antibodies. (B) Dose response of increasing concentrations of NT-3 incubated with live *T. cruzi* parasites and $TrkC^{Fc}$. Bound $TrkC^{Fc}$ was calculated by scanning densitometry, with each sample standardized against its parasite wash control. (Inset) A representative blot from the experiment is shown. Experiments in A and B were done three times, with similar results; error bars show SD.

3.3 The Parasite Protein PDNF is Responsible for *T. cruzi*-TrkC Binding.

PDNF binds and activates TrkA. Following the Trk binding pattern of NT-3, it may be that *T. cruzi* also interacts with TrkC through the same parasite molecule that interacts with TrkA. This possibility would fit well with NT-3 interactions with Trk receptors because based on the pull-down assay using live parasites *T. cruzi*, like NT-3, appears to bind TrkC to a greater extent than TrkA (Fig. 1A). Whether PDNF is the parasite molecule that binds TrkC was tested by coimmunoprecipitation and *T. cruzi*-TrkC competition assays. PDNF, purified from *T. cruzi* culture supernatants by affinity chromatography (Scudder et al., 1993), was incubated with $TrkC^{Fc}$, $TrkB^{Fc}$, and $FGFR^{Fc}$. Receptor-PDNF complexes, if any, were pulled down on protein G-Sepharose (via the Fc arm of the chimeric receptors) and evaluated by Western blotting with a monoclonal antibody (TCN-2) specific for PDNF (Prioli et al., 1990). We found that $TrkC^{Fc}$ specifically immunoprecipitates PDNF (Fig. 3A), indicating that PDNF is a TrkC ligand. Additionally, soluble PDNF inhibits $TrkC^{Fc}$ binding to the outer membrane of *T. cruzi* in a dose-dependent manner (Fig. 3B), further indicating that PDNF is a TrkC ligand. To determine whether additional *T. cruzi* proteins bind TrkC, we repeated the pull-down experiments using whole *T. cruzi* lysate, probing with Chagasic serum. The results showed no evidence for TrkC-binding *T. cruzi* molecules other than PDNF (data not shown).

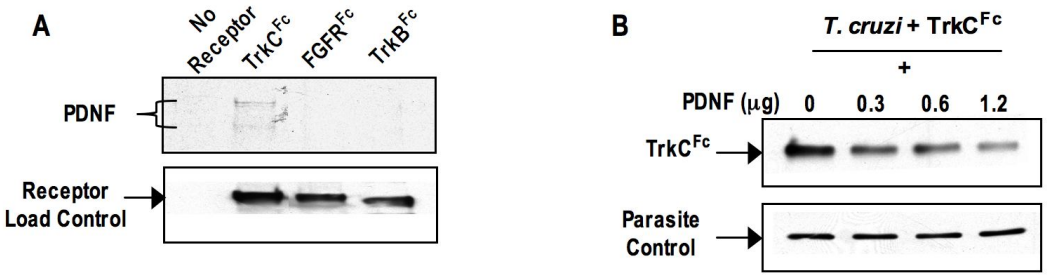


Fig. 3 PDNF binds TrkC.

(A) Receptors (1 μg) were incubated with PDNF (1 μg) and mixed with protein G-Sepharose to pull down Fc-receptors. Coimmunoprecipitated PDNF was revealed by Western blotting using TCN-2 monoclonal antibody. Total receptor was revealed using anti-human IgG antibodies. The additional blot shows 1 μg PDNF visualized by a Western blot developed using TCN-2 monoclonal antibody. (B) Parasites were incubated with 1 μg of $TrkC^{Fc}$ and increasing concentrations of PDNF. Parasites were washed and evaluated for bound $TrkC^{Fc}$. Bound $TrkC^{Fc}$ was calculated by densitometry, with each sample standardized against its parasite wash control, as revealed with chagasic serum (one of several bands shown). Experiments in A and B were done three times, with similar results.

3.4 TrkC Mediates *T. cruzi* Infection *In vitro* and *In vivo*.

Cytoadhesion, such as *T. cruzi* binding to the cell-surface receptor TrkC, is the first step involved in cellular invasion. After finding that *T. cruzi* binds TrkC, we next wanted to determine whether TrkC can mediate cellular invasion. To do this, we used both a genetic and an immunohistochemical approach. First, we took advantage of the commonly used rat pheochromocytoma neuronal cell line (PC12) that lacks Trk receptor expression, PC12-NNR5 (NNR5) cells to determine whether transfection with TrkC increases invasion susceptibility. NNR5 cells were used because they do not express Trk receptors, are readily transfectable and are capable of functional responses to neurotrophins (when transfected with Trk receptors) (Green et al., 1986). Full-length

TrkC and TrkB (ECD plus transmembrane domain plus intracellular kinase domain) were cloned from human brain RNA and expressed in the cells using a bicistronic dsRed mammalian expression vector. NNR5 cells transfected with TrkB, TrkC, or empty vector (EV) were cultured in selective media and sorted by FACS to obtain cell populations with similar expression levels of dsRed and, by extension, of transfected receptors (Fig. 4A). Such sorting was performed to facilitate comparisons between the cell lines stimulated with various agonists. We then tested whether transfected cells respond appropriately to physiological ligands. To evaluate Trk receptor function we used two classic parameters: 1) MAPK Erk1/2 kinase phosphorylation status and 2) cellular differentiation (neurite extension). MAPK Erk1/2 kinase phosphorylation is key for Trk-dependent neurite extension, cell differentiation, and cell survival (Huang and Reichardt, 2003). Neurite extension is an easily visualized product of neurotrophin-induced neuronal differentiation where cells extend projections called neurites. By both criteria, we found that NNR5 cells transfected with a given Trk receptor responded appropriately and specifically to their corresponding ligands (Fig. 4B and C).

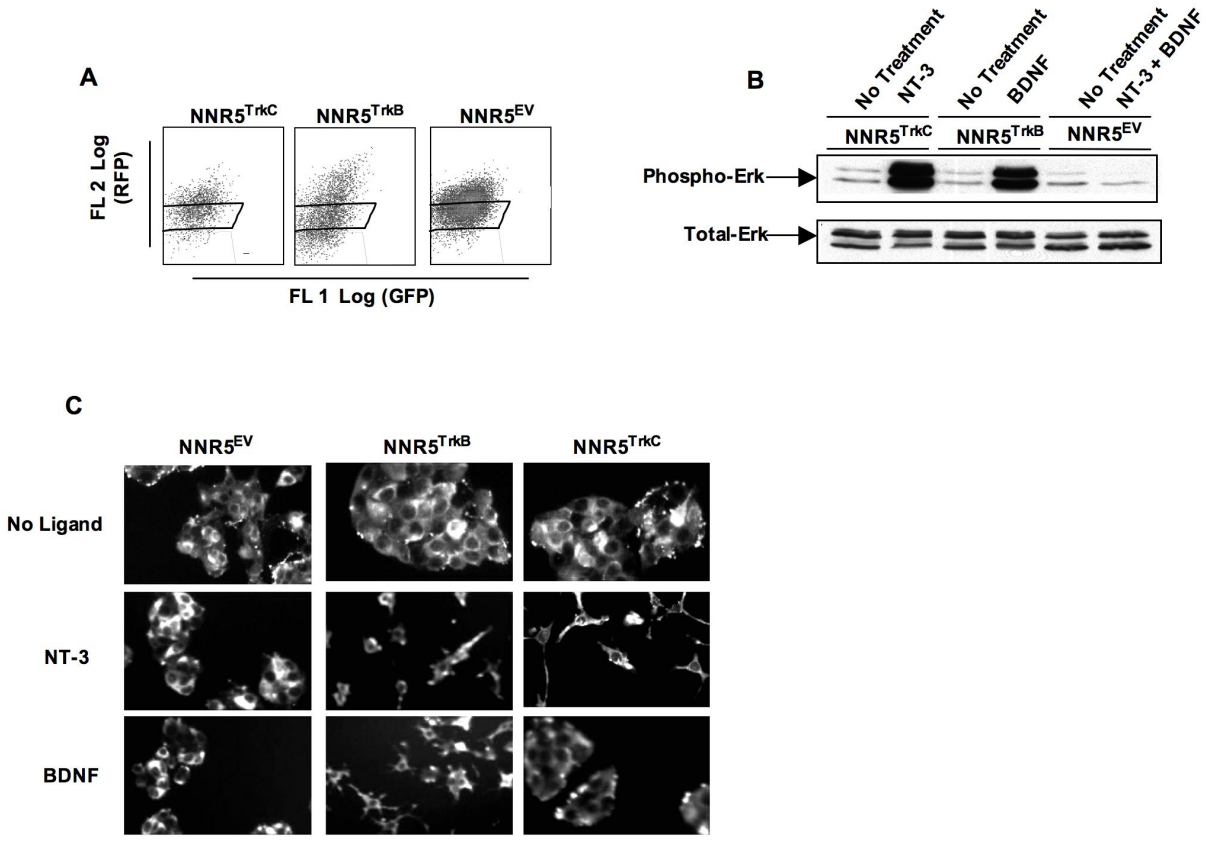


Fig. 4 Trk-deficient PC12-NNR5 (NNR5) cells transfected with TrkC, TrkB, and EV respond appropriately to corresponding ligands.

TrkB and TrkC were cloned from human RNA into the bicistronic mammalian expression vector pIRES-dsRed, which was used to transfect NNR5 cells. (A) $NNR5^{TrkC}$, $NNR5^{TrkB}$, and $NNR5^{EV}$ cells were sorted by FACS for dsRed expression to obtain similar expression levels; rectangles represent the cells selected from each population. The x and y axes represent channels that detect green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively. FL, fluorescence. (B) P-Erk was examined by Western blotting with transfected cells cultured in serum-free medium overnight and treated with NT-3 or BDNF (100 ng/ml) for 12 min at 37℃. The upper and lower bands in each blo t represent Erk1 (MAPK3) and Erk2 (MAPK1), respectively. (C) Transfected cells were cultured in medium with or without NT-3 (100 ng/ml) or BDNF (100 ng/ml) for 3 days, fixed, probed with anti-neurofilament primary antibody and Alexa 488 secondary antibody, and imaged (magnification, 20X) to visualize neurite extension. Experiments were repeated multiple times at various points to ensure that cells maintained correct responsiveness.

Next, cell lines were infected with parasites to determine whether TrkC-transfected NNR5 cells ($NNR5^{TrkC}$) were more susceptible to infection compared to NNR5 cells transfected with TrkB, empty vector (EV) or non-transfected (WT) cells. Cells were plated in 96-well plates in triplicate, incubated with parasites for 2 hours and then the remaining extracellular parasites were rinsed away. The infected cells were cultured for two days to allow for parasite division, fixed and stained with an acid/base stain and then infected cells were counted by light microscopy (40X). Invasion increased in $NNR5^{TrkC}$ cells by ~85% compared to EV controls whereas no significant change was found in the TrkB transfectants (Fig. 5A). Invasion in $NNR5^{TrkC}$ cells was inhibited when α-TrkC antibodies were incubated with the cells prior to infection, but not when they were incubated with α-TrkB antibodies (Fig. 5B), further confirming the specificity of TrkC for invasion. In addition, invasion was also inhibited in $NNR5^{TrkC}$ cells when they were incubated with the parasite protein TS/PDNF, suggesting TS/PDNF is the parasite molecule responsible for mediating invasion through TrkC (Fig. 5B). These data are in agreement with the previous data that show *T. cruzi*/PDNF binds TrkC but not TrkB.

We then examined whether TrkC to mediates invasion in a glial cell line using an immunohistochemical approach. To do this, we used cultured human Schwann cells, which express TrkC and TrkB but not TrkA (Fig. 8C inset). We used Schwann cells because they are highly parasitized by *T. cruzi* and are the most common glial cells in the PNS. Using the same *in vitro* invasion assay as previously described, we incubated the plated Schwann cells with neurotrophins prior to infection to determine whether they could inhibit infection. We found that NT-3 inhibited invasion but neurotrophins that do not bind TrkC (NGF, BDNF) did not (Fig. 5C). In addtion, when parasites were incubated with $TrkC^{Fc}$ and $TrkA^{Fc}$ receptors prior to infection, invasion of Schwann cells

(Fig. 5D) was also inhibited. This inhibition, was specific because $TrkB^{Fc}$ and $FGFR^{Fc}$ did not inhibit invasion (Fig. 5D). Although TrkA is not expressed by Schwann cells, $TrkA^{Fc}$ inhibition further confirms that the parasite molecule PDNF/TS mediates invasion through TrkC because TrkA also binds the parasite molecule PDNF/TS. These findings suggest that invasion of Schwann cells is mediated through TrkC but not through TrkB because competition for *T. cruzi* binding to the cell-surface TrkC receptor blocked invasion.

The data thus far demonstrate that *T. cruzi* can invade cultured nerve tissue cells through TrkC. We next wanted to determine whether TrkC can also mediate infection *in vivo*. Previous experiments in mice showed that $TrkA^{Fc}$ can block infection in mice (de Melo-Jorge and PereiraPerrin, 2007), presumably in a fashion similar to the *in vitro* data seen above (Fig. 5D). However, with our recent data that suggest *T. cruzi* invades cells through both TrkA and TrkC through the parasite surface protein TS/PDNF, the contribution of each receptor towards infection in mice is not defined because $TrkA^{Fc}$ would inhibit all invasion mediated by TS/PDNF. To determine whether TrkC and/or TrkA mediate infection *in vivo*, mice were infected subcutaneously in the footpad with 5000 Tulahuen parasites mixed with 2μg of BSA or α-TrkA, α-TrkB or α-TrkC antibodies. If, for example, TrkC mediates infection, α-TrkC antibodies should block infection. This model of acute infection mimics how infection is thought to frequently occur in humans: parasites penetrate skin, infect cells and replicate locally and then spread to the other tissues. After allowing for local cellular invasion and replication, mice were sacrificed three days after parasite inoculation and parasitism in the footpad was quantified by qPCR. We found that α-TrkA and α-TrkC antibodies block infection in mice, but α-TrkB antibodies did not (Fig. 5E). This is the first result that suggests both TrkC- and TrkA-mediated cellular invasion specifically contribute to acute *T. cruzi* infection of mice.

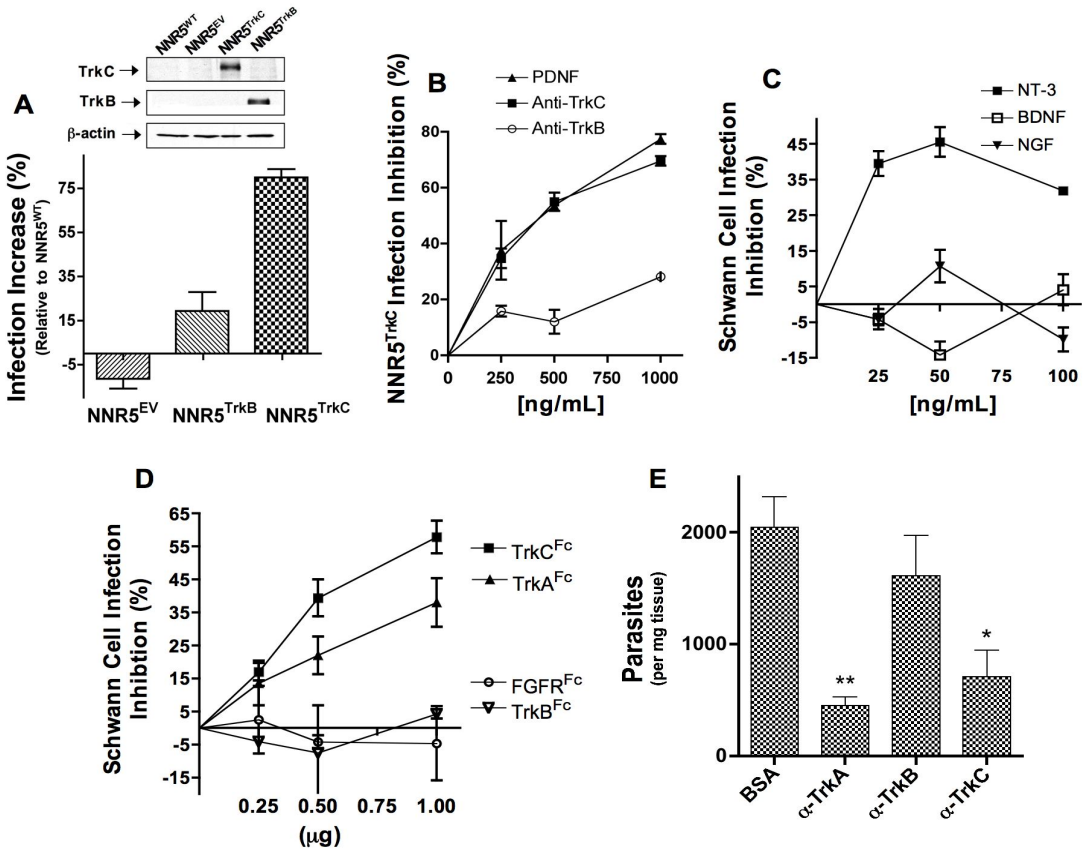


Fig. 5 TrkC mediates *T. cruzi* infection *in vitro* and *in vivo*.

For *in vitro* infections (A-D), cells were plated in 96-well plates in triplicate (3 x 10^3/well), infected with parasites (3 x 10^4/well) and infection was quantified by light microscopy 2 days later. (A) TrkC expression increases infection in NNR5 cells. Infection in transfected NNR5 cell lines were compared to non-transfected NNR5 ($NNR5^{WT}$) cells. (Inset) Western blot of transfected cell lines. (B) Infection in $NNR5^{TrkC}$ cells is blocked with PDNF and TrkC antibodies. $NNR5^{TrkC}$ cells were incubated with PDNF or specific Trk antibodies were for 1 hour prior to infection. (C) The TrkC ligand, NT-3, blocks invasion in Schwann cells, which express TrkB and TrkC. Schwann cells were incubated with Trk ligands for 1 hour prior to infection. (D) Soluble recombinant TrkC receptor blocks infection in Schwann cells. Parasites were pre-incubated with soluble Trk^{Fc} receptors and then used to infect cells. Error bars represent the SEM between three independent experiments. (E) TrkC antibodies block *T. cruzi* infection in mice. Mice were infected via subcutaneous footpad injection of 5000 Tulahuen parasites mixed with 2μg of specific Trk antibodies or BSA, and footpad parasitism was quantified 3 days later. Error bars represent SD of mice from 3 independent experiments; (*) indicates p=0.011 and (**) indicates p=0.009 compared to BSA treated mice.

3.5 TrkC Signaling is Required for Cellular Invasion

The process of cellular invasion requires coordinated intracellular signaling events such as actin and lysosome mobilization and PI 3-kinase and Ca^{2+} signaling (Reddy et al., 2001; Woolsey and Burleigh, 2004; Woolsey et al., 2003). Thus, it may be that TrkC-mediated invasion requires TrkC receptor activation. To determine whether TrkC signaling is required for TrkC-mediated cellular invasion, we first determined whether *T. cruzi* and PDNF can activate TrkC signaling using the same criteria as seen in Fig. 4. First, we probed whether PDNF activates TrkC-dependent Erk1/2 signaling by treating $NNR5^{TrkC}$ cells with PDNF (150 ng/ml for 12 min) or *T. cruzi* (10^7 trypomastigotes/ml for 15 min) and examining whether such treatment promoted the phosphorylation of Erk1/2 by Western blotting. We found that both PDNF and *T. cruzi* activate Erk kinase (Fig. 6A and B) in a dose-dependent manner (Fig. 6C). In contrast, purified PDNF and live *T. cruzi* did not activate Erk in $NNR5^{TrkB}$ and $NNR5^{EV}$ cells (Fig. 6A and B). Furthermore, the observed P-Erk increase in the $NNR5^{TrkC}$ cells was inhibited by K252a, a selective pharmacological inhibitor of Trk signaling (Fig. 6B) (Berg et al., 1992), further suggesting that *T. cruzi* and PDNF specifically activate TrkC signaling.

Next, to further examine PDNF-dependent TrkC activation, transfected NNR5 cells were plated and grown in media in the presence or absence of PDNF (250 ng/ml) or NT-3 (100 ng/ml) for 72 h. Cells were then evaluated for neurite extension by fluorescence microscopy. Without the addition of PDNF or NT-3, $NNR5^{TrkB}$, $NNR5^{TrkC}$, and $NNR5^{EV}$ cells grew into clusters of small, round cells. However, the treatment of the cells with PDNF resulted in neurite outgrowth in $NNR5^{TrkC}$ cells but not in $NNR5^{TrkB}$ or $NNR5^{EV}$ cells (Fig. 6D). This finding further suggests that PDNF activates TrkC signaling.

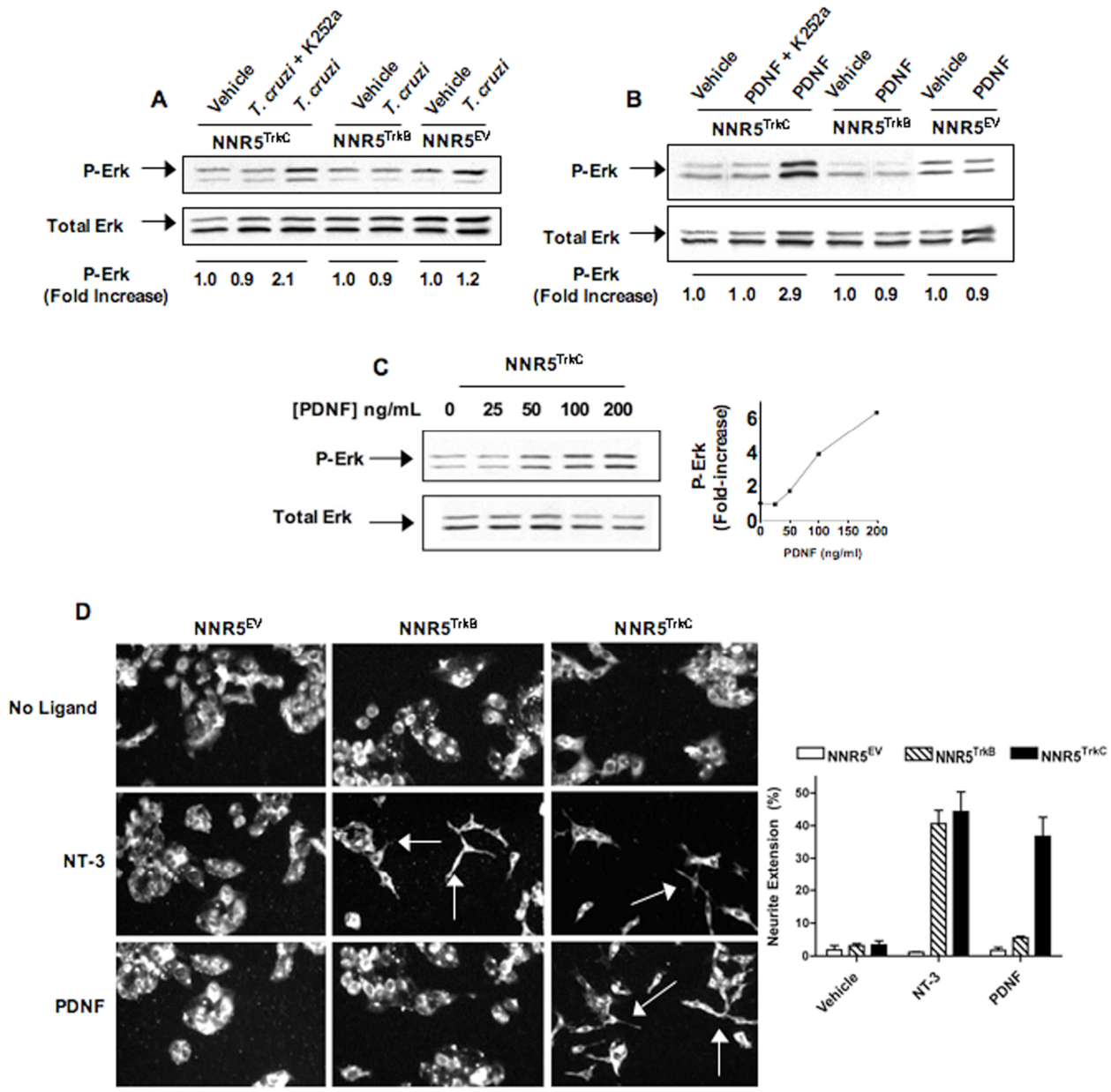

A
Vehicle
T. cruzi + K252a
T. cruzi
Vehicle
T. cruzi
Vehicle
T. cruzi
NNR5TrkC
NNR5TrkB
NNR5EV
P-Erk
Total Erk
P-Erk (Fold Increase)
1.0 0.9 2.1 1.0 0.9 1.0 1.2
B
Vehicle
PDNF + K252a
PDNF
Vehicle
PDNF
Vehicle
PDNF
NNR5TrkC
NNR5TrkB
NNR5EV
P-Erk
Total Erk
P-Erk (Fold Increase)
1.0 1.0 2.9 1.0 0.9 1.0 0.9
C
NNR5TrkC
[PDNF] ng/mL 0 25 50 100 200
P-Erk
Total Erk
P-Erk (Fold-increase)
0 2 4 6
0 50 100 150 200
PDNF (ng/ml)
D
NNR5EV
NNR5TrkB
NNR5TrkC
No Ligand
NT-3
PDNF
NNR5EV
NNR5TrkB
NNR5TrkC
Neurite Extension (%)
0 10 20 30 40 50
Vehicle
NT-3
PDNF

Fig. 6 *T.cruzi*/PDNF activates TrkC signaling.

(A) Cells were cultured in serum-free medium overnight, treated with the Trk-specific inhibitor K252a (1 μM) for 1 h where indicated, and then treated with 10^6 parasites or not treated for 12 min at 37℃. Cell lysates were ana lyzed by Western blotting to evaluate P-Erk and total Erk. (B) Similar to A, but plated cells were treated with PDNF (200 ng/ml) for 12 min. (C) PDNF dose-response treatment of $NNR5^{TrkC}$ as described above (B) with accompanying graph. Phosphorylation (increase) was calculated by scanning densitometry. Each P-Erk band was standardized against its total Erk band and then standardized against vehicle (DMEM), which was arbitrarily set to 1. Experiments in A to C were done twice, with similar results obtained. (D) $NNR5^{TrkC}$, $NNR5^{TrkB}$, and $NNR5^{EV}$ cells were plated and cultured for 2 to 3 days with PDNF (250 ng/ml) or NT-3 (100 ng/ml). Cultures were then fixed, probed with anti-neurofilament primary antibody and Alexa 488 secondary antibody, and imaged (magnification, 20X) to visualize neurite extension; arrows point toward neurites. (Inset) Neurite extension was quantified for three individual experiments; error bars represent the standard errors of the means between the three experiments, and 50 or more cells were counted for each condition in each experiment. Neurite extension (percent) is the number of cells with at least one neurite with a length greater than 100% of the diameter of the cell body divided by the total number of cells.

After determining that *T. cruzi*/PDNF can activate TrkC signaling, we next wanted determine whether signaling is necessary for invasion. In *in vitro* experiments similar to those in Fig. 5A-D, $NNR5^{TrkC}$ cells were plated in 96-well plates and incubated with specific small molecule signaling inhibitors before cells were incubated with parasites. K252a, a Trk-specific tyrosine kinase inhibitor and AG879, a tyrosine kinase inhibitor, both inhibited *T. cruzi* invasion in a dose-dependent manner (Fig. 7A and B). AG879-treatment of cells resulted in high levels of cell death above 250nM concentrations. Erk 1/2 phosphorylation is a major signaling pathway for cell survival and differentiation, yet it has not been determined whether *T. cruzi* invasion is dependent on Erk signaling. We

found that the MAC inhibitor, UO126, which inhbibts of Erk phosphorylation, inhibited *T. cruzi* invasion of NNR5^{TrkC} cells (Fig. 7C). This result further confirms the importance of TrkC signaling for cellular invasion and is the first result that suggests that the Erk signaling pathway is important for *T. cruzi* invasion. In addition, an inhibitor of the insulin-like growth factor receptor-1 (IGFR-1) did not inhibit invasion (Fig. 7D), which suggests that the inhibition of invasion by K252a, AG879 and UO126 is specific.

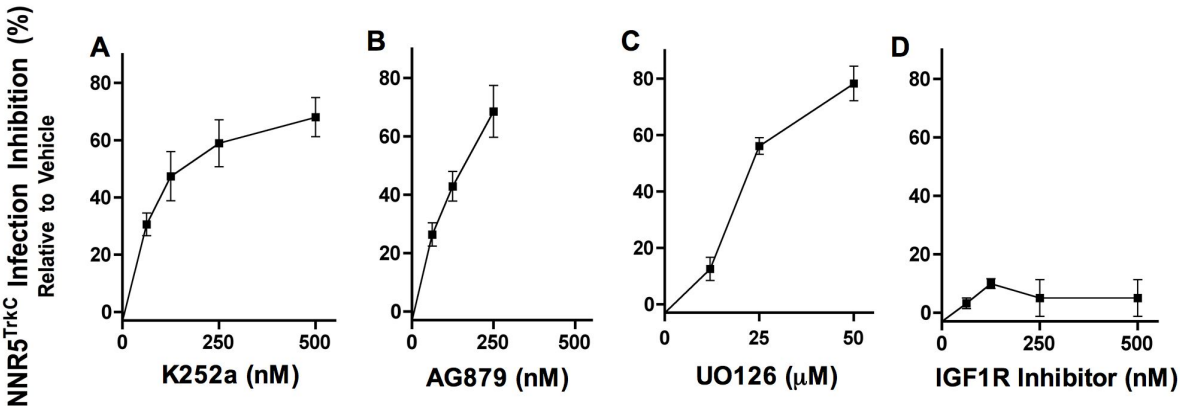


Fig. 7 TrkC signaling is necessary for *T. cruzi* invasion of NNR5^{TrkC}.
NNR5^{TrkC} cells were plated in 96-well plates in triplicate (3 x 10^3/well), pre-incubated with vehicle or indicated concentrations of specific signaling inhibitors 1 hour before infection, infected with parasites (3 x 10^4/well) and cellular infection was quantified 2 days later. Error bars represent the SEM between three independent experiments.

3.6 Invasion Through TrkA and TrkC Results in More Robust Intracellular Division.

TrkA and TrkC receptors are neurotrophic receptors that mediate cell survival, and, as our results indicate, *T. cruzi* has evolved mechanisms that exploit the receptors for cellular invasion. The activation of Trk trophic signaling could potentially benefit the parasite by promoting the health of its intracellular environment. Said another way, Trk signaling could promote parasite health within the cell. One such effect of this may be that invasion through TrkC and/or TrkA could result in an increased rate of parasite intracellular division. To address this hypothesis, we first determined whether *T. cruzi*-induced TrkC signaling promotes the survival of cultured nerve tissues cells, again using $NNR5^{TrkC}$ and Schwann cell cultures.

To determine whether PDNF promotes the TrkC-dependent survival of neuronal cells, we induced apoptosis in the NNR5 transfectants by growing the cells in serum-free medium for 3 days in the absence or presence of PDNF (250 ng/ml). Cell death was assessed by fluorescence microscopy using the Hoechst 33343-PI assay. While most $NNR5^{TrkC}$ cells died in serum-free medium, PDNF rescued ~70% of the serum-starved cells (Fig. 8A and B). In contrast, PDNF did not rescue $NNR5^{TrkB}$ and $NNR5^{EV}$ transfectants (Fig. 8A and B). This result suggests that PDNF treatment protects a neuronal cell line from cell death in a TrkC-dependent manner.

To determine whether the survival action of PDNF extends to glial cells, we grew a human Schwann cell line in serum-free medium for 3 days without and with PDNF (250 ng/ml), with or without an antibody specific for TrkC or TrkB. Preliminary experiments showed that the commercial TrkC antibodies reacted with TrkC and not TrkA and TrkB and that the TrkB antibodies were selective for TrkB (not shown). In addition, preliminary experiments confirmed that human Schwann cells express TrkB and TrkC (Fig. 8C, inset) but not TrkA (not shown). We found that PDNF potently

promoted the survival of Schwann cells (Fig. 8C, compare the serum bar with the serum-free and PDNF bars). Because PDNF binds TrkC but not TrkB and because Schwann cells do not express TrkA, this result suggests that Schwann cell protection is mediated by PDNF recognition of TrkC, and this was confirmed by inhibition with Trk-specific antibodies. Thus, the PDNF-induced protection of Schwann cells was blocked by a TrkC-specific antibody ($P < 0.01$) but not by a TrkB-specific antibody (Fig. 8C).

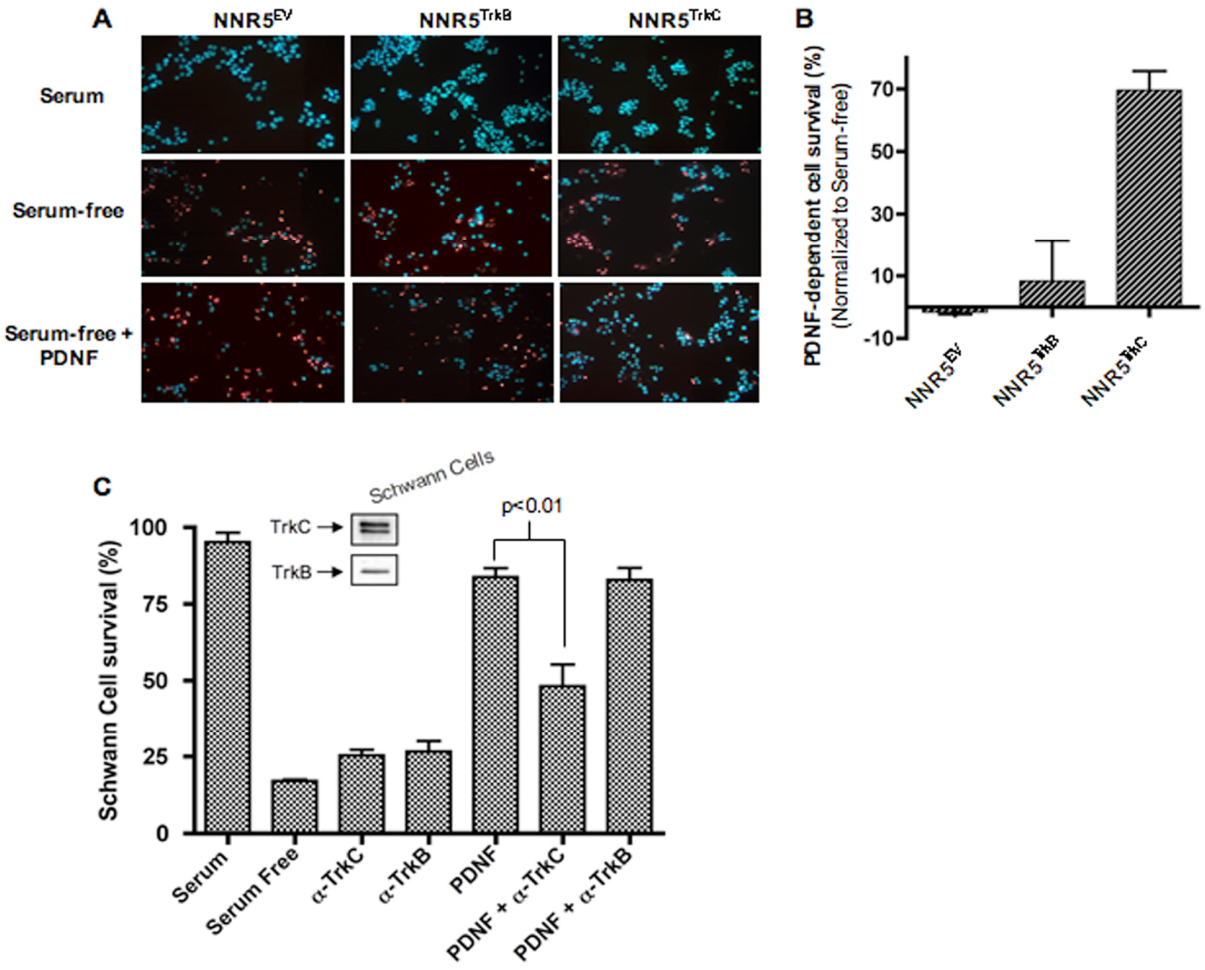


Fig. 8 PDNF promotes TrkC-dependent neuronal and glial cell survival.

(A) NNR5^{TrkC}, NNR5^{TrkB}, and NNR5^{EV} cells were plated in triplicate and cultured for 3 days in 1% FCS, serum-free DMEM, or serum free-medium supplemented with PDNF (250 ng/ml). Cells were stained with propidium iodide and Hoechst nuclear staining reagents and counted by fluorescence microscopy (magnification, x20). (B) Graph of data for experiment shown in A with PDNF survival normalized against serum-free survival. Error bars represent the standard errors of the means between experiments; an average of three experiments were performed, with each point in triplicate. (C) Schwann cells were plated in triplicate and then cultured under conditions as indicated: 1% FBS or serum free with or without α-TrkC (1 μg/ml), α-TrkB (1 μg/ml), PDNF (250 ng/ml), or a combination. (Inset) Western blot of lysate from Schwann cells. Error bars represent the variation between experiments. P values (analysis of variance) were calculated using Prism software. An average of two experiments were performed, each point in triplicate.

As discussed previously, one effect of invasion through the trophic receptors TrkA and/or TrkC may be that the invasion would result in more rapid amastigote intracellular division. To test this possibility, we first needed to create a NNR5 cell line transfected with TrkA to allow for comparison with $NNR5^{TrkC}$ cells. To do this, all steps in the process including the cloning strategy, transfection, cell culture and sorting with FACS were identical to that used when creating $NNR5^{TrkC}$ cells. After creating $NNR5^{TrkA}$ cells, using our *in vitro* invasion assay (see Fig. 5A-D), we infected $NNR5^{TrkC}$, $NNR5^{TrkA}$ and $NNR5^{EV}$ cells. Two days after infection the total number of parasites within each infected cell was counted (counting at least 40 infected cells). The average number of amastigotes in one infected $NNR5^{TrkA}$ or $NNR5^{TrkC}$ cell two days after infection was nearly double ($p=0.001$) compared to a $NNR5^{EV}$ cell (Fig. 9B). A possible explanation of this result is that there is not an increased rate of parasite division in TrkA and TrkC cells, but instead that the cells were initially invaded by multiple parasites. To determine whether this is the case, we counted intracellular parasites 24 hours after infection. At this point, a parasite is just completing its transformation into an amastigote and may have undergone ~1 replication. 24 hours after infection there was no significant difference between the infected cell lines (Fig. 9A), which would suggest the increased number of amastigotes seen by day 2 is due to an increased rate of cellular division. This is an impressive outcome because it suggests that although *T. cruzi* has multiple methods of cell entry (note that $NNR5^{EV}$ are still infected), specific invasion through TrkA or TrkC in a relatively small number of cells would result in hundreds more parasites in a short amount of time compared to cells that do not express TrkA or TrkC. Indeed, this is exactly what was found (Fig. 9C). Thus, TrkA and TrkC expression on a cell results in a dual benefit for *T. cruzi*: 1) increased susceptibility for cellular invasion and 2) more robust parasite division.

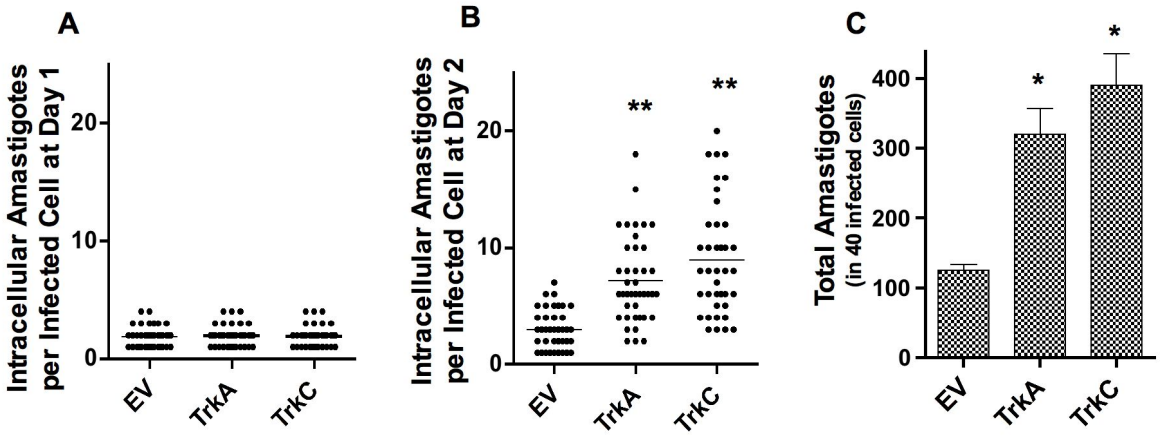


Fig. 9 TrkA- and TrkC-mediated invasion results in an increased rate of *T. cruzi* intracellular division *in vitro*.

Transfected NNR5 cells lines were plated in 96-well plates in triplicate (3 x 10^3/well) and infected with parasites (3 x 10^4/well). The number of intracellular amastigotes in 40 infected cells (from 3 separate wells) were counted 1 day (A) and 2 days (B) after infection. Each point on the graph represents the number of amastigotes within one cell. (A and B) Graph shows a representative experiment of three independent experiments. (C) Bar graph shows the total number of amastigotes in 40 infected cells 2 days after infection; error bars represent the SEM between three experiments. (**) Indicates p=<.001 for TrkA and TrkC compared to EV in (B); (*) indicates p=<.01 for TrkA and TrkC compared to EV in (C).

3.7 Mice Have a Specific Loss of TrkC-expressing Cells in their Colon During Acute *T. cruzi* Infection

The results thus far demonstrate that *T. cruzi* can invade cells through TrkC, that invasion mediated by TrkA or TrkC results in more robust intracellular growth and that both TrkA and TrkC help mediate infection in mice. Next, we wanted to determine what

role *T. cruzi*-Trk interactions play during the subsequent stages of infection. As acute infection in mammals proceeds, parasites spread from the initial inoculation site through the blood to infect cells in nearly all tissues. During this time, cells of the PNS, which can variably express TrkA, TrkB and/or TrkC, are often destroyed. To study whether *T. cruzi*-Trk interactions play a role in these processes we studied nerve tissue cells in the ENS of the colon because it is large, easily accessible and disease-relevant. In agreement with previous data (Esteban et al., 1998; Sternini et al., 1996), we found TrkB and TrkC were expressed in the murine colon, but not TrkA (RT-PCR data, not shown).

To examine infection in the colon ENS, we started by determining the course of colon parasitism during infection. Mice were infected using a subcutaneous inoculation of 5000 Tulahuen parasites in the hind footpad. To determine the time-course of infection in our model we counted parasites in the blood of infected mice by fluorescent microscopy. In addition, we sacrificed mice weekly for ~5 weeks, collected colon tissue, extracted DNA and determined colon tissue parasitism by qPCR during the course of acute infection. As parasites spread to tissues distant from the initial inoculation site, parasitemia peaked at 11 dpi (Fig. 10A). Colon parasitism peaked about one week later at 18 dpi and was nearly undetectable by 32 dpi (Fig. 10A).

T. cruzi preference for invasion of TrkC-expressing cells over TrkB-expressing cells (Fig. 5A-D) led us to suspect that TrkC-expressing cells in the colon may be preferentially destroyed compared to TrkB cells. The interconnected nature of the neural network in colon would suggest that initial destruction of some cells could lead to the subsequent destruction of associated cells. Therefore, to address our hypothesis regarding differential destruction, we evaluated TrkB- and TrkC-expressing cells at points close to when colon parasitism peaks during acute infection. Mice were infected via the footpad as described above and sacrificed when parasites are first detectable and at peak colon parasitism, 11 dpi and 18 dpi, respectively. After collecting and

washing the colons of infected and uninfected mice, we used two strategies to look at TrkB and TrkC cell populations: qPCR and immunohistochemistry.

First, using qPCR and primers specific for housekeeping genes expressed by neurons (HuD) and glial cells (S100b), we found a roughly 50% decrease in mRNA of these cell populations at 18 dpi in the colon (Fig. 10B, E and F). This suggests that roughly 50% of the neurons and glia cells are dying in the colon, which is in agreement with previous results (Maifrino et al., 2005), because housekeeping genes maintain steady levels in living cells. The same technique using primers specific for TrkC and TrkB reveals a specific and significant decrease of TrkC but not TrkB mRNA in infected versus uninfected mice (Fig. 10B, C and D) at 18 dpi. This result could suggest that 1) *T. cruzi* down-regulates *nTrk3* (TrkC) gene expression or 2) TrkC-expressing cells are dying, which results in the loss of TrkC mRNA expression.

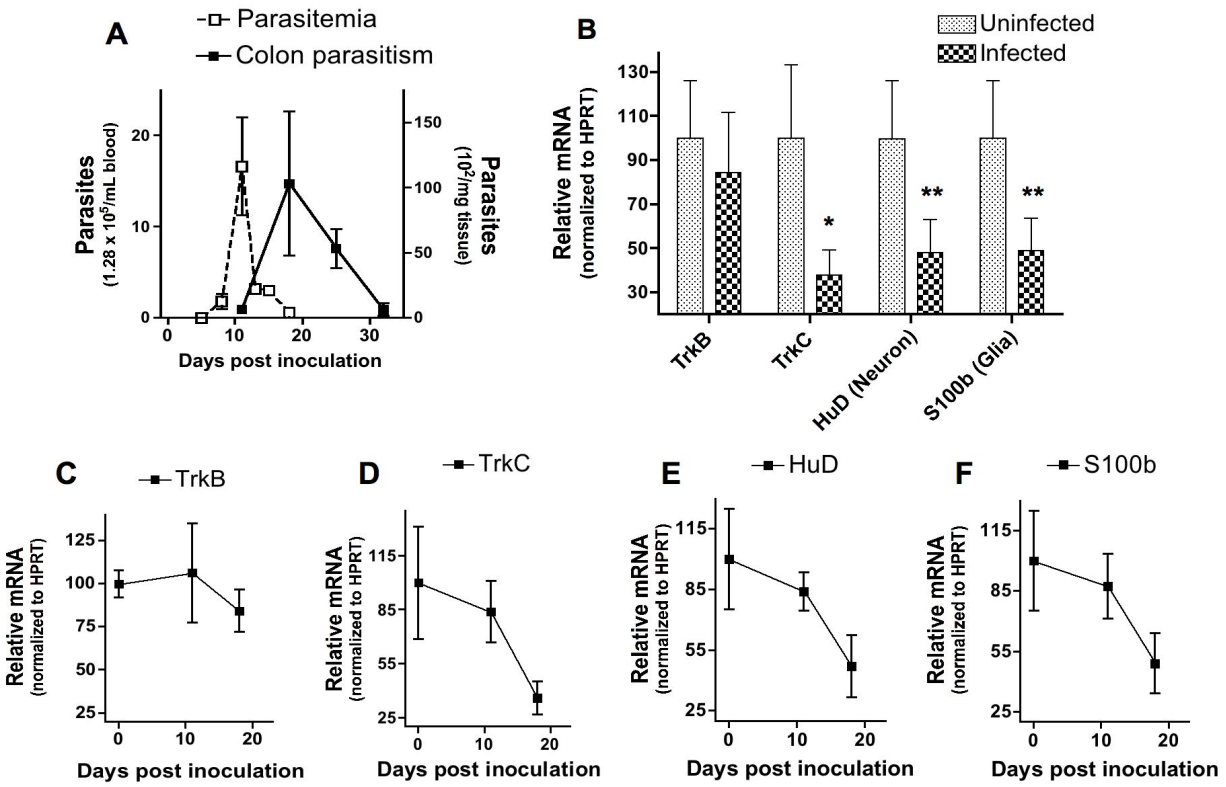


Fig. 10 Specific decrease of TrkC mRNA in the colon of mice infected with *T. cruzi*.

C57BL/6 mice were subcutaneously inoculated with 5 x 10^3 Tulahuen parasites or vehicle (PBS). At specified time points, mice were sacrificed and colon tissue was harvested. (A) Colon parasitism peaks at 18 days post inoculation (dpi), roughly 1 week after peak parasitemia. Parasites in the blood were counted by light microscopy and colon parasitism was calculated by qPCR at indicated points; each point represents 5-8 mice. (B) Specific decrease of TrkC mRNA expression in infected mice. cDNA from the colon of mice was analyzed by qPCR using primers specific for TrkB, TrkC, HuD and S100b. HuD and S100b are housekeeping genes specific to neurons and glia cells, respectively. (C-F) Time-course of infection as in (B); 0 dpi represents uninfected mice. Error bars represent the SD between 5-8 mice from two independent experiments. (*) signifies p=<0.001 and (**) signifies p=<0.01 of infected compared to uninfected mice.

To further evaluate whether TrkC-expressing cells are indeed dying in the colon of infected mice we used immunohistochemistry techniques. Mice were infected as described and infected and uninfected mice were sacrificed at specified time points. Colon tissue was harvested, cleaned and frozen in OTC medium. Unfixed frozen tissue was then cryosectioned and evaluated by light and fluorescent microscopy. In agreement with previous data, we first confirmed that TrkC expression was limited to the myenteric and submucosal plexuses (Fig. 11B). In keeping with the time-course of our infection (Fig. 10A), inflammatory infiltrates were easily visible at the point of peak tissue parasitism (Fig. 11A). During the process of cell death gene regulation can precede death and cell clearance. Therefore, to help determine at what stages this cell death was occurring, we harvested tissue at 18 dpi, 25 dpi and 32 dpi and evaluated whether and when cell death was occurring in infected mice using TUNEL staining assay. In infected mice TUNEL positive cells were detected at 18 dpi and 25 dpi with staining nearly undetectable by 32 dpi (Fig. 11C). It is notable that in areas where there are several TUNEL positive cells there were also clusters of inflammatory cells (see Fig. 11D, 18 dpi, area demarcated by red arrows). Uninfected mice had little to no TUNEL positive cells (Fig. 11C). These results suggest that cell death in the colon occurs via apoptosis (at least in part) and that this cell death is occurring roughly 18-25 dpi.

Next, colon tissue was evaluated at 18 and 32 dpi using α-Tuj (a marker of neurons), α-GFAP (a marker of glial cells) and α-TrkC antibodies to evaluate specific cell populations during infection. In agreement with the TUNEL assay, cells that stain positive for neuron or glial markers are greatly diminished by 32 dpi (Fig. 11D) in infected mice compared to uninfected contols. Furthermore, α-TrkC staining shows that there is a roughly 60-65% decrease of TrkC-expressing cells in the colon of mice infected with *T. cruzi* (Fig. 11D, bar graph), an amount that is comparable to the specific decrease in TrkC mRNA. Loss of TrkC expression in addition to cell death (TUNEL assay) and death of glial and neuronal cells (decresased house-keeping genes and staining) suggest that the decrease in TrkC expression is reflective of TrkC cell death.

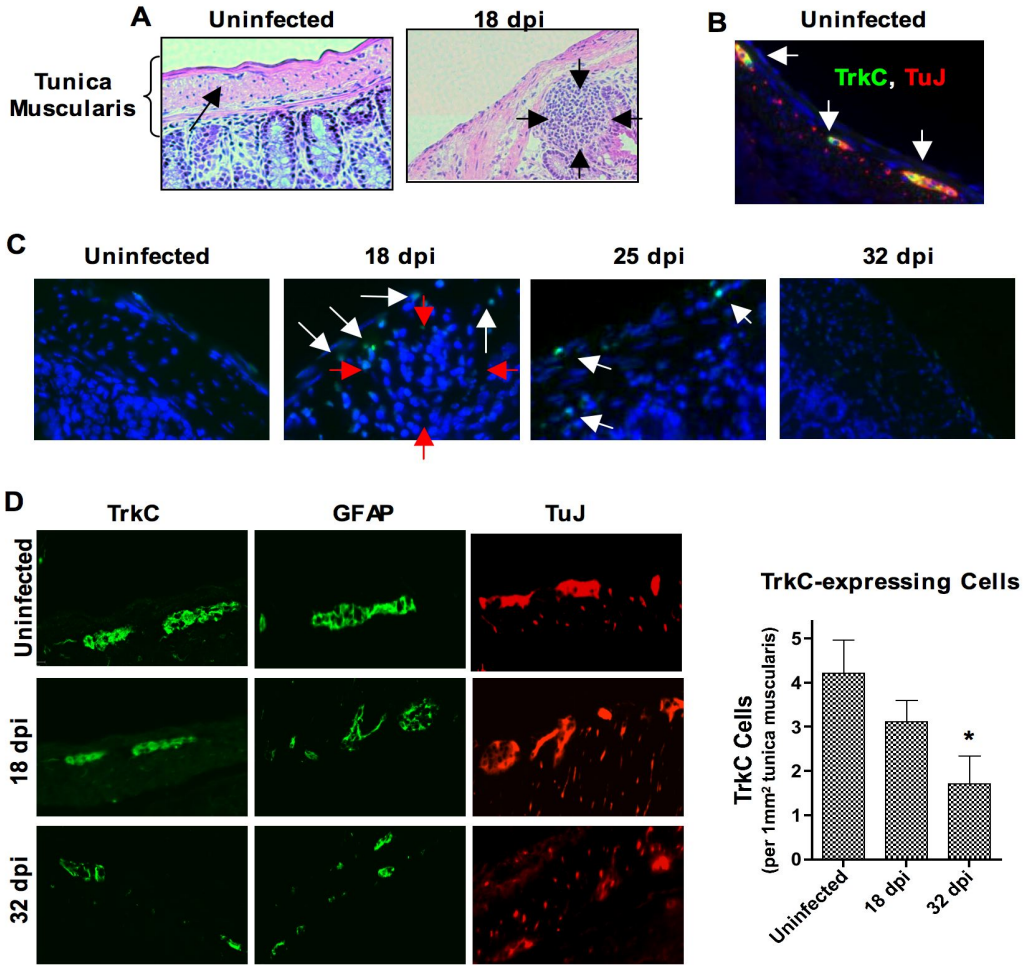


Fig. 11 TrkC-expressing cells are destroyed in the colon of mice infected with *T. cruzi*.

Mice were infected as in Fig. 10. Uninfected and infected mice were sacrificed at indicated points, colon tissue was preserved in OTC, cryosectioned, stained and imaged (40X). (A) Inflammation in colon tissue of infected mice. Tissue was stained with H&E; arrow points to neuron bodies in myenteric plexus (Uninfected) and demarcates inflammatory infiltrate (18 dpi). (B) TrkC expression in uninfected colon. Tissue was costained with DAPI (blue) and α-TrkC (green) and α-Tuj (red, neuron) antibodies. (C) Apoptosis in infected colon. Tissue from various points during infection was stained with TUNEL (green) and DAPI. White arrows point to TUNEL+ cells; red arrows demaracte inflammatory infiltrate. (D) Cell death in the colon of infected mice. Tissue was stained with α-TrkC (green), α-GFAP (green, Glia cell) and α- Tuj (red, neuron) antibodies. (Inset) TrkC-expressing cells were counted in 10-20 images from multiple sections of tissue from 3 mice for each group; graph shows mean and SD; (*) signifies $p = 0.02$.

3.8 TrkC Cell Death Correlates with Increased Colon Size in Mice Infected with *T. cruzi*.

Humans infected with *T. cruzi* have acute neuron death in the colon and can develop megacolon in the chronic stages of disease. Studies show that megacolon development is a gradual process that is subclinical for decades and is preceded by constipation and increased bowel content transit time (de Oliveira et al., 1998). We hypothesized that something similar may also occur in mice after neuron death in the colon during acute infection. Such changes could include aperistalsis and the accumulation of feces in the colon. The finding that bowel transit time increases in mice during acute *T. cruzi* infection supports this hypothesis (de Oliveira et al., 2008). To determine whether such changes in the colon occur, we removed the colon from mice at various points during acute infection and weighed the entire colon (just distal the cecum to the anus) without removing any contents. During the first 2-3 weeks of infection there was very limited change in colon size or appearance in infected mice (Fig. 12A). However, by 25 dpi the colon weight of infected mice doubled and is significantly greater ($p=0.0001$) than that of uninfected mice (Fig. 12B). At this point, there is also a clear difference in the appearance of colons from infected versus uninfected mice. Uninfected mice have smooth, non-tortuous colons with few and well-demarcated feces (Fig. 12C). In contrast, infected mice (at 25 and 32 dpi) generally have tortuous colons entirely full of fecal matter with small sites of hemorrage (Fig. 12C).

We next used our qPCR method to quantify a longer time-course for TrkC cell loss (Fig. 12D) to compare TrkC cell loss to colon size increase over time. At peak colon parasitism (18 dpi), TrkC cells begin dying (Fig. 12D and 11E) and colon weight increase follows by about 1 week (Fig. 12E). In addition, we found that colon size in each mouse correlates with the amount of cell death of TrkC-expressing cells (as quantified by

qPCR) (Fig. 12F). These results are the first to document and quantify an increased colon size in mice infected with *T. cruzi*.

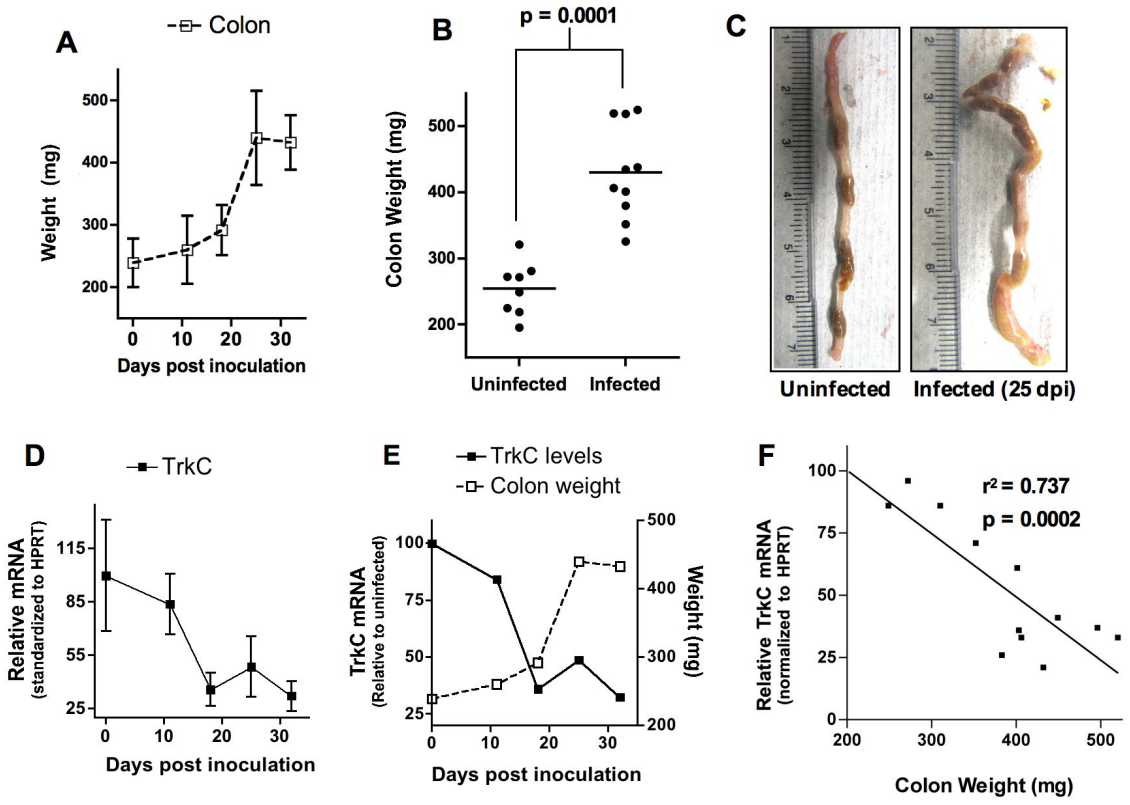
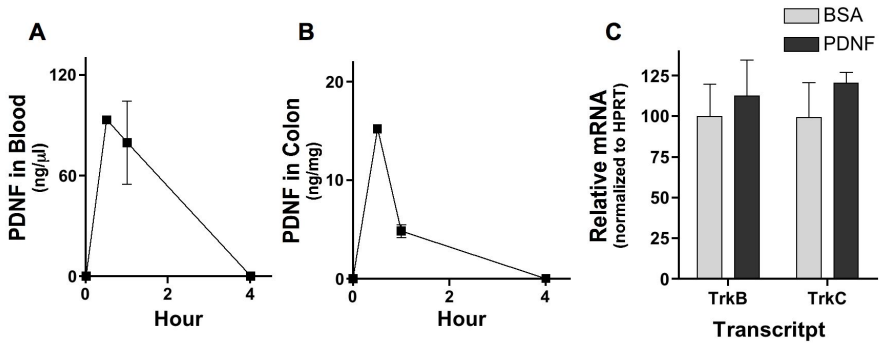


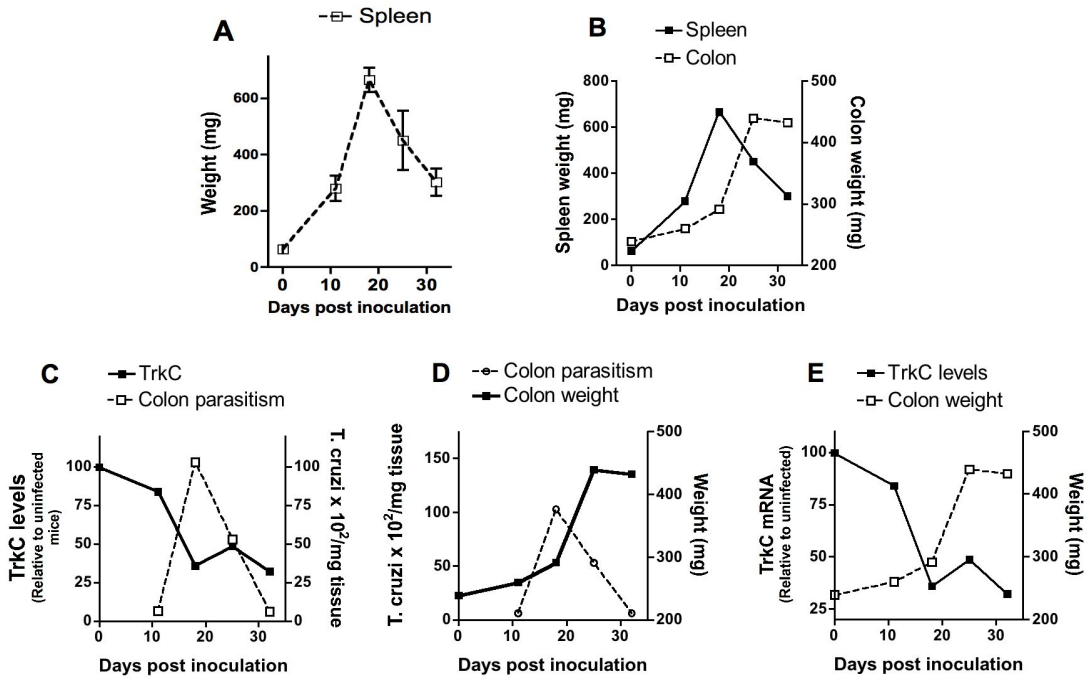
Fig. 12 TrkC cell death correlates with increased colon size in mice infected with *T. cruzi*.

Mice were infected as in Fig. 10. Mice were sacrificed at indicated times (11, 18, 25 and 32 dpi) and the colons were weighed (A); time point 0 represents the average of the uninfected mice. (B) Colon weight at 25 dpi; each point represents the weight of the colon from individual mice from two independent experiments. (C) Photos of uninfected and infected colons from mice 25 dpi. (D) Relative TrkC-expressing cells were calculted by qPCR as in Fig. 10. (E) Graphs (A) and (D) are combined without error bars. (F) Each point represents relative TrkC level and colon weight from infected (25 and 32 dpi) and uninfected mice from (A). Error bars represent SD.



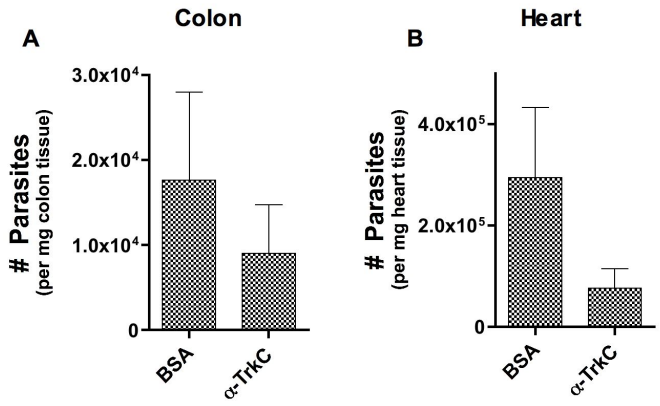
Supplemental Fig. 1 TrkC and TrkB genes in the colon are not regulated by PDNF

(A and B) PDNF distributes to blood and colon tissue. PDNF (50μg) was injected into the peritoneum of C57Bl/6 mice at hour 0. Blood and colon tissue was collected at indicated time points and analyzed for PDNF. (C) PDNF does not regulate TrkB or TrkC transcript levels. PDNF (50μg) or BSA (50μg) was injected into the peritoneum of mice for two days; mice were sacrificed 4 hours after injections on day 2 and TrkB and TrkC transcripts were measured by qPCR.



Supplemental Fig 2. TrkC cell death and colon enlargement occur subsequent to *T. cruzi* colon invasion.

C57Bl/6 mice were subcutaneously inoculated with 5000 Tulahuen parasites or PBS. Mice were sacrificed at indicated times (11, 18, 25 and 32 dpi) to determine colon weight, spleen weight, colon TrkC expression levels and colon tissue parasitism. (A) At noted points during infection spleens were weighed; error bars represent SD of at least 5 mice from 2 experiments. (B-E) Comparative plots are shown of previous data without error bars to facilitate comparison of the timing of events that occur during acute *T. cruzi* infection in mice.



Supplemental Fig. 3 α-TrkC antibodies block infection of colon and heart tissue in mice already infected with *T. cruzi*.

Mice were infected as in Fig. 10 with 5000 parasites via the footpad. After waiting 3 days for the infection to be established, mice were injected intraperitoneally with 10 μg of either α-TrkC antibodies or BSA daily for 10 days. Colon (A) and heart (B) tissue was harvested from mice that were sacrificed at 18 dpi and tissue parasitism was quantified by qPCR. Error bars represent the variation between 2 mice (BSA) and SD between 3 mice (α-TrkC) from one experiment.

CHAPTER 4. DISCUSSION

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4.1 TrkC Mediates *T. cruzi* Invasion.

T. cruzi cellular invasion is a process that entails a relatively large eukaryotic parasite (2 x 20μm) entering mammalian cells, which can have average diameters of 10-100μm. Upon cell entry, the parasite contained within a membrane-bound vesicle differentiates into an amastigote (2 x 2μm), escapes into the cytoplasm and replicates by binary fission (Tyler and Engman, 2001). Since it was proposed in the early 1990's that the process of cellular invasion may be mediated by specific receptors, a variety of host and parasite molecules involved in the complicated process of invasion have been defined (see Introduction, Section 1.8). By employing multiple strategies for cellular invasion, *T. cruzi* is similar to other eukaryotic parasites such as *Leishmania* and also to simpler pathogens such as the virus HIV. *Leishmania* inhabits macrophages, and, to invade only a single cell type, can use at least three separate surface receptors (complement receptor 1 and 3 and mannose/fucose receptor) (Handman and Bullen, 2002). HIV, which also has a very limited repertoire of host cells in comparison to *T. cruzi*, uses at least four host molecules that aid in the process of invasion (CD4, CXCR4, CCR5 and mannose-binding C-type lectin) (Smith and Helenius, 2004). A challenge in interpreting this work, and the work shown here, is to define what such molecular interactions contribute to invasion. Some surface molecules, such as mucin on gut epithelium, may be relevant for *T. cruzi* attachment, yet not mediate cellular invasion. However, if host cell mucin production is blocked, the likely effect would be decreased parasite invasion. One reasonable, and incorrect, interpretation of these data could be that mucin mediates cellular invasion. Another example of the complications of interpreting invasion data is seen in *T. cruzi* interaction with the surface receptor TGF-β

receptor II (TGF-β RII). Inhibition of TGF-β RII signaling results in decreased parasite cellular invasion (Ming et al., 1995). However, a *T. cruzi* molecule that binds TGF-β RII could never be defined, and competitive inhibition of the receptor with an activating ligand also increases cellular invasion. These data suggest that TGF-β RII signaling can facilitate invasion, yet the parasite may not use the receptor itself to guide cellular entry. An explanation could be that TGF-β RII signaling could mediate cell membrane changes that make invasion more permissible or that additional receptors work in conjunction with TGF-β RII.

In the work here, we have been able to define both a host receptor (TrkC) and the parasite molecule that binds the receptor (TS/PDNF) that appear to mediate invasion. Specific over-expression of TrkC on TrkC-deficient cells results in increased invasion (Fig. 5A), suggesting TrkC expression promotes cellular invasion. NT-3, which activates TrkC signaling, inhibits invasion in TrkC-expressing cells (Fig. 5C). This suggests that *T. cruzi* invasion depends on *T. cruzi* contacting TrkC itself, not simply TrkC signaling activation. Similarly, invasion in TrkC cells is also inhibited using soluble $TrkC^{Fc}$ (theoretically through binding parasite PDNF). This result suggests that *T. cruzi* needs to contact cell surface TrkC. An alternative would have been that parasite engagement with TrkC could trigger parasite changes that allow it to invade through a non-TrkC mechanism. This is a reasonable possibility because other organisms such as HIV go through this exact process (Smith and Helenius, 2004). These points are subtle, but they help to more clearly define how TrkC contributes to cellular invasion.

The mechanism of TrkC-mediated invasion was further defined using specific small molecule signaling inhibitors. Previous reports show that actin rearrangement, calcium signaling and PI3-kinase signaling are important for *T. cruzi* invasion (Reddy et al., 2001; Woolsey and Burleigh, 2004; Woolsey et al., 2003). TrkC can mediate each of

these processes (Huang and Reichardt, 2003). K252a, a Trk specific inhibitor that blocks the initial autophosphorylation of the receptors and all downstream signaling, inhibits invasion in NNR5^{TrkC} cells (Fig. 7A). This suggests invasion is dependent on TrkC signaling. A commonly studied pathway activated by TrkC ligation involves Erk 1/2 phosphorylation. Erk 1/2 activation has not been previously defined as important in cellular invasion, but we found that inhibition of Erk 1/2 phosphorylation inhibits invasion (Fig. 7C). This is an interesting result because Erk 1/2 has been shown to regulate actin organization (Han et al., 2007), which is linked to cytoskeletal rearrangement and could therefore participate in the cellular invasion, as my data suggest it does.

4.2 Significance of TrkC as an Invasion Receptor

T. cruzi can invade nearly all nucleated cells. Although Trk receptor expression has been documented on a growing number of cell types (Shibayama and Koizumi, 1996; Vega et al., 2003), it is clear that the complex eukaryote has evolved multiple mechanisms for cell entry. *T. cruzi* invasion of NNR5^{EV} cells (Fig. 5A) is a clear example that Trk receptors are not the only receptors that *T. cruzi* uses to invade cells. However, the *in vivo* experiments in Figs. 5 and 10-12, which I will discuss below, suggest that both TrkA and TrkC play a prominent role in infection in mice. What Trk receptors features may be important for this role? I discuss such features within two groupings: Trk signaling and Trk localization on specific tissues.

An additional way to further examine the role TrkA and TrkC play during murine infection would be to infect mice that lack the genes that code for TrkA and TrkC. Our hypothesis would be that TrkA- and TrkC-deficient mice would be resistant to infection. These experiments could be pursued, although with much difficulty because TrkA and TrkC knockout mice die very early postnatal (all mice die from 1-3 weeks) and have severe defects in their PNS and CNS (Klein, 1994).

4.2a TrkC Receptor Signaling

T. cruzi invasion and intracellular division is arguably a stressful process for the host cell. To counteract this stress, it may be that *T. cruzi* activates TrkC signaling, which includes signaling pathways such as Erk 1/2 and PI3-kinase activation that promote cell survival (Huang and Reichardt, 2003). In support of this idea, data in Fig. 8 show that *T. cruzi*/PDNF protect neurons and glial cells from cell death via TrkC activation (other work showed similar results for TrkA) (Chuenkova and PereiraPerrin, 2004). In stark contrast, imagine the ability of *T. cruzi* to survive and divide intracellularly if during the process of invasion it activated receptors such TNF-α, FAS or p75. These receptors are members of the TNF superfamily of receptors that mediate cell death. p75 is a particularly interesting example because it is a low-affinity receptor for neurotrophins. If neurotrophins bind p75, it is plausible—if not likely—that *T. cruzi* could also engage the death receptor p75. Such *T. cruzi* binding and activation of p75 could serve as an explanation for the relatively large amount of cell death seen in the PNS. However, we have been unable to find evidence that suggests *T. cruzi* binds to p75 (Chuenkova and PereiraPerrin, 2004). Considering these data from the evolutionary perspective of *T. cruzi*, it makes sense that an intracellular organism would not survive long if it activated cell death signaling cascades upon cell entry.

As *T. cruzi* engagement of TrkA and TrkC induces protective signaling, we reasoned that such an action may create an intracellular environment that is also more beneficial to *T. cruzi*. One result from such trophic effects could be increased parasite division. Our results showed that parasites divided more quickly in $NNR5^{TrkA}$ and $NNR5^{TrkC}$ cells compared to $NNR5^{EV}$ cells (Fig. 9). Although we presume that this is dependent on Trk signaling, it is difficult to test experimentally because the initial invasion process also depends on Trk signaling (Fig. 7). To speculate what may

contribute to the increased rate of division, it is key to consider what *T. cruzi* needs to 1) first convert into an amastigote and 2) what amastigotes need to divide. My results show that at 1 day post infection there are an equal number of amastigotes inside $NNR5^{TrkA}$, $NNR5^{TrkC}$ and $NNR5^{EV}$ cells (Fig. 9A). Thus, it seems that the process of conversion into an amastigote and exit into the cytoplasm, although it may be different in the three cells lines, is not contributing to differential parasite division seen when comparing cells that have an established infection. This suggests that the different number of parasites seen by 2 dpi (Fig. 9B) is likely due to the process of division, which starts ~1 day after invasion.

One promising hypothesis of how TrkC signaling results in increased parasite division relates to *T. cruzi* dependence on glucose for both differentiation and division (Engel et al., 1987; Tyler and Engman, 2001). Trk activation induces Akt phosphorylation, which is a signaling pathway known to upregulate cell surface glucose transporters (Jacobs et al., 2008). Therefore, since *T. cruzi* activates TrkA and TrkC, there may be a subsequent upregulation of glucose transporters that would bring more glucose into the intracellular environment, making more glucose available for *T. cruzi* division. This could be experimentally tested by determining whether 1) *T. cruzi* (in $NNR5^{TrkC}$ versus $NNR5^{EV}$ cells) can upregulate glucose transporters; 2) Akt inhibitors can inhibit this process; and 3) depletion of glucose from the media eliminates this response.

Another potentially beneficial aspect of invasion through TrkA or TrkC is that upon ligation, Trk receptors are endocytosed. Other tyrosine kinase receptors, such as EGF, are also endocytosed, but Trk receptor endocytosis differs in important ways that are relevant to *T. cruzi* invasion. Trk endocytosis involves Rac-dependent macroendocytosis whereas EGF depends on clathrin-dependent endocytosis (Valdez et al., 2007). Clathrin-mediated endocytosis results in cellular incorporation of small

vesicles of 100 nm diameter, a diameter that is theoretically far too small to contain a *T. cruzi* parasite. Macroendocytosis, however, is a process that results in membrane ruffling, invagination and pinching to form endosomes 0.5 to 5.0 μm in diameter, a size that is much more compatible with parasite containment (Falcone et al., 2006). Although macroendocytosis often leads to endosome fusion with lysosomes, Trk vesicles are thought to be maintained as an intracellular signaling endosome (not fusing with lysosomes), which creates long-term Erk kinase signaling (Campenot and MacInnis, 2004). Sustained signaling could be beneficial to the parasite, but this point is of interest because *T. cruzi*-containing vesicles need to fuse with lysosomes to promote *T. cruzi* escape into the cytosol where the parasite divides. Thus, although there is at least one point of difference between *T. cruzi* invasion and Trk endocytosis, the mechanism of Trk endocytosis gives Trk receptors a unique ability to mediate cellular invasion. It would be interesting to determine whether *T. cruzi* invasion is dependent on Rac signaling and whether *T. cruzi* and TrkC are located within a single vesicle after invasion.

4.2b TrkC Receptor Localization

The concept of tissue tropism is a complicated issue in Chagas' disease because *T. cruzi* has the ability to invade almost all mammalian cells. What then controls specificity of cellular invasion and/or destruction? Is there any? My data suggest that although any given cell can be infected, *T. cruzi* does show preference. Therefore the issue of tropism can theoretically be applicable, but only to a certain degree. If two identical cells only differ by their expression of TrkC (or TrkA) versus TrkB or no Trk receptor, then the cell that expresses TrkC is at least 2X as likely to be invaded (Fig. 5A). During the course of an infection, *T. cruzi* goes through many rounds of invasion, replication, escape, invasion, et cetera. One can easily imagine the scenario where over

multiple rounds of invasion there would be an exponentially greater number of TrkC-expressing cells that are infected, even though many other cells that don't express TrkC would also be infected.

TrkC is a promising receptor for mediating tissue tropism because it is expressed on cells in disease relevant tissues. TrkC is necessary for the normal development of the heart and the GI tract. One example that is relevant to Chagas' disease is Hirschsprung's disease (HD). HD is a disease of congenital megacolon caused by lack of nervous tissue development in the most distal segments of the colon. Although some reports link this disease with defects in GDNF (glial-derived neurotrophic factor), there are several reports that link Hirschspung's disease with TrkC/NT-3 deficiencies (Fernandez et al., 2009; Ruiz-Ferrer et al., 2008; Sanchez-Mejias et al., 2009). In a related fashion, it may be that *T. cruzi* invades and destroys these same cells that do not develop in HD, with the similar subsequent effect of megacolon development. Of even greater importance than TrkC expression during development is TrkC expression in adult tissue. In adult mammalian tissue, cardiomyocytes express TrkC (but not other Trk receptors), and many cells throughout the PNS and ENS express TrkC as well as TrkA and TrkB.

In addition to the gut and heart, the CNS is another disease-relevant area where TrkC is expressed. The young and the immunosupressed can die of fulminant CNS infection. Many infectious pathogens do not cause brain damage because they cannot gain access to the CNS through the blood brain barrier, which suggests *T. cruzi* can cross the blood brain barrier (BBB), although the mechanisms involved in this process are not clear. CNS endothelial cells are a key cell that contributes to the formation of the BBB, and they express TrkC (Takeo et al., 2003). One possibility is that *T. cruzi* crosses the BBB through invasion of such TrkC-expressing CNS endothelial cells.

4.3 Infection in Mice is Blocked Using TrkC- and TrkA-Specific Antibodies.

Mechanisms of cellular invasion are easier to study and dissect in cultured cells, but to determine whether invasion through TrkC or TrkA contributes to mammalian infection, we determined whether α-TrkC and α-TrkA antibodies inhibit infection in mice. To do this, TrkC- or TrkA-specific antibodies were injected with parasites into the footpad of mice. α-TrkB antibodies or BSA were also used as controls. To allow enough time for cellular invasion and parasite replication, mice were sacrificed after three days and footpad parasitism was quantified by qPCR. Both α-TrkC and α-TrkA antibodies inhibited invasion (Fig. 5E), but TrkB and BSA did not. It appears that TrkA may contribute to infection in the footpad to a greater extent than TrkC because TrkA antibodies block infection to a greater degree compared to TrkC antibodies. This may be surprising because data in Fig. 1A would suggest that *T. cruzi* has greater binding affinity for TrkC compared to TrkA. An obvious interpretation of this is that there may be more TrkA expressing cells within the footpad that are relevant for infection. Candidate Trk expressing cells that may be important for infection could include sensory neurons, Schwann cells, dendritic cells and muscle cells. This is the first experiment that suggests specific invasion through TrkC and TrkA plays a role in infection in mice.

This experiment also suggests that inhibition of invasion via TrkC and TrkA could be a successful strategy for mitigating *T. cruzi* infection in humans. The experimental model we used, with the administration of an inhibiting reagent and parasites together, would not be relevant to prevent human infection. We therefore designed an experiment in which antibodies were injected after infection was established. α-TrkC antibodies were injected into the peritoneum daily for 10 days starting 3 days after initial infection. Mice were sacrificed 18 dpi and colon and heart tissue was analyzed for tissue parasitism.

Compared to daily injections of BSA, α-TrkC antibodies decreased colon and heart parasitism (Supplemental Fig. 3). This experiment only included a total of 5 mice and the numbers are not statistically significant. It will be important to use another antibody (TrkB) or pre-immune IgG as a control if additional experiments are done. However, this is a very promising result for a number of reasons. First, it provides further evidence that TrkC-mediated invasion is important in colon tissue infection. Second, as noted before, cardiomyocytes express TrkC. This result would suggest that TrkC-mediated invasion contributes to infection in heart tissue, which is particularly important because heart disease is the leading cause of death in Chagas' disease patients. Third, this result suggests that the targeted inhibition of invasion through TrkC, even after infection is established, may mitigate disease burden.

4.4 Destruction of TrkC-Expressing Nerve Tissue Cells in the Colon of Mice Infected with *T. cruzi*.

There are two major areas of pathology in mammals infected with *T. cruzi*: the heart and the GI tract. Destruction and fibrosis of the heart muscle, in conjunction with destruction of the segment of the PNS that helps regulate the heart leads to cardiomegaly, the leading cause of death in Chagas' disease. Within the GI tract, death of cells in the enteric nervous system occurs during acute infection and can result in the subsequent development of gut aperistalis and megacolon. My project focused on the colon because 1) colon disease etiology is better defined compared to heart disease, and it is clearly linked to nerve tissue death; 2) TrkC is expressed in the nervous tissue of the colon; and 3) the ENS is large and easily isolated. However, because the majority of labs that study *T. cruzi* infection in mice focus on the heart, I had to do more initial work to define our model and determine the time-course of colon infection. After infecting C57BL/6 mice via subcutaneous inoculation of 5000 parasites (or vehicle for

control mice) in the hind footpad, parasitemia peaked at 11 dpi. Parasite division is thought to start at the initial inoculation site (here, the footpad) then spread to distant organs via the blood. It is likely that parasites enter the blood to infect sites distant the initial infection by 3-4 dpi, undergo another round of infection and division and then re-enter the blood, peaking at 11 dpi. Colon parasitism first reaches detectable levels by 11 dpi, peaks at 18 dpi and decreases linearly to nearly undetectable levels by 32 dpi (Fig. 10A).

Because *T. cruzi* preferentially infects TrkC-expressing cells compared to TrkB-expressing cells (Fig. 5A), we thought there may be a subpopulation of neurons in the CNS—those that express TrkC—which are destroyed. The underlying presumption here is that there is limited overlap in expression of TrkC and TrkB in ENS cells of the colon. Although coexpression of Trk receptors in the colon has not been tested directly, neurons in other areas of the PNS such as the dorsal root ganglia (DRG) neurons have limited overlap in Trk receptor expression; reports find anywhere from 0 to 35% of TrkC cells have either TrkA or TrkB coexpression (Karchewski et al., 1999; Mu et al., 1993; Wright and Snider, 1995).

After determining the course of colon parasitism, we next examined what was occurring with TrkC-expressing cells using both qPCR and immunohistochemistry. qPCR revealed a decrease in housekeeping genes specific for neurons and glial cells in infected mice compared to uninfected controls. Using qPCR to analyze tissue is a valuable strategy because it is less biased than taking images and counting cells and it also allows you to examine a much greater percentage of the total tissue. In addition, tissue analysis is quicker and cheaper. Using primers specific for TrkC and TrkB, we also found a specific decrease in TrkC expression in infected mice compared to uninfected controls. Although a decrease in the levels of housekeeping genes suggest that there is a decreased number of cells that express those specific genes (neurons

and glial cells, here), TrkC and TrkB are not housekeeping genes. Therefore, this qPCR data suggest that *T. cruzi* could either be downregulating TrkC gene expression or that TrkC-expressing cells are dying.

To differentiate between these possibilities, we examined several sections of colon tissue from at least 3 infected and 3 uninfected mice by fluorescent microscopy using TrkC-specific antibodies. The number of TrkC-expressing cells was decreased by 18 dpi, and, by 32 dpi, there was about a 60% loss of TrkC-expressing cells ($p = 0.02$) in the colon of infected mice compared to uninfected controls (Fig. 11D). These data suggest that TrkC cells are dying, which is in agreement with interpretation of the observed decrease in TrkC mRNA being reflective of cell death. In addition, we have not been able find *T. cruzi* down regulating Trk receptors in cultured nerve tissue cells (Caradonna, 2009); on the contrary, *T. cruzi* and PDNF upregulate Trk receptors in CNS nervous tissue and cultured cells (Caradonna, 2009). Because PDNF can regulate Trk gene expression in brain tissue, we took the further step to see if PDNF can regulate gene expression in the ENS. We found that although PDNF was reaching colon tissue after intraperitoneal injections, PDNF did not regulate Trk gene expression (Supplemental Fig. 1). This finding is in agreement with interpreting the specific decrease in TrkC mRNA in infected mice as suggestive of TrkC cell loss.

This is the first such data that suggest *T. cruzi* targets a specific sub-population of nerve tissue cells in the colon for destruction. However, these findings are in agreement with previous experiments. To be clear, although it had not been previously tested, limited evidence supports our findings that TrkC-expressing cells are specifically destroyed during *T. cruzi* infection. According to data from DRG neurons, neurons of the PNS can be divided into three main populations with respect to expression of TrkA, TrkB or TrkC. Within the DRG, those that express TrkC are the largest neurons. TrkA-expressing neurons are the smallest and TrkB expressing neurons are of intermediate in

size (Mu et al., 1993). This is of interest because in the cardiac ganglia of mice infected with *T. cruzi*, there is significant neuron death and the remaining neurons have a smaller than average diameter (Rodrigues et al., 2002). If TrkC neurons are those that are dying (and the cardiac ganglia neuron size to Trk relationships are similar to those in the DRG), then the remaining neurons might be those that have a smaller diameter.

4.5 Mechanisms of Cell Death in the Colon of Mice Infected with *T. cruzi*

Our data suggest that TrkC-expressing cells are specifically destroyed in the colon of mice infected with *T. cruzi* (Figs. 10 and 11). TUNEL staining suggests that cellular apoptosis is at least one mechanism involved with the cellular death seen in the colon of infected mice (Fig. 11C). However, the mechanisms involved with the induction of apoptosis (or other possible forms of cell death) are not clear. In my introduction, I enumerated 4 proposed mechanisms of cell death that are debated to varying degrees in Chagas' disease research: 1) Immunopathology (immune response out of proportion to what is needed to control the parasite); 2) Autoimmunity; 3) Neurotoxin; and 4) Death from parasite invasion and escape. Below I will discuss how, if at all, my data reflect on these proposed mechanisms of cell death.

Cell death in the colon appears to be an ongoing process that starts after 11 dpi and is almost entirely undetectable by 32 dpi (Fig. 11C). Two things are particularly striking about this relatively narrow window of cell death. First, it is interesting that where there were inflammatory infiltrates (like that seen in Fig. 11A, 18 dpi) there were also large numbers of apoptotic cells (Fig. 11C, 18 dpi, red arrows). Second, the window of cell death perfectly correlates with *T. cruzi* presence in the colon (Figs. 10A, 11C and Supp. Fig. 2C), nothing before 11 dpi and everything nearly gone by 32 dpi.

4.5a Immunopathology and Autoimmunity Cell Death Hypotheses

With regards to immunopathology or autoimmunity, our data do not agree with those models of cell death. The caveat is that our experiments are only carried out to 32 dpi. Looking at the window of disease that we have, TUNEL staining found in close proximity to inflammatory infiltrates (Fig. 11C, 18 dpi, red arrows) agrees with the notion that apoptosis could be immune-mediated. CD8 T-cells are important for controlling *T. cruzi* infection (Gutierrez et al., 2009) and could be directly inducing apoptosis of infected cells (or uninfected cells, which would be considered immunopathology). A relevant point to consider is that inflammatory infiltrates are present when colon tissue parasitism is peaking. This supports the notion that the inflammatory response, at least during acute infection, is targeted against *T. cruzi* and not self. In addition, it would be difficult to say that the degree of inflammation is out of proportion to infection because the inflammatory infiltrates appear to peak at 18 dpi and are gone by 32 dpi, which is the exact course of tissue parasitism. qPCR using primers specific for T cells could help quantify this. If inflammation was specific for self or out of proportion to what is needed, one might expect inflammatory infiltrates (and likely continuing cell death) to be present at 32 dpi after parasites have decreased to nearly undetectable levels. To further examine the inflammatory response, the spleen was also removed and weighed during the course of acute infection. Increased spleen size, splenomegaly, can be a marker of infection and inflammation. The spleen size also rises and falls in unison with parasite levels (Supplemental Fig. 2A), further suggesting the immune response is appropriate. As such, it does not appear that these data are in agreement with autoimmunity or immunopathology as a mechanism of cell death during acute infection.

4.5b Invasion-Mediated Cell Death Hypotheses

Our *in vitro* data show that *T. cruzi* preferentially invades TrkC-expressing cells compared to TrkB-expressing cells. In addition, mice appear to have a specific loss of TrkC-expressing cells in their colon compared to TrkB-expressing cells. A simple explanation of this may be that cells are invaded and those cells die, as invaded cultured cells invariably die. *In vivo*, such cell death could be secondary to parasite invasion and escape or secondary to parasite invasion and CD8+ T-cell-induced death. Both mechanisms are similar in that both result in the specific death of an infected cell. However suggestive these data may be, we do not precisely show that only invaded cells are those that are dying. There are additional microscopy stainings that would be useful in addressing these issues: co-staining with TUNEL and TrkC; co-staining with TrkC, *T. cruzi* and TUNEL; staining with a marker for CD8 T-cells. The immune component of disease in the colon was not pursued, but the other experiments were pursued for months to no avail. One problem was initially finding a TrkC antibody functional in immunohistochemistry. Finally, one worked but only under very restrictive tissue processing procedures, which did not work with TUNEL staining. I used human Chagasic serum to stain for intracellular amastigotes, but the background was so high that finding parasites was difficult. A purified antibody specific for amastigotes could be very beneficial in these experiments.

4.5c Neurotoxin-Mediated Cell Death Hypotheses

In the 1960's Franz Koberle completed some of the most thorough autopsies of Chagas' disease victims (Koberle, 1968). From this work he developed the hypothesis that *T. cruzi* secretes a neurotoxin that devastates PNS neurons. The popularity of the hypothesis has waxed and waned, but still remains 50 years later because there is often a paucity of parasites compared to the extensive amount of cellular death. The ability to

detect parasites in the tissue of infected animals and patients has increased (for example, using qPCR), yet it is still an accepted view that there are far fewer infected cells within the nervous system compared to the amount of cell death. As I discussed, I had limited resources, time and ability to detect parasites by microscopy in infected colon tissue, although the qPCR data shows that parasites are clearly there. Thus, I mentioned above, in going forward, parasite detection would be an important tool to develop.

4.5d TrkC-Induced Apoptosis Cell Death Hypotheses

An additional consideration regarding the specific death of TrkC cells in the colon relates to new work that shows TrkC is a dependence receptor (Tauszig-Delamasure et al., 2007). Unlike TrkA and TrkB, under stressful conditions, the intracellular domain of TrkC is cleaved by caspases and contributes to an irreversible cascade that leads to cell death. This could relate to the process of cell death in the colon (and other infected TrkC-expressing tissues) in two ways. First, it could contribute to cell death in an infected cell itself. However, it seems like this would at most simply speed up the process of cell death, not induce a greater amount of death in TrkC cells. Second, considering the interdependent network of neurons and glial cells in the ENS, infection could disrupt homeostasis of the ENS. Even without infection of every TrkC-expressing cell, there could be a sufficient amount of stress to induce the death response mediated by TrkC. One could even imagine that nearby inflammation could be a sufficient stress on cells to contribute to this. This possibility has something akin to the neurotoxin hypothesis because both are ideas that respond to the notion that there are a limited number of infected cells in comparison to the large amount of cell death. In addition, this hypothesis also relates to the idea that disruption of interconnected neural network could greatly affect cells beyond one infected cell. The best example of such a possibility

would be seen with the destruction of glial cells that support neurons: when glial cells die, so too perish neurons.

4.6 TrkC Cell Loss Correlates with Increased Colon size in Mice Infected with *T. cruzi*.

Megacolon is a major complication of Chagas' disease resulting from destruction of nervous tissue in the ENS. Although fulminant megacolon (a surgical bowel with blocked intestines that have ballooned to many times their normal size) only occurs during the chronic stages of the disease, colon disease in Chagas' disease patients is a gradual process (Madrid et al., 2004; Oliveira et al., 1980; Rezende Filho et al., 2005). Constipation is a common complaint in Chagas' disease patients long before megacolon develops (Rezende Filho et al., 2005; Sanchez-Guillen Mdel et al., 2006). Thus, it is not surprising that we found gross changes in the colon (Fig. 12C) when we were initially determining the course of colon parasitism during infection. What is particularly surprising is that others have not described this finding. One explanation for this may be that the parasite and/or mouse strain could give different results. In addition, the majority of *T. cruzi* studies focus on the heart because heart pathology is the leading cause of death in Chagas' disease patients. To quantify the obvious changes in the colon of infected compared to uninfected mice, mice were sacrificed and their colons were weighed. Colon size increased very slightly over the first weeks of infection, but doubled in size by 25 dpi and remained increased a week later (Fig. 12A). This change in size was significant ($p = 0.0001$) and colon size correlates directly with TrkC cell loss, as quantified by loss of TrkC mRNA (Fig. 12B and F).

A strength of these experiments that examine TrkC-expressing cells in the colon of infected mice is the well-defined time-course of events. When are parasites in the colon? When does cell death occur? When does colon size increase? To more easily

compare the course of events and discuss what they suggest, I included previously-shown data without error bars in Supplemental Fig. 2. After initial inoculation of parasites, colon tissue parasitism peaks at 18 dpi, which is when the initial decrease of TrkC mRNA and cell death begins (Sup. Fig. 2C and Fig. 11C and D). Looking at Sup. Fig. 2E, it is clear that the increase in colon size does not occur immediately when parasites peak, but there is a one week delay; colon size increases only after TrkC cells are dying. To contrast this with a marker for systemic inflammation, I also removed the spleen and weighed it to quantify splenomegaly. Splenomegaly peaks at 18 dpi and decreases linearly in a fashion similar to tissue parasitism (Sup. Fig. 2A). This is in contrast to the increased colon size, which increases at a later point and does not appear to decrease even after parasites are cleared from the colon. I raise the point to suggest that the data are in more agreement with increased colon size being secondary to nerve tissue pathology and not secondary to general inflammation. These are the first such experiments to show that *T. cruzi* infection in mice leads to an increased colon size, and it appears that this increase in colon size is secondary to TrkC-specific cell death. To go further it would be nice to see if the colon size stayed increased over a greater length of time; we would predict the size would stay increased unless there is nervous tissue regeneration in mice.

4.7 Model of Colon Pathology During Acute *T. cruzi* Infection

Thus, our model of colon pathology during acute *T. cruzi* infection is that *T. cruzi* preferentially invades TrkC-expressing cells in the ENS of the colon. This leads to the initial specific loss of infected TrkC cells and possibly to the subsequent loss of additional cells that may be dependent on those cells or affected by associated inflammation. The mechanism of cell death includes apoptosis, and, whether cell death is directly induced by parasite burden or by secondary inflammation, both mechanisms

appear to depend on the presence of *T. cruzi*. This is a key point in the field of Chagas' disease research: my data suggest that *T. cruzi* presence is necessary for tissue destruction during acute disease because cell death occurs subsequent to *T. cruzi* presence in the colon and cell death decreases to undetectable levels by the time *T. cruzi* is also below detectable levels. Furthermore, my results raise the intriguing possibility that the beginning stages of megacolon may occur during acute infection and are secondary to TrkC-specific cell death. A key issue our model does not explain, however, is why then would it take 30 years for humans to develop megacolon if the vast majority of tissue damage occurs during acute infection when *T. cruzi* burden is high?

4.8 Model of Chronic Chagas' Disease

Many results, including those shown here, suggest that the majority of tissue damage and cell death in Chagas' disease occurs during acute infection (Rassi et al.). It is not clear then why megacolon develops decades after acute infection. As the parasite is never fully cleared from the GI tract, heart and other organs, it is possible that there is a continuous low-level turnover of cellular infection. Therefore, one possibility for why it takes 30 years to develop symptomatic Chagas' disease is that low-level tissue destruction—possibly of TrkC-expressing cells—needs 30 years to accumulate the amount of damage that results in symptoms. Could blocking invasion via TrkC—even during the chronic stages of disease—decrease the possibility of megacolon development? Another possibility is that as the immune system wanes with age there may eventually be poorer and poorer parasite containment. This could reach a point that sufficient tissue damage accumulates and that is when symptoms appear.

One additional hypothesis relates to the neuron and glial death that occurs naturally with age. Mice, rats, sheep, guinea-pigs and humans lose neurons in their intestines as they age (Gabella, 1987; Phillips and Powley, 2007). Rodent work

suggests this is specific to the cholinergic neurons and the greatest losses occur in the distal segments of the GI tract (Phillips and Powley, 2007). This appears to be a common aspect of aging in mammals and has been linked to peristalsis problems and constipation. As an example of how this relates to Chagasic megacolon, we could theoretically imagine that an average person needs 25% of their neurons in their gut to avoid megacolon. When that person is infected with *T. cruzi*, they may lose ~50% of their neurons. This leads to some minor symptoms and signs. However, after 30 years of additional progressive neuron loss, that number of neurons finally decreases to only 25% of the original. Thus, colon function, which has progressively worsened, is now almost entirely compromised and fulminant megacolon ensues.

It is probable that there is a combination of factors that contribute to megacolon development. Destruction of the ENS during acute infection seems likely to be one of the major factors due to the amount of tissue damage that can occur. As the years pass, it may be that cells of the ENS continue to die with age and with low-level *T. cruzi*-induced tissue destruction. GI tract function continues degrading until finally enough nerve tissue is lost that fulminant disease develops. Considering that TrkC cells can fail to develop in Hirschsprung's disease and TrkC cells are destroyed in Chagas' disease, it may be an important strategy to consider TrkC-specific cell renewal/regeneration experiments. Such experiments could explore whether tissue regeneration is possible in the ENS using resident gut neural progenitor cells (Kawaguchi et al.; Silva et al., 2008), neurotrophin treatment (or PDNF possibly) or stem cell therapy.

CHAPTER 5. REFERENCES

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