Identification and characterization of a novel population of murine colonic Sca-1+ cells and implications in Chagas disease

A thesis

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Abstract

Chagas disease, a chronic degenerative condition caused by the protozoan parasite *Trypanosoma cruzi*, infects an estimated 10 million people worldwide, mostly in Latin America where it is endemic. The disease has two phases, acute and chronic. Although acute infection is characterized by a high parasite burden in the blood and other tissues, parasitism is kept in check by innate and adaptive immunity. However, heavy tissue parasitism coupled with a strong immune response elicits tissue destruction, especially in the heart, colon, and esophagus. Nevertheless, most patients progress from acute infection to the chronic phase without permanent tissue damage or sequela, except for less than 5%, who succumb to fulminating myocarditis and encephalomyelitis. Patients acutely infected with *T cruzi* progress to the chronic indeterminate phase, characterized by extremely low tissue parasitism and absence of symptoms and tissue damage. After many years or decades, about 30% of patients in the indeterminate phase progress to life-threatening pathology in the heart (cardiomegaly) and 10% in the gastrointestinal tract (megaesophagus and megacolon).

The time course of Chagas disease progression led us to hypothesize that *T cruzi* helps the host repair infected tissues by expressing factors such as Parasite Derived Neurotropic Factor (PDNF) that act in concert with endogenous host repair mechanisms. Much attention has been given to the emerging concept that adult stem cells help tissue repair by paracrine signaling, such as through upregulating expression of anti-inflammatory cytokines and growth

factors. In support of our hypothesis, recent results in our lab demonstrate that PDNF stimulates self-renewal and survival of reparative Sca-1+ cardiac progenitor/stem cells (CPCs), and enhances secretion of the anti-inflammatory cytokine TSG-6. However, the heart is by no means the sole organ in Chagas disease to suffer severe insult, and given that specific tissue-resident stem cells are being identified throughout the body, it stands to reason that a similar population of progenitor/stem cell exists in the colon with similar reparative properties. **My project seeks to isolate a novel population of Sca-1+ cells in the colon of naïve mice, characterize the morphology and surface markers, and profile cellular interactions with *T cruzi*.**

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

BDNF – Brain Derived Neurotrophic Factor

CCC – Chronic Chagasic Cardiomyopathy

CNS – Central Nervous System

CD[#] – Cluster of Differentiation molecule [#]

c-Kit (CD117, SCFR) – tyrosine protein kinase Kit (Stem Cell Growth Factor Receptor)

COX-2/PTGS2 – Prostaglandin-endoperoxide synthase 2

CPC – Cardiac Progenitor Cell

CSC – Colonic Sca-1+ Cells

DMEM – Dulbecco's Modified Eagles Medium

FCS – Fetal Calf Serum

GI- Gastro-Intestinal

IL-[#] – Interleukin [#]

ISC – Intestinal Stem Cell

MAPK/ERK – Mitogen-Activated Protein Kinase/Extracellular signal-regulated Kinase

MCP-1 – Monocyte Chemotactic Protein -1

MOI – Moiety of Infection

MSC – Mesenchymal Stem Cell

NGF- Nerve Growth Factor

NT-3 – Neurotrophin 3

p75 – Pan-neurotrophin receptor

PBS – Phosphate Buffered Saline Solution

PI3K/AKT – Phosphoinositide 3-kinase/protein kinase B

Sca-1 –Stem Cell Antigen 1

SDF-1 – Stromal Cell Derived Factor 1

(s)PDNF – (short) Parasite-Derived Neurotrophic Factor

TGF-β - Transforming Growth Factor β

TNF-α - Tumor Necrosis Factor α

Trk – Tropomyosin-related kinase

TS – *Trans*-sialidase

TSG-6 – TNF stimulated gene 6

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Chapter 1: Introduction

1.1 History of Chagas Disease

1.1a. Discovery

In 1909, Brazilian physician Carlos Chagas documented his discovery of a new human disease, which would rightfully come to bear his name. The discovery of Chagas disease marks the first and only time in the history of modern medicine that a single scientist described in great detail both the full clinical picture and complete transmission cycle, including identification of vector, host, reservoir, and novel infectious organism, of the first documented human case of a previously unrecognized infectious disease (Coura & Borges-Pereira, 2010; Rassi, Rassi, & Marin-Neto, 2010).

While working on malaria control in the Brazilian state of Minas Gerais, Chagas encountered a hematophagous triatomine beetle known colloquially as the kissing bug, since it often fed on the face of the local population as they slept (Coura & Borges-Pereira, 2010). Chagas' intimate knowledge of malaria made him acutely aware of the ability of blood-sucking insects to transmit infectious disease. Therefore, he examined the intestinal contents of the bug and found a new species of trypanosomes, which he christened *Trypanasoma cruzi* after his friend, mentor, and director Oswaldo Cruz. Since Chagas lacked the laboratory facilities in the railroad car which doubled as both his living quarters and mobile laboratory, he sent a few infected bugs to Cruz with instructions to let them feed

on naïve marmosets (Coura, Viñas, & Junqueira, 2014). Three weeks later, Cruz observed copious amounts of trypanosomes within the monkeys' blood, prompting him to summon Chagas back to the city to proceed with animal experiments immediately. Within 6 months, Chagas confirmed triatomine vector transmission and establishment of *T cruzi* infection in domestic (guinea pigs and dogs) as well as peridomestic (mice and rabbits) animals known live with or within close proximity to humans throughout brazil(Steverding, 2014). Armed with this novel information on transmission of the parasite in the laboratory, Chagas returned to his post in Mineas Gerais to search for any natural cases in animals or humans. He first located an infected cat who was often seen in and around the local dwellings. Within one of these homes was a febrile two-year-old girl named Berenice with hepatosplenomegaly and unilateral periorbital edema (Miles, 2004). Chagas observed trypanosomes in her blood and made history publishing, under sole authorship, three simultaneous papers detailing the morphology and cyclical development of *T cruzi* in the intermediate (triatomines) and definitive (mammals) hosts, the course of transmission and infection in laboratory animals (domestic and peridomestic), and an in-depth description of the acute form of the disease from the first documented human case (Chagas, 1909a,b,c). In the years to follow, Chagas continued his devoted research on the newly discovered disease.

By 1922 he had identified both a domestic and wild cycle of Chagas disease in nature, described the clinical manifestations of both the acute and chronic

disease, and published complete descriptions of the etiological, pathological, and epidemiological features of the disease. In light of his impressive accomplishments, Chagas was nominated multiple times for the Nobel Prize in Physiology and Medicine. In 1913, Charles Richet won the award over Chagas for his work on anaphylaxis, however, when Chagas was nominated again in 1921, the prize went unawarded that year and it is widely speculated that Chagas was overlooked due to politics and personal jealousies. A few colleagues tried to right this injustice with a nomination for the 1935 Nobel Prize, but Chagas died before the inquiry could be completed (Coura et al., 2014; Miles, 2004). There remains a push today for the first ever awarding of a posthumous Nobel Prize to Carlos Chagas for the complete discovery of a previously unknown human disease.

1.1b Origins

Despite the human history of Chagas disease beginning in 1909, the natural history of the disease has much more ancient origins. Advancements in molecular biology techniques, such as highly sensitive PCR, has allowed paleoparasitologists to definitively prove 9,000-year-old to 12,000-year-old mummies from various areas spanning as far north as the Texas border, to the southern tip of Chile, to be infected with *T cruzi* at the time of their death (Araújo, Jansen, Reinhard, & Ferreira, 2009; L. F. Ferreira et al., 2000). This challenged the classical theory that humans first encountered *T cruzi* about 6,000 years ago after the Andean population began a sedentary life style and domesticated

guinea pigs. Rather, evidence now indicates infection is as old as human presence in the Americas. Paintings dating over about 26,000 years and attributed to the first inhabitants of America, are found in caves with large populations of active triatomine species, providing evidence for very early interaction within human history, (Luiz F. Ferreira, Jansen, & Araújo, 2011)and supporting the theory of infection occurring in prehistory during times of human encroachment on wild cycles of *T cruzi* transmission within triatomine ecosystems (Araújo et al., 2009).

Technological advancements also allowed for the phylogenetic analysis of the trypanosome genus comparing 18S RNA sequences. Using this method, it was determined that salivarian trypanosomes (transmitted through saliva, i.e. *T. brucei*, the causative agent of African Sleeping Sickness) diverged from stercorarian trypanosomes (fecal transmission, i.e. *T. cruzi*, the causative agent of Chagas disease) nearly 100 million years ago when Africa separated from the continent of South America, Antarctica, and Australia (Araújo et al., 2009; Luiz F. Ferreira et al., 2011). This lead to the southern super continent hypothesis, which suggests *T cruzi* evolved in isolation in early terrestrial mammals on the landmass composed of the future South America, Antarctica, and Australia. However, recent findings have discovered that evolutionarily, *T cruzi* is phylogenetically related to a trypanosome only found in bats, resulting in the 'bat seeding' hypothesis for parasite evolution (Hamilton, Teixeira, & Stevens, 2012). To further support this theory, another trypanosome, initially discovered in bats,

was actually determined through phylogenetic relationships to be a previously unknown subgroup of *T cruzi* (Lima, Espinosa-Álvarez, Pinto, et al., 2015; Pinto et al., 2015). In recent months there have been documented cases of this 'new' subgroup, called Tcbat, infecting humas (Lima, Espinosa-Álvarez, Ortiz, et al., 2015).

1.1c Epidemiology

Chagas' disease is becoming the next emerging global public health threat. The World Health Organization estimates approximately 10 million people are infected with *Trypanosoma cruzi*, the majority of them living in the 18 endemic countries of Latin America (Clayton, 2010). The annual incidence is 200,000 cases, with nearly 10,000 deaths per year, and an at risk population of almost 100 million (Moncayo & Silveira, 2009). A high rate of emigration from endemic countries to places all over the world makes Chagas' disease a global concern; over 300,000 people living in the United States are estimated to be infected, and further transmission through blood transfusions and organ transplants is possible (Bern & Montgomery, 2009). In addition to humanitarian concerns, a high financial burden on the global economy is associated with Chagas' disease. A recent computational model estimated medical and disability costs of the disease to be $7.2 billion per year (B. Y. Lee, Bacon, Bottazzi, & Hotez, 2013). No vaccine currently exists against *T cruzi,* and treatment options remain limited, and until recently, thought only efficacious in the acute phase.

1.2 *Trypanosoma cruzi*

1.2a Life Cycle

T cruzi is an obligate intracellular, flagellated, protozoan parasite with a uniquely identifying ‘extra’ piece of circular DNA within the cytoplasm known as a kinetoplast (Araújo et al., 2009). Both insect and mammalian developmental stages are present within the life cycle of the parasitic organism, with replicative and non-replicative forms of each. Bloodstream trypomastigotes from a mammal enter the triatomine vector after a blood meal and travel to the midgut, where they differentiate into epimastigotes capable of replication through binary fission (Coura & Borges-Pereira, 2010). The epimastigotes continue to the hindgut and are then transformed into infectious metacyclic trypomastigotes, which are released into the feces after a subsequent blood meal by the insect. Metacyclic trypomastigotes are highly motile and can enter the mammalian host through mucus membranes or abrasions in the skin (Longo & Bern, 2015). After entry into a host cell, the trypomastigote enters the phagolysosome and alters the pH, rendering the organelle inactive. The sequential differentiation to amastigotes triggered by the acidic environment causes the breakdown of the phagolysosome and permits variable rounds of replication within the cytoplasm of the cell (Miles, 2004). At a certain point, replication is halted and the amastigotes revert back to the non-replicative, highly-motile trypomastigotes which burst from the cell into the surrounding extracellular matrix. These trypomastigotes are now able to

infect adjacent cells or enter the bloodstream where they can travel to other tissues or be ingested by another triatomine vector (Coura et al., 2014).

1.2b Transmission

Chagas disease can be transmitted to over 150 species including humans, domestic animals, and wild animals such as raccoons, possums, marsupials, and armadillos through the feces of reduviid bugs of the subfamily *Triatominae* (Longo & Bern, 2015). Over 130 unique species of triatomines have been identified, however only a handful are competent vectors of *T cruzi*: *Triatoma infestans, Rhodnius prolixus,* and *Triatoma dimidiate* represent the most important vectors for human transmission (Rassi et al., 2010). Both adult sexes of triatomines have five nymphal stages, all of which can harbor and transmit *T cruzi*.

For millions of years, *T cruzi* has been enzootic in the New World, with an established sylvatic/wild transmission system sustaining endemic infection levels in nature (Luiz F. Ferreira et al., 2011). As humans began arriving to the Americas and interacting with triatomine ecosystems, ‘accidental’ human infections triggered an ongoing anthropozoonosis. As human society progressed, a sedentary life style and the need for vast amounts of space caused deforestation and the destruction of both wild life and the forest habitat. Consequently, triatomines that had lost shelter and food sources began colonizing the areas surrounding human dwellings, as well as the dwellings

themselves. Triatomine species adapted nicely to their new niche, finding novel food sources in humans and domesticated animals. This established three intercommunicating transmission cycles still present to this day: the wild/sylvatic cycle that exists in nature between the vector and wild mammal reservoirs such as the armadillo; the peridomestic cycle which involves the infection of animals, such as bats and mice, which tend to live in close proximity to dwellings, allowing for potential interaction between vector and humans; and the domestic cycle, which brings the vector within the human living space, either through pets or the actual nesting of vectors within human dwellings (Coura et al., 2014). In addition to vectoral mechanisms, Chagas disease can be transmitted through blood transfusions and organ transplants, during childbirth, and through ingestion of vector or contaminated food (Rassi et al., 2010).

1.3 Chagas disease progression and pathogenesis

1.3a Acute Phase

Following initial infection with *T cruzi*, a patient enters the acute phase of Chagas disease. Overt signs of infection can be easily identified in this stage depending on the portal of entry: a local induration termed a Chagoma forms when the parasite enters the bite wound or similar abrasion, while entry through the periorbital membrane creates a unilateral periorbital edema called Romaña's sign (Longo & Bern, 2015; Rassi et al., 2010). Although most patients infected will experience no symptoms at all, a febrile, flu-like illness, including chills, nausea, hepatosplenomegaly, and enlarged lymph nodes, is common after an incubation

period of one to two weeks. Severe complications of acute infection are rare, but include myocarditis and meningoenchphalitis, which could lead to death (Coura & Borges-Pereira, 2010). Use of trypanosomal chemotherapies such as Benznidazole and Nifurtimox leads to complete parasite clearance and cures around 80% of patients, however parasitism levels lower significantly and symptoms resolve without treatment in 4 to 8 weeks (Steverding, 2014).

Acute infection is characterized by robust parasitemia and tissue parasitism throughout the body such as the heart, GI, and the CNS. Control of *T cruzi* is critical for host survival and depends on both innate and acquired immune responses (Fabiana S. Machado et al., 2012). Activation of innate cells such as macrophages, NK cells, or neutrophils through *T cruzi* protein interactions with TLRs, leads to induction of pro-inflammatory cytokines IL-12, IFN-γ, and TNF-α. Specifically, IL-12 production by *T cruzi*-stimulated macrophages leads to IFN-γ production through the activation of NK cells as well as the induction of CD4+ Th1 cells (Kayama & Takeda, 2010). Subsequently, IFN-γ, with TNF-α as a secondary signal, activates the infected macrophage to produce nitric oxide, which has direct trypanocidal activity, in a paracrine manner (F. S. Machado et al., 2000). In this way, organ and tissue damage can be caused by both the parasite itself, as well as the immune-inflammatory response of the host to the presence of the parasite. Therefore, an opposing anti-inflammatory/regulatory, Th1-inhibiting response, including IL-10 and TGF-b is vital to host survival, as demonstrated by the development of an endotoxin shock syndrome in IL-10

knock out mice infected with *T cruzi* (Hölscher et al., 2000). Increased IFN-γ production and parasite killing observed after neutralization of endogenous IL-10 lead to the hypothesis of IL-10 as a potent inhibitor of IFN-γ during murine *T cruzi* infection (Cardillo, Voltarelli, Reed, & Silva, 1996). Evidence suggests that a balance must exist between Th1 pro-inflammatory and Th2 anti-inflammatory/regulatory cytokines, specifically IFN-γ and IL-10, in order to both control infection and ensure survival during the acute phase of *T cruzi* infection.

1.3b Chronic Phase

Despite the spontaneous resolution of acute infection, all patients not treated with anti-trypanosomals (and 20% that do receive treatment), enter the chronic phase and remain infected for life (Longo & Bern, 2015). Most patients are asymptomatic and considered to have indeterminate Chagas disease, which is characterized by low parasitemia, positive serum antibodies, and normal ECG and radiology exams of the chest, esophagus, and colon (Coura & Borges-Pereira, 2010). Most patients will never develop a clinically relevant disease, but remain in the asymptomatic indeterminate phase for life. However, 30-40% of infected individuals will progress to the symptomatic, determinate phase of Chagas disease, years to decades after initial infection. Chronic symptomatic Chagas disease manifests as megasyndromes of the heart (chronic Chagasic cardiomyopathy) and GI tract (megacolon and megaesophagus) (Rassi et al., 2010). Although the use of anti-trypanosomals in the chronic phase is becoming

more appreciated and encouraged, treatment in the chronic phase is usually limited to palliative care and alleviation of symptoms (Steverding, 2014)

Although the exact pathogenesis of chronic Chagas disease has yet to be fully elucidated, the balance between immune-mediated parasite containment and damage to host tissues through inflammation, demonstrated to be critical in survival of the acute phase, is thought to determine progression and severity in disease. It is thought that persistence of parasites is necessary for development of chronic disease, however whether the most important determinant is tissue damage elicited directly from parasite factors, or indirectly triggered through parasite-driven immunopathology or even autoimmune mechanisms is unclear (Rassi et al., 2010). Studies have shown the involvement of adaptive immunity in the chronic form of Chagas disease, including both humoral and cell-mediated immunity. Patients with chronic disease have both diagnostic and effector antibodies within their sera, indicative of a robust B cell response (Gazzinelli, Pereira, Romanha, Gazzinelli, & Brener, 1991; Fabiana S. Machado et al., 2012). Additionally, both CD4+ and CD8+ T cells have been observed to be necessary for keeping parasite replication in check during the chronic phase (Longo & Bern, 2015). CD4+ T cells have been identified as major cytokine producers during chronic infection, with the cytokine type being predominantly expressed correlating with disease. For example, patients with chronic Chagas heart disease have CD4 cells shown to produce pro-inflammatory cytokines such as IFN-γ, while patients clinically in the indeterminate phase secreted anti-inflammatory IL-10 (Fabiana S. Machado et al., 2012)

1.4 Chronic disease manifestations and pathophysiology

1.4a Chronic Chagasic Cardiomyopathy (CCC)

Around 30% of patients will develop chronic heart pathology, which is the most frequent and severe chronic manifestation. CCC develops many years or even decades after initial infection and is characterized by combined dilated and hypertrophic cardiomyopathy, resulting in an enlarged heart (cardiomegaly) (Longo & Bern, 2015). Sudden death can occur from arrhythmias caused by conduction defects and thromboembolic phenomena originating from a characteristic ventricular apical aneurysm (Coura et al., 2014). As the heart enlarges, congestive heart failure can develop, resulting in a very poor prognosis. Heart transplant is the only cure for late stage heart failure in CCC, but is not a practical therapy because of the financial burden and difficulty in securing appropriate hearts.

1.4b Gastro-Intestinal Megasyndromes

A striking neuronal depopulation through destruction of intramural autonomic ganglia in the colon, esophagus, or both, cause the chronic megasyndromes of the GI tract in chronic Chagas disease. Peristalsis of the bowels progressively worsens and the GI sphincters begin to lose function, causing the areas directly proximal to balloon due to retention of contents, thus creating an enlarged “mega” organ (de Oliveira, Troncon, Dantas, & Menghelli, 1998; Matsuda, Miller, & Evora, 2009). Patients with megaesophagus suffer from dysphagia,

odynophagia, regurgitation, aspiration, and epigastric pain, which can lead to malnutrition in severe cases (de Oliveira et al., 1998; Rassi et al., 2010). Manifestations of megacolon include prolonged constipation, abdominal distention, and occasional fecaloma or volvulus which can cause large bowl obstruction (Longo & Bern, 2015). Besides the esophagus and colon, chronic GI megasyndromes have been reported in the stomach, small intestine, gall bladder, and bile ducts. Additionally, chronic manifestations can include GI motor disorders such as achalasia of the cardia, disturbances in gastric emptying, and altered intestinal transit (Coura, 2007; Matsuda et al., 2009).

Chronic Chagas GI disorders are much less common than CCC, with only about 10% of patients affected, predominately in the Southern Cone: Argentina, Bolivia, Chile, Paraguay, Southern Peru, Uruguay, and parts of Brazil (Longo & Bern, 2015). Current treatment is palliative in nature and surgical intervention is often necessary for late stage megasyndromes. GI syndromes are characterized by a destruction of the nervous plexus with impairment of both excitatory and inhibitory neurons, pointing to a clear neurogenic pathophysiology in this form of chronic Chagas disease.

1.5 Parasite-Derived Neurotrophic Factor (PDNF)

1.5a Discovery and Expression

In 1983, Mercio Perrin discovered a developmentally-regulated *T cruzi* neuraminidase (TCNA), abundantly expressed on the infective (trypomastigote)

form of *T cruzi*, that can remove terminal sialyl residues from oligosaccharide moieties creating an epitope for binding of the plant lectin peanut agglutinin and was suggested to be important for the pathogenicity of the parasite (Pereira, 1983). In addition to its neuraminadase activity, TCNA also catalyzed the transfer of sialic acids to terminal galactosyl units of glycoproteins on the surface of the protein, thus the enzyme was renamed trans-sialidase (TS) (Schenkman, Pontes de Carvalho, & Nussenzweig, 1992). Most recently, its role as a parasite-derived neurotrophic factor (PDNF) was established (section 1.5c), and therefore, this acronym will be exclusively used moving forward.

PDNF is detected on almost 100% of trypomastigotes prior to, and immediately following, the release of parasites from infected cells (Rosenberg, Prioli, Mejia, & Pereira, 1991). However, once present in the extracellular compartment, the amount of PDNF+ trypanosomes drops to approximately 20%, which remain the most infective population (Pereira, Zhang, Gong, Herrera, & Ming, 1996). This dynamic expression of PDNF means isolation of a “pure” population of PDNF+ is not possible. In its native form, PDNF belongs to a group of over 14,000 TS proteins with overlapping homology and function, known as the trans-sialidase family, making laboratory generated knock downs of the protein not experimentally feasible (Campetella, Sánchez, Cazzulo, & Frasch, 1992; El-Sayed et al., 2005).

PDNF consists of an N-terminal catalytic domain, connected through a lectin-like region to a C-terminus with a variable number of 12 amino acid repeats in tandem (long tandem repeat, LTR) (M. Chuenkova, Pereira, & Taylor, 1999; Pereira, 1983). Protein stability, as determined by neuraminidase/trans-sialidase activity, is dependent on the length of the tandem repeat, which retains PDNF in the serum for up to 35 hours (Buscaglia, Alfonso, Campetella, & Frasch, 1999), allowing ample time for interaction with any number of host cells and molecules.

1.5b Virulence factor

Increased PDNF expression and activity on highly pathogenic *T cruzi* type II isolates as compared to the more innocuous, sylvatic type I strains, suggests a direct link between PDNF and parasite virulence (Risso et al., 2004). Accordingly, several functions of PDNF have been identified that mediate interactions between the parasite and host cells and dampen host cell immunity (dC-Rubin & Schenkman, 2012). PDNF reportedly induces thrombocytopenia via desialylation of platelet membranes (Tribulatti, Mucci, Van Rooijen, Leguizamón, & Campetella, 2005), yet also triggers apoptosis in thymocytes and mature T cells (Mucci, Risso, Leguizamón, Frasch, & Campetella, 2006), demonstrating the multi-factorial role that PDNF enzymatic activity plays in maintaining infection. Independent of its enzymatic activity, PDNF disrupts complement-mediated lysis through inhibition of complement factor B binding to C3b on the surface of the trypanosome (Beucher & Norris, 2008; Norris, Bradt, Cooper, & So, 1991).

PDNF has also been implicated in attachment and invasion of host cells during infection. Through the TS enzymatic activity of PDNF, sialylated mucins on the outer surface of the parasite mediate attachment to host cells and evasion of the immune system by masking parasite epitopes (Pereira-Chioccola et al., 2000). Additionally, PDNF can function through direct, non-enzymatic protein-protein interactions, such as through binding the neurotrophin receptors TrkA and TrkC, but not TrkB (Marina V. Chuenkova & PereiraPerrin, 2004; Weinkauf & Pereiraperrin, 2009). Binding to these receptors results in parasite internalization into the expressing cell, as implicated by the inhibition of cellular infection *in vitro* and decrease of parasitism *in vivo* after Trk receptor blockade (de Melo-Jorge & PereiraPerrin, 2007; Weinkauf, Salvador, & Pereiraperrin, 2011)

1.5c Neurotrophic factor

In addition to invasion, PDNF also activates TrkA and TrkC with neurotrophic consequences. It was initially suggested that PDNF was a neurotoxin responsible for the destruction of neurons observed in chronic disease, given its nature as a virulence factor. However, quite paradoxically, PDNF prevented degeneration and promoted neural cell survival through activation of the pro-survival PI3K signaling pathway and its downstream signaling component Akt, to subsequently enhance transcription of the anti-apoptotic factor Bcl-2 (M. V. Chuenkova, Furnari, Cavenee, & Pereira, 2001; M. V. Chuenkova & Pereira, 2000). Because these properties were very similar to those seen by the prototypical neurotrophin NGF, PDNF was identified as a Trk receptor ligand.

Although there are three members of the Trk family, PDNF has been demonstrated to bind and activate only TrkA and TrkC. These receptors are widely expressed on neural cells such as neurons and glial cells, as well as non-neural cells like cardiac fibroblasts, cardiomyocytes, epithelial cells, and dendritic cells (Marina V. Chuenkova & PereiraPerrin, 2004; Weinkauf & Pereiraperrin, 2009). Activation of the Trk downstream signaling pathways PI3K/Akt and Ras/MAPK/Erk by PDNF led to enhanced survival, resistance to oxidative stress, and differentiation of different cell types such as neurons, glial cells, and cardiac cells (Aridgides, Salvador, & PereiraPerrin, 2013b; Marina V. Chuenkova & Pereira, 2003; Marina V. Chuenkova & PereiraPerrin, 2004; Marina V. Chuenkova & Pereiraperrin, 2011). Given the similarities in Trk receptor signaling between endogenous neurotrophins and PDNF, the parasite-derived molecule is often referred to as an NGF or NT-3 mimic.

Recent studies in our lab show that PDNF differentially stimulates cardiac myocytes and fibroblast cells in infection, confers protection against oxidative stress, and upregulates the chemokine MCP-1 (Aridgides, Salvador, & PereiraPerrin, 2013a; Aridgides et al., 2013b). Recent unpublished results also show that PDNF activates cardiac stem cells (Salvador, Aridgides and PereiraPerrin, in preparation). These new findings raise the possibility that *T. cruzi*, via PDNF, facilitates repair of infected tissues in the heart by expanding and activating resident progenitor/stem cells.

1.6 Stem Cells

1.6a Definition and Classifications

By definition, stem cells are undifferentiated cells with the capacity for self renewal and the ability to differentiate into multiple cell types upon proper stimulation (Deans & Moseley, 2000). This supposed indefinite self renewal distinguishes stem cells from all other types of dividing cells in mammals (Fuchs & Chen, 2012). For classification purposes, stem cells are broadly broken into two categories: embryonic stem cells isolated from the inner cell mass of blastocysts (Thomson et al., 1998), and somatic, tissue-resident stem cells found in various adult tissues (Kobolak, Dinnyes, Memic, Khademhosseini, & Mobasheri, 2015). Stem cells are then further classified by their potency, or multidifferentiative potential. Embryonic cells have the ability to differentiate into embryonic and extraembryonic cell types, which can propagate a viable organism, and are therefore the only stem cells with totipotency (Thomson et al., 1998). Adult, tissue resident stem cells have a more limited differentiation potential and are considered multipotent, oligopotent, or unipotent, depending on the diversity of germ layers and types of cells generated during differentiation (Easterbrook, Fidanza, & Forrester, 2016; Sancho, Cremona, & Behrens, 2015; Visweswaran et al., 2015).

With advancements in molecular biology techniques, a cellular profile of putative surface markers has become the accepted determinant for classification and identification as a stem cell population. Although a fair amount of dissent exists in

the literature, significant effort has been put into developing a definitive stem cell surface marker signature. A large portion of adult, tissue-resident stem cells fall under classification as Mesenchymal Stem Cells (MSCs) with CD271, CD44, and CD117/cKit as typical markers (Álvarez-Viejo, Menéndez-Menéndez, & Otero-Hernández, 2015; Kobolak et al., 2015). Other markers, such as Lgr5 and Bmi1 have been demonstrated as markers of specific subsets of intestinal crypt forming stem cells (Yan et al., 2012). Sca-1 was initially thought to be found exclusively on hematopoietic precursor cells, but recent studies have found subpopulations of stem/progenitor Sca-1+ cells within tissues of the heart, kidney, lungs, and bladder (Hishikawa et al., 2015; Hittinger et al., 2013; Lilly et al., 2015; H. Wang et al., 2014).

1.6b Mesenchymal Stem Cells (MSCs)

Morphologically fibroblast-like and adherent in culture, MSCs have been identified in a wide variety of adult tissues and suggested to be involved in tissue maintenance and repair, regulation of hematopoiesis, and immunomodulatory host responses (Kobolak et al., 2015; Q. Wang, Ding, & Xu, 2016). Lineage studies have determined MSCs are pluripotent with the ability to differentiate into osteoblasts, chondrocytes, and adipocytes (Jiang et al., 2002; Pittenger et al., 1999). Various studies have elucidated anti-apoptotic (Li, Zhang, & Wang, 2008), anti-fibrotic (Mias et al., 2009), and pro-angiogenic (Sorrell, Baber, & Caplan, 2009) functions of MSCs, in addition to the widely noted immunomodulatory properties (Q. Wang et al., 2016). The pro-inflammatory cytokine IFN-γ, in

tandem with another inflammatory signal from TNF-α, IL-1α, or IL-1β, directly triggers production of nitric oxide (NO) from MSCs, which suppresses responsiveness of T cells within the immediate environment (Ren et al., 2008). It has also been demonstrated that MSCs possess and signal through TLR receptors, which is thought to promote self renewal (Pevsner-Fischer et al., 2007)

1.6c Intestinal Stem Cells (ISCs)

As the field of adult tissue resident stem cells grows, more and more functionally specific stem cells are being identified in various organs. In the intestines, the two distinct clonogenic precursors for crypt development have been identified and extensively studied in culture. These cells were given the names crypt-based columnar cells (CBCs) and +4 cells based on their appearance and position within the crypt (Sato & Clevers, 2013; Yan et al., 2012). CBCs are characterized by the marker Lgr5, which is a Wnt target gene, and are highly active cells that persist for life, while their progeny include all differentiated cell linages of the epithelium (Sato et al., 2009). Bmi1 is the surface marker of +4 cells, which are quiescent cells found in close proximity to Paneth cells in the upper crypt and are resistant to radiation (Yan et al., 2012). Both Notch and Wnt signaling pathways are required for growth of these stem cells, with most groups using the ligands R-spondin and noggin (Sancho et al., 2015; Sato & Clevers, 2013; Yan et al., 2012).

1.6d Cardiac Progenitor Cells (CPCs)

CPCs exist in various subsets characterized by the expression of several stem cell markers, the most prominent being Sca-1 and c-Kit (CD117) (Valente, Nascimento, Cumano, & Pinto-do-Ó, 2014). CPCs are capable of producing substantial amounts of paracrine molecules, such as stromal-cell derived factor-1α (SDF-1), which activates CXCR4 and mediates acute cardioprotection through the Jak2/STAT3 signaling, observed to improve cell growth and inhibit apoptosis (Huang et al., 2011). In recent years, our lab has demonstrated the Sca-1+ population of CPCs to have impressive immunomodulatory effects both *in vivo* and *in vitro*, with implications in Chagas disease. sPDNF was demonstrated to potently enhance proliferation and trigger secretion of anti-inflammatory cytokines such as TSG-6. In a chronic model of Chagas heart disease, sPDNF was shown to reduce fibrosis and ameliorate disease severity (Salvador, 2014).

1.7 Summary and Goals

Chronic Chagas disease is preceded by acute infection, characterized by high parasitemia and parasitism of most tissues such as the heart, GI tract, liver, pancreas, skin, and central nervous system. Intense tissue parasitism elicits innate and adaptive immunity that results in tissue destruction. However, tissue injury is repaired and no sequelae remains after tissue parasitism subsides, yet the disease progresses to the chronic phase. The potential extent of tissue destruction in acute infection is indicated by the fact that a small proportion of acutely infected patients

(less than 5%) cannot cope with tissue injury, and die of acute myocarditis and encephalomyelitis.

The observation that acutely *T cruzi*-infected tissues recover without permanent tissue damage led us to hypothesize that *T cruzi* interacts with the mammalian host to promote tissue regeneration and prolong the life span of both the infected host and the invading protozoan parasite.

Our lab has identified several molecules that can potentially promote tissue healing. The most studied of which is parasite-derived neurotrophic factor (PDNF), identified in our lab as a neuraminidase. Earlier studies in the lab showed that PDNF mimics the neurotrophins nerve growth factor (NGF) and neurotrophin-3 (NT-3) in that PDNF binds and activates NGF and NT-3 receptors TrkA and TrkC (Marina V. Chuenkova & PereiraPerrin, 2004; de Melo-Jorge & PereiraPerrin, 2007; Weinkauf & Pereiraperrin, 2009; Weinkauf et al., 2011). As a result, PDNF binding to TrkA and/or TrkC triggers cell survival mechanisms, including protection of cardiomyocytes against oxidative stress (Aridgides et al., 2013b).

More recently, studies in our lab performed by Ryan Salvador demonstrate that PDNF promotes expansion and survival of cardiac progenitor/stem cells (CPCs). **This finding further supports the hypothesis that PDNF is an agent employed by *T cruzi* to help repair acutely infected host tissues**. However, the heart is by no means the sole organ in Chagas disease to suffer severe insult, and given that

specific tissue-resident stem cells are being identified throughout the body, it stands to reason that a similar population of progenitor/stem cell exists in the colon with similar reparative properties. **My project seeks to isolate a novel population of Sca-1+ cells in the colon of naïve mice, characterize the morphology and surface markers, and profile cellular interactions with *T cruzi*.**

Chapter 2: Materials and Methods

2.1 Mice

All mice were female C57BL/6 (stock number: 000664) aged 6-8 weeks purchased from Jackson Laboratories (Bar Harbor, ME). Mouse work was approved by the Institutional Animal Care and Use Committee and Department of Laboratory Animal Medicine of Tufts University School of Medicine (Protocol B2013-28).

2.2 Primary cell isolation

2.2a Cardiac progenitor cells (CPCs)

Primary CPCs were isolated by positive selection based on the expression of the Sca-1 marker, as previously described (Salvador, 2014). In short, freshly harvested murine hearts were subjected to a digestion solution (Hank's Balanced Salt Solution with Type II Collagenase and DNase), gentleMACS^{TM} Dissociator (Miltenyi Biotec), and Red Blood Cell Lysis Buffer in order to create a single-cell suspension, in accordance with the protocol available from Miltenyi. An anti-Sca-1 microbead kit (#130-092-529, Miltenyi Biotec) was applied to the murine single-cell cardiac suspension to isolate stem cells by positive selection: Cells were magnetically immunolabeled with subsequent incubations of anti-Sca-1-FITC and anti-FITC microbeads prior to separation and elution using a magnetic column (MACS Column and Separator). CPCs were maintained in CPC medium containing DMEM (66.2%), IMDM (8.1%), Ham's F-12 (16.2%), FCS (5.9%)

horse serum (1.25%), B27 supplement (0.5%), 1.75mM Glutamax, penicillin/streptomycin (1X), 0.025 mM NEAA, 2.25 mM Sodium pyruvate, basic fibroblast growth factor (FGF, R&D 3139-FB/CF, 20ng/ml), epidermal growth factor (EGF, R&D 2028-EG, 6.25 ng/ml), thrombin (R&D, 2196-SE, 0.25U/ml), cardiotrophin-1 (R&D, 438-CT/CF, 1ng/ml), insulin (Sigma, 0.25μg/ml), and 0.025 mM β–mercaptoethanol.

2.2b Colonic Sca-1+ Cells (CSCs)

Freshly harvested colons were harvested from naïve mice and flushed with cold PBS to removed all fecal matter. The colon was then fileted open, rinsed twice in PBS and cut into small pieces. A single cell suspension was then created using a protocol modified slightly from the cardiac single cell suspension used in section 2.2a: the digestion media contained Collagenase types I&II and twice the amount of DNase, incubation time for digestion was extended to 1 hour, and RBC lysis buffer was not used. Magnetic immunolabeling for isolation of Sca-1+ cells within the colonic cell suspension proceeded identically to CPC isolation.

2.3 Basal Expression of surface markers

Primary CSCs were seeded in triplicate at 2.5 x 10^5 cells per well of a 6 well plate in CPC media, allowed to adhere overnight, and serum deprived in DMEM/0.5% FBS for 5 hours. Following deprivation, cell monolayers were rinsed with PBS and collected in Trizol for qPCR analysis.

2.4 qPCR analysis

RNA was isolated from Qiazol™ (Trizol, Qiagen) lysates of cellular monolayers or liquid nitrogen snap-frozen colon tissue samples dissociated by a Tissue-Tearor mechanical homogenizer (Biospec Products, Inc.) in the usual manner, and cDNA was synthesized using the Quantitect Reverse Transcription kit (Qiagen) according to manufacturer's instructions. Gene transcripts were amplified using specific primers and normalized to the housekeeping gene HPRT, using SYBR Green (Qiagen), and, if needed, expressed relative to control samples (Livak & Schmittgen, 2001). Primers used are summarized in Table 1.

2.5 PDNF purification

PDNF was cloned from the *T cruzi* Silvio X-10/4 strain (GenBank accession number AJ002174), and an N-terminal short form of PDNF (sPDNF) that contains Trk-binding sites and a 6-His-tag was expressed in BL21(DE3) bacteria by IPTG (isopropyl β–D-1-thiogalactopyranoside) induction, and purified by Ni++-affinity chromatography, according to published procedures (M. Chuenkova et al., 1999). sPDNF purity was assessed by SDS-PAGE, where it migrates at 68 kDa. If sPDNF was pure, it was quantified by densitometry after staining with Coomassie blue, otherwise it was re-purified. sPDNF was filter-sterilized through 0.22μm pores and kept at 4 degrees Celsius in PBS.

2.6 Cumulative Growth

CSCs were plated in triplicate at a density of $1x10^5$ cells per well of a six well plate. For experiments involving sPDNF two groups were plated: the control (Veh-co) group received PBS, while the other was given 2μg/ml sPDNF each time the media was changed. At confluency, prior to passaging, each well was counted individually and recorded for future calculations of cumulative growth. Cells were replated at the above density and the process was repeated for as many passages the culture was viable.

2.7 sPDNF stimulation

Primary CSCs were seeded in triplicate at 2.5×10^5 cells per well of a 6 well plate in CPC media and allowed to adhere overnight. Cultures were serum starved in DMEM/0.5% FBS for 2 hours and treated with 2μg sPDNF for 3 hours. Following stimulation, cell monolayers were rinsed with PBS and collected in Trizol for qPCR analysis.

2.8 sPDNF administration *in vivo*

sPDNF was diluted in 200μl sterile endotoxin-free PBS and injected via the tail vein into 6-8 week old female C57BL/6 mice at a concentration of 75μg. For the time course experiment, mice were given a single dose of sPDNF or PBS and sacrificed at 1.5, 3, 5, 8 and 24 hours after injection. For the investigation of cytokine expression, mice were injected three times with sPDNF or PBS vehicle control at times 0, 3 hrs, and 24 hrs, and were sacrificed 24 hours after the final

injection. After sacrifice in both experimental designs, mice were perfused by intracardiac injection of 5ml ice cold PBS and the colon was harvested and flushed with PBS to remove feces. Colons were flash frozen in liquid nitrogen, and stored at -80 degrees Celsius prior to processing by qPCR (time course) or used immediately for isolation of primary CSCs (cytokine expression).

2.9 *T cruzi* culture *in vitro*

Infection experiments were performed with *T cruzi* Tulahuen strains, which were propagated in Vero cells. This strain has been used in *in vitro* studies of adhesion and cell invasion and are often used *in vivo* for the ability to cause both acute and chronic infection in mice. Free swimming infective trypomastigotes were harvested from Vero cell supernatants (5-7 days) by initial low speed centrifugation (500 x g, 5 min) to remove host cells and debris. Parasites are then pelleted by high speed centrifugation (1,200 x g, 15 min), and resuspended in DMEM/0.1% FCS to be used in *in vitro* experiments.

2.10 *T cruzi* Infection

Primary CSCs were seeded in groups of four at 2.5 x 10^3 cells per well in a 96 well plate in DMEM/10% FBS and allowed to adhere overnight. Medium was changed to DMEM/1% FCS and cells were infected with *T cruzi* Tulahuen strain at MOIs (multiplicity of infection) of 0, 1, 5, 10, and 20 for 3 hours, at which time parasites were rinsed away and cells were incubated in DMEM/0.1% FBS. After 2 and 3 days, the infection was stopped and cellular monolayers were stained

with Diff-Quick and number of infected cells were counted using an Olympus IX70 microscope and uninfected cell size in infected monolayers was measured with the SPOT program.

2.11 qPCR primers

Table 2.1 qPCR Primer Sequence

Gene	Forward Primer	Reverse Primer
Lineage Markers		
CD4	CTTCGCAGTTTGATCGTTTTGAT	CCGGACTGAAGGTCACTTTGA
CD8	AAGAAAATGGACGCCGAACTT	AAGCCATATAGACAACGAAGGTG
CD11b	GGCTCCGGTAGCATCAACAA	ATCTTGGGCTAGGGTTTCTCT
Stem Cell Markers		
Sca-1	TCCTGGGTACTAAGGTCAACG	CTCCATTGGGAACTGCTACATT
CD29	ATGCCAAATCTTGCGGAGAAT	TTTGCTGCGATTGGTGACATT
CD34	ATCCCCATCAGTTCCTACCAAT	TGGTGTGGTCTTACTGCTGTC
CD44	TCGATTTGAATGTAACCTGCCG	CAGTCCGGGAGATACTGTAGC
CD90/Thy1	TGCTCTCAGTCTTGCAGGTG	TGGATGGAGTTATCCTTGGTGTT
CD105	CCCTCTGCCCATTACCCTG	GTAAACGTCACCTCACCCCTT
CD117/cKit	GGCCTCACGAGTTCTATTTACG	GGGGAGAGATTTCCCATCACAC
CD133	CTCCCATCAGTGGATAGAGAACT	ATACCCCCTTTTGACGAGGCT
CD271/p75	CCTATGGCTACTACCAGGACGAG	CACAACCACAGCAGCCAAGAT
Lgr5	CCTACTCGAAGACTTACCCAG	GCATTGGGGTGAATGATAGCA
Bmi1	ATCCCCACTTAATGTGTGTCCT	CTTGCTGGTCTCCAAGTAACG
Lrig1	TTGAGGACTTGACGAATCTGC	CTTGTTGTGCTGCAAAAAGAGAG
Cytokines		
TSG-6	CGTCTCGCAACCTACAAGCA	GGTATCCGACTCTACCCTTGG
IL-1Ra	GCTCATTGCTGGGTACTTACAA	CCAGACTTGGCACAAGACAGG
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGC
IDO	CAAAGCAATCCCCACTGTATCC	ACAAAGTCACGCATCCTCTTAAA
COX-2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
IL-22	TTTCCTGACCAAACTCAGCA	TCTGAATGTTCTGGTCGTCA
Trk Receptors		
TrkA	CGCTGAGTGCTACAACCTTC	GAAAGTCCTGCCGAGCATTC
TrkB	AGCCCTGGTATCAGCTATCG	GGGTGTGGATGCTCTTGATG
TrkC	TACCTGGCTTCCCAGCACTTTG	GTGTCCTCCCACCCTGTAGTAATC
Housekeeping		
HPRT	CAGCGTCGTGATTAGCGATGATG	CGAGCAAGTCTTTCAGTCCTGTC

Chapter 3: Results

3.1 Isolation and characterization of colonic Sca-1+ cells

3.1a Novel Sca-1+ cell population isolated from murine colon

Tissue-resident, adult stem and progenitor cells have been identified in virtually every tissue type in the body (Deans & Moseley, 2000) . Previous work in our lab has focused on a Sca-1+ subset of cardiac progenitor cells (CPCs) with potential immunomodulatory and tissue repair properties. Besides the heart, the Sca-1 marker has been used to identify and characterize stem/progenitor cell subsets in lung, kidney, adipose tissue, and most recently the bladder (Chen et al., 2015; Hishikawa et al., 2015; Hittinger et al., 2013; Lilly et al., 2015; Valente et al., 2014). Given that chronic Chagas disease causes pathology of the GI tract in addition to the heart, we reasoned that a population of Sca-1+ cells with stem/progenitor cell properties reside within the colon of mice.

With slight modifications to the CPC isolation protocol for efficient digestion of colonic tissue, I successfully isolated Sca-1+ cells from the colon of naïve mice (**Fig. 3.1A**), which we have termed colonic Sca-1+ cells (CSCs). Upon observation, this population can be described as a heterogeneous mixture of adherent fibroblast-like cells. When juxtaposed with CPCs in culture, CSCs are morphologically similar and practically indistinguishable at the initial plating (**Fig. 3.1**)

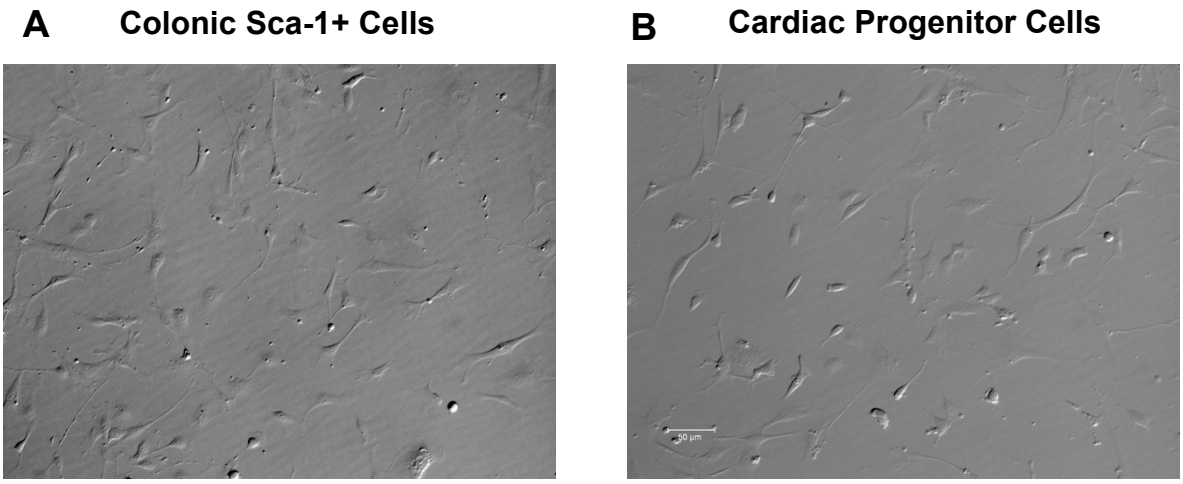

Fig. 3.1 Sca-1+ cells isolated from the colon look similar in morphology to known Sca-1+ CPCs in the heart of naïve mice.
Fresh colons (A) and hearts (B) were harvested from naïve mice. A single cell suspension was established from each tissue and Sca-1+ cells were isolated through positive selection immunolabeling and magnetic bead separation. Cells were plated and maintained in culture with our lab-developed CPC media. Pictures in A and B were taken cultures in passage one, one-day post plating using an Olympus IX70 microscope at 40x and represent typical cultures of the respective cell populations.

3.1b mRNA expression profile of colonic Sca-1+ cells *in vitro*

Much of the current work in adult stem cell biology centers around classification and functional characterization of stem/progenitor cell subsets based on identifiable markers used to isolate cellular populations. Theoretically, unique combinations of surface markers can be used to identify specific subpopulations of adult stem/progenitor cells within various tissues, such as mesenchymal, endothelial, and organ specific stem cells. All stem cells, no matter the origin, do not possess lineage-specific markers. However, elucidation of conclusively identifying positive markers (or combination) for stem/progenitor cells for classification purposes requires more attention as significant overlap in putative markers exists between types of stem/progenitor cells. For example, Sca-1, CD34, and c-Kit are found in high levels in hematopoietic stem cells (Bradfute, Graubert, & Goodell, 2005; Sidney, Branch, Dunphy, Dua, & Hopkinson, 2014, p. 34), but are increasingly being identified as markers for mesenchymal stem cells within a profile that may also include CD271, CD29, and CD105, among others depending on subset(Álvarez-Viejo et al., 2015; Deans & Moseley, 2000; Kobolak et al., 2015). On the other hand, markers such as Lgr5, Bmi1, and Lrig1 give rise to colonic crypts and are being studied as unique populations of obligate epithelial intestinal stem cell markers (Yan et al., 2012; Zhang & Huang, 2013). Defining a profile of surface markers on CSCs will help classify our novel intestinal cell subset.

Freshly isolated primary CSCs were plated after the initial passage and assessed for basal expression of various surface markers through qPCR analysis (**Fig. 3.2)**. Table 3.1 displays a comprehensive list of the markers tested, grouped by classification, and includes generalized relative expression levels as compared to HPRT, highlighting the heterogenous nature of CSCs in culture.

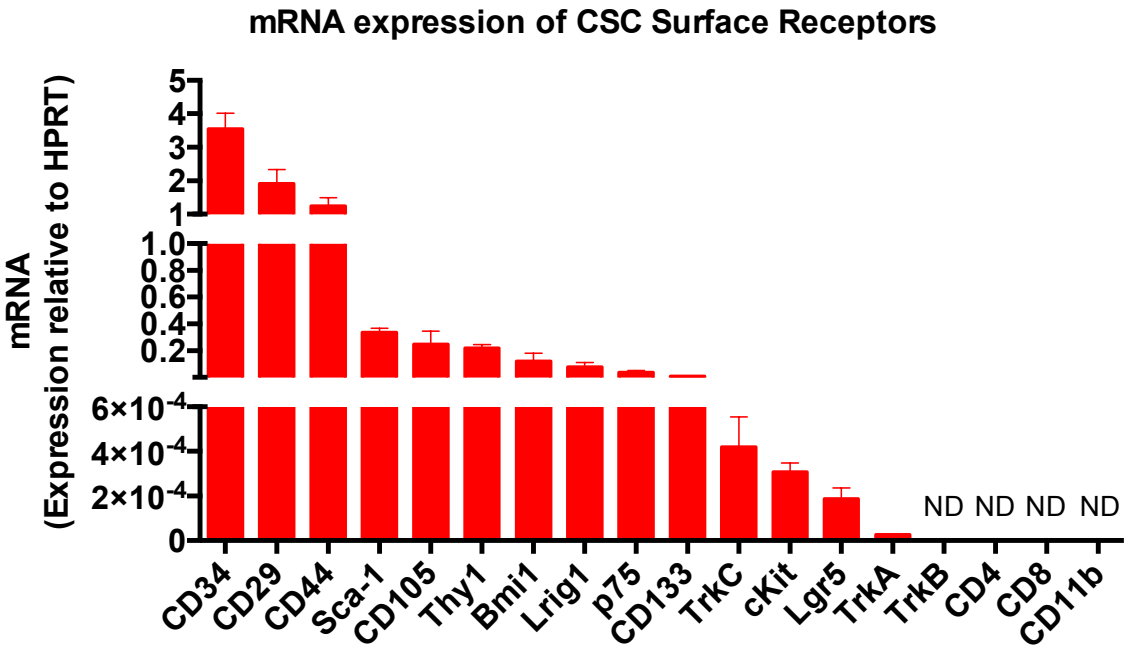

Fig. 3.2. Basal transcriptional expression profile in naïve murine CSCs.
Colonic Sca-1+ cells (CSCs) were isolated from the colon of naive C57/BL6 mice and grown in culture with CPC media. At Passage 1, $2x10^5$ cells were plated in a six well plate (3 per condition). 24 hours after plating, the CPC media was replaced with DMEM/0.5% FBS to deprive the cells of serum for 5 hours, after which, trizol was added to the cellular monolayers and RNA was isolated. CDNA was synthesized and transcripts of the genes were measured in duplicate by qPCR analysis and graphed above as expression relative to the housekeeping gene HPRT. ND=not detected. Each bar represents the mean ± SEM mRNA expression of 2 independent experiments with different CSC isolates. The graph represents a composite of multiple qPCR experiments with identical levels of HPRT.

Table 3.1 mRNA profile of CSC surface markers

mRNA Expression Level	
Lineage Markers	
CD3	ND
CD4	ND
CD8	ND
B220	ND
CD11b	ND
MSC/ Progenitor Markers	
CD29	High
CD34	High
CD44	High
CD90/Thy1	Medium
CD105	Medium
CD117/cKit	Low
CD133	Medium
CD271	Medium
Sca-1	Medium
ISC Markers	
Bmi1	Medium
Lgr5	Low
Lrig1	Medium
Neurotrophin Receptors	
TrkA	(very)Low
TrkB	None
TrkC	Low

ND= not detected; high=relative expression>1;
Medium= relative expression <1; >.006;
low=relative expression <.006

3.1c Colonic Sca-1+ cells maintain population through self renewal

By definition, a stem cell has the clonogenic capacity for maintenance of an undifferentiated population indefinitely through self renewal until signaled for differentiation into one of a number of potential cell types depending on the organ/tissue of origin (Fuchs & Chen, 2012). CPCs have reportedly been maintained in culture up to passage 50 without any morphological or physiological alterations (H. Wang et al., 2014).

To determine the self renewal capacity of our CSCs, freshly isolated cells were grown in culture and the cumulative growth was tracked from passage 0. Murine CSCs cultured *in vitro* with CPC media (described in section 2.2a) demonstrated a high proliferative capacity for 6 passages while maintaining consistent fibroblast-like morphology (**Fig. 3.3**). Subsequent passages produced cultures with negative growth rates and morphologically altered cells that no longer reached total confluency (data not shown).

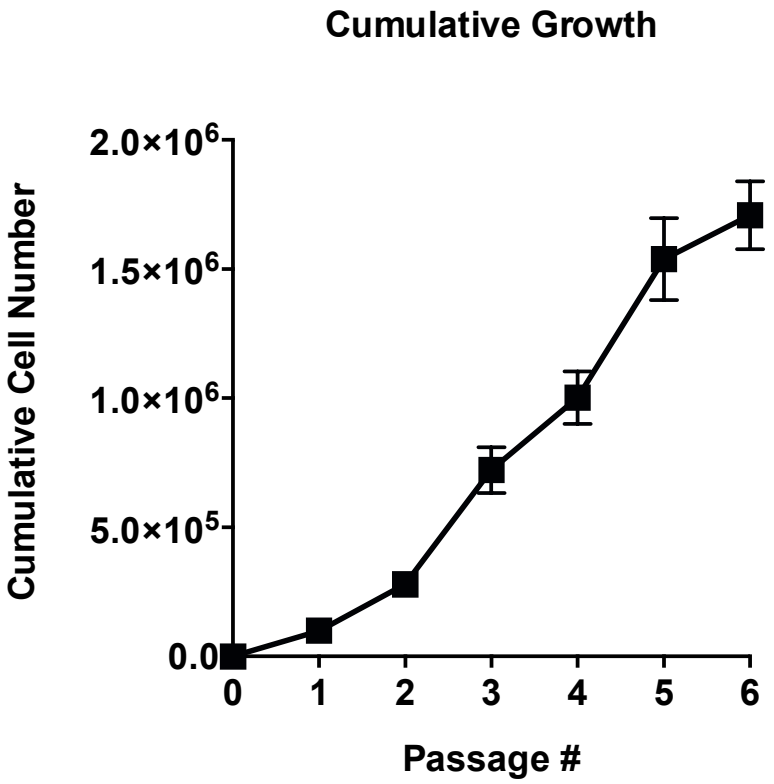

Fig. 3.3. sPDNF stimulates growth of Colonic Sca-1+ Cells (CSCs).
Sca-1+ cells were isolated from the entire colon by magnetic bead affinity chromatography from naive (uninfected) C57/BL6 mice (CSCs) and cultured in a complex media developed in the lab. Cells were plated in triplicate and grown to confluency before being passaged the indicated number of times, which was approximately 4 days in culture. Points represent the mean ± SEM cumulative growth in number of cells for three individual wells per passage This graph is representative of 4 independent experiments using different primary CSC isolates.

3.2 *T cruzi*-PDNF interaction with colonic Sca-1+ cells

3.2a sPDNF profoundly enhances growth of CSCs *in vitro*

Work in our lab by Ryan Salvador has demonstrated the ability of sPDNF to potently enhance the proliferative capacity of CPCs both *in vitro* and *in vivo*, implicating sPDNF as a stem cell mitogen (Salvador, 2014). Given the self renewal capacity of CSCs, we sought to determine if sPDNF would have the same effect on this novel population, as CPCs. To test this, CSCs were maintained in culture with or without the addition of 2μg sPDNF to the media and cell counts were collected for calculation of cumulative growth at each passage and comparison between groups. When grown in media conditioned with sPDNF, CSCs demonstrated a profound enhancement in growth, as measured by cumulative growth (**Fig. 3.4**).

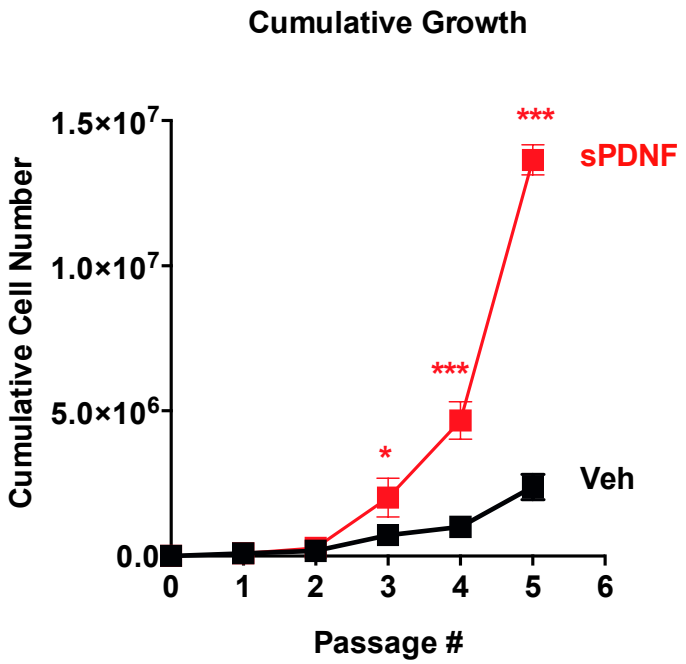

Fig 3.4. sPDNF stimulates growth of Colonic Sca-1+ Cells (CSCs).
Sca-1+ cells were isolated from the entire colon by magnetic bead affinity chromatography from naive (uninfected) C57/BL6 mice (CSCs), cultured in a complex media developed in the lab. Cells were plated in two groups, one receiving media with 2 μg/ml sPDNF and the other PBS (Veh), and grown to confluency before being passaged for the indicated times, which was approximately 4 days in culture. Points represent the mean ± SEM cumulative growth in number of cells for three individual wells per group, per passage The graph is representative of 3 independent experiments with different primary isolates of CSCs. *$P < .05$ and ***$P < .001$, as compared to Veh with the student's T test

3.2b *In vitro* stimulation with sPDNF upregulates immunomodulatory cytokine expression in colonic Sca-1+ cells

Tissue-resident adult stem cells are known to produce multiple cytokines with immunomodulatory properties, such as TSG-6 and IL-10 (Chen et al., 2015; R. H. Lee et al., 2009; N. Wang et al., 2012). Current theories suggest that stem/progenitor cells can be programmed for specific phenotypes, akin to the development of induced pluripotent stem cells (iPSC) (Q. Wang et al., 2016). Based on the mRNA data in Figure 3.3, we have transcriptional evidence of the expression of the neurotrophin receptors, TrkA and TrkC in CSCs. sPDNF is known to both invade and signal through these receptors (Marina V. Chuenkova & Pereiraperrin, 2011), and given the protein's mitogenic properties, could potentially skew the immunologic potential of CSCs to an immunomodulatory phenotype.

To investigate the possibility of sPDNF activating an immunomodulatory phenotype in CSCs, cell stimulation assays were performed and mRNA levels of anti-inflammatory cytokines were measured. In short, freshly isolated CSCs were plated at high density in two groups of triplicate and allowed to adhere overnight. Both groups were subjected to a two-hour serum deprivation prior to a three-hour stimulation with either PBS (Veh) or 2μg/ml sPDNF (sPDNF). RNA was isolated from the cellular monolayer of each group and mRNA expression levels, measured through qPCR, were compared between the groups. Naïve CSCs

stimulated with a dose of sPDNF upregulated mRNA expression of anti-inflammatory cytokines such as TSG-6, IL-22, IL-1Ra, IL-10, and IDO (**Fig. 3.5**)

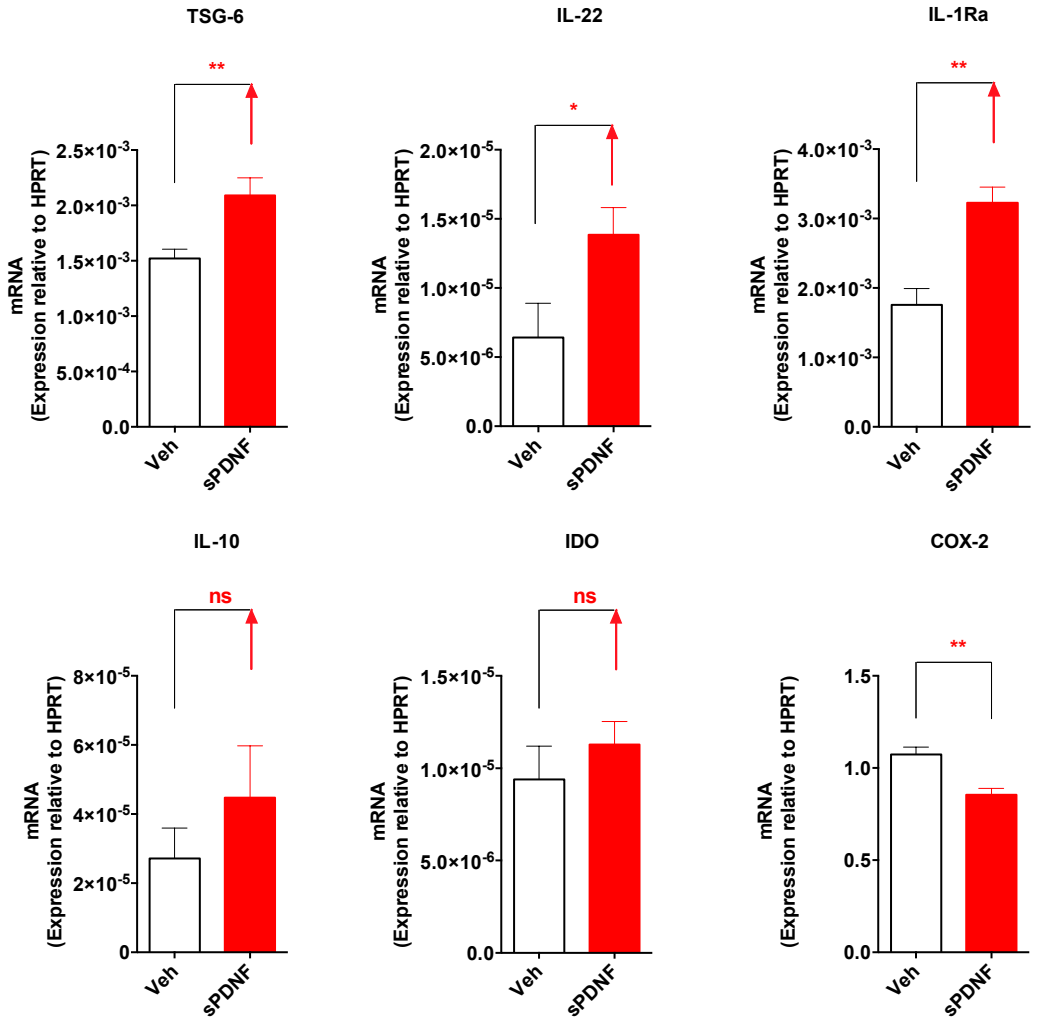

Fig 3.5. sPDNF increases the expression of anti-inflammatory cytokines in colonic Sca-1+ cells

CSCs were isolated from naive C57/BL6 mice using affinity chromatography. At passage 1, cells were plated in a six well plate (3 wells per group) at a density of 2.5x10^5 cells and incubated over night. The following day, the cells were deprived of serum (0.5%FBS-DMEM) for 2 hours, after which one group was stimulated with 2ug/ml sPDNF for 3 hours. Trizol was applied to the cellular monolayers from each group, RNA was isolated, cDNA was synthesized and transcripts of the above genes were analyzed in duplicate by qPCR. The mean ± SEM Ct values from each group were normalized to HPRT and are represented by the bars in the graphs above from 3 individual experiments using different CSC isolates. *P < 0.05 and **P < 0.01. ns = not significant.

3.2c sPDNF stimulates colonic Sca-1+ cells *in vivo*

Primary cells grown in an *ex vivo* culture system do not always display the same properties or functionality that are true to nature in a closed and complete system, highlighting the necessity of *in vivo* studies to delineate biological importance. Our lab demonstrated the ability of CPCs to respond to sPDNF in culture systems (*in vitro*) and in naïve, acute, and chronic mouse studies (*in vivo*) (Salvador, 2014). Therefore, we wondered if we could mirror these *in vitro* results of increased proliferation and activation of immunolodulatory cytokines with sPDNF stimulated CSCs *in vivo*.

In order to assess the possibility of expansion of CSCs *in vivo*, we performed a kinetic analysis to determine transcriptional expression of Sca1 over a time course of 24 hours. Upregulation of Sca1 began at 3 hours, peaked at 8 hours, and was still slightly increased at 24 hours post injection with sPDNF (**Fig. 3.6)**. Given this apparent expansion of Sca-1+ cells in the colon, we next sought to determine if these cells were activated *in vivo*, using an increase in transcript expression as a positive readout. Mice were injected intravenously with sPDNF (or PBS for control group) three times over a twenty-four-hour period (times 0, 8, and 24hrs) and sacrificed twenty-four hours following the final injection. Freshly harvested colons were used to isolate CSCs, which were expanded in culture and analyzed by qPCR for immunomodulatory cytokines to suggest activation. *Ex vivo* cultured CSCs displayed an upregulated expression profile of immunomodulatory cytokines after *in vitro* stimulation of sPDNF (**Fig. 3.7**). Taken

together, these results suggest an expansion and stimulation of CSCs *in vivo* after an intravenous treatment regimen of sPDNF, supporting the *in vitro* results reported in sections 3.2a and 3.2b.

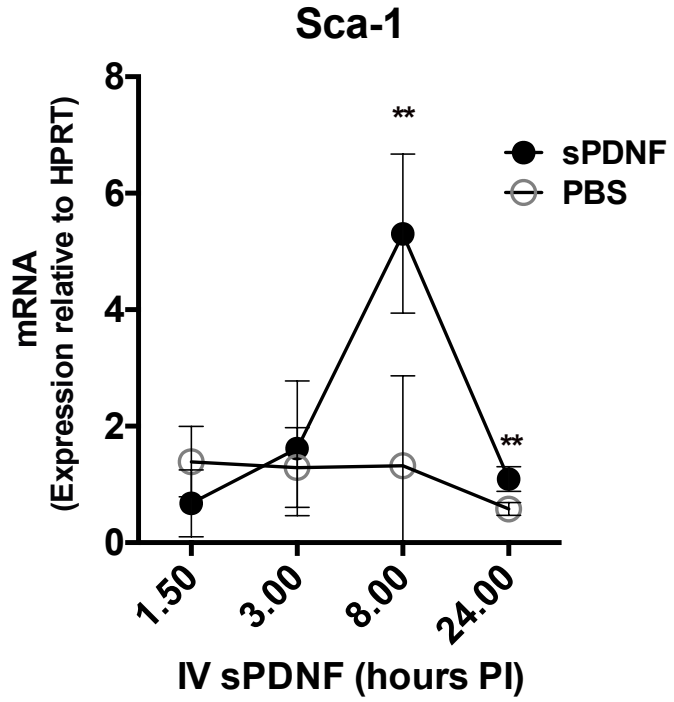

Fig. 3.6 A single IV injection of sPDNF upregulates Stem Cell Antigen-1 (Sca-1) over the course of 24 hours, peaking at 8 hours, in the colon of naive mice.
Naive C57BL/6 mice (three per group) were intravenously injected with a single dose of PBS or sPDNF (75μg/mouse). Mice were sacrificed 1.5, 3, 8, and 24 hours post injection and the colons were harvested. Total colonic RNA was isolated and cDNA was synthesized. Transcripts of Sca-1 were quantified in duplicate by qPCR for each time point of both groups and normalized to the housekeeping gene HPRT. Points on the graph represents the mean ± SEM of Sca1 mRNA expression relative to HPRT. Each time points represents an individual experiment where n=2. ***P* < .01, as compared to PBS group, measured by the student's T test.

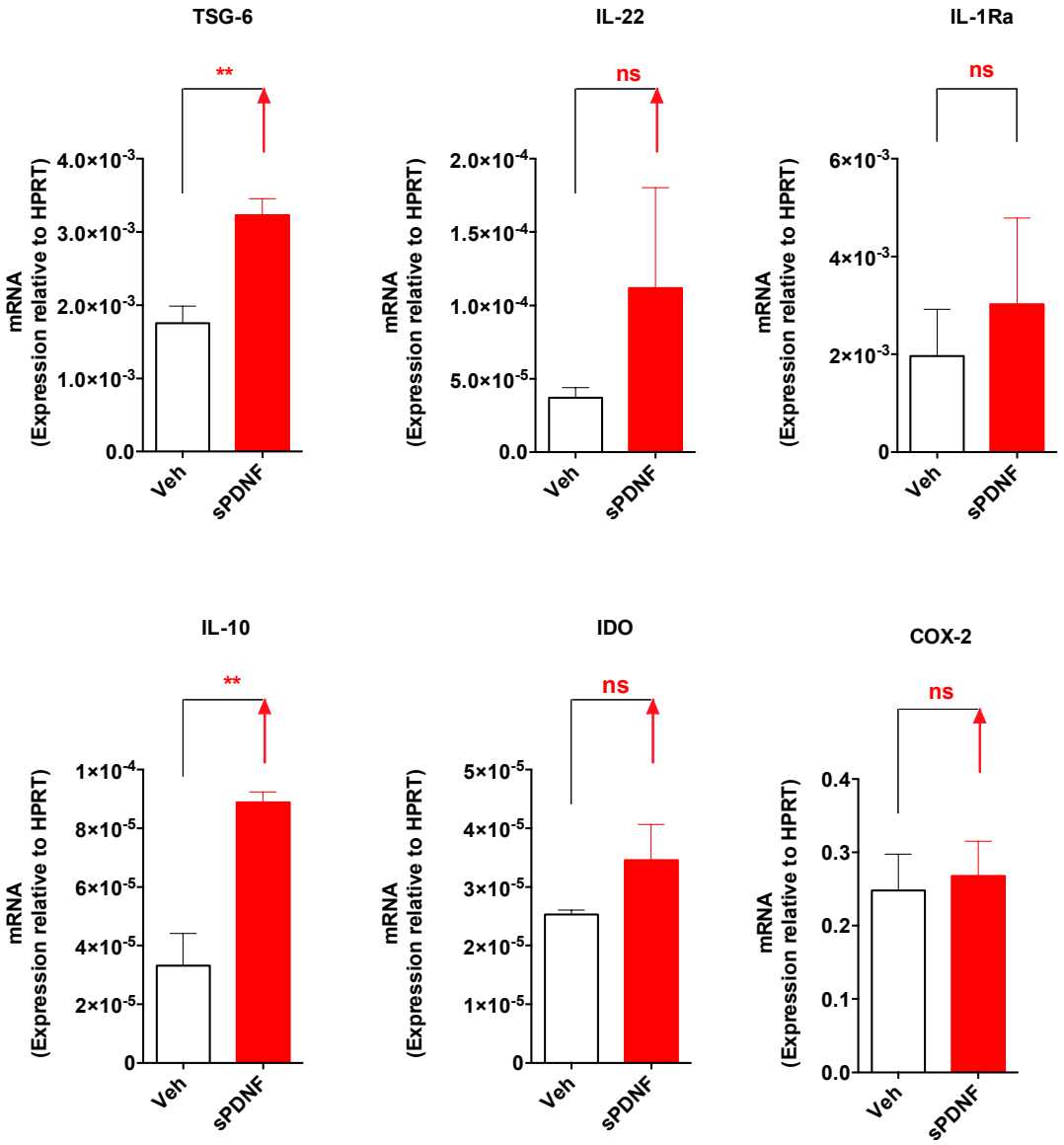

Fig. 3.7. Intravenous administration of sPDNF upregulates expression of anti-inflammatory cytokines in colonic Sca-1+ cells (CSCs)

C57/BL6 mice (three per group) were intravenously injected with PBS or 75ug/mouse sPDNF at times 0, 8, and 24 hours. Mice were sacrificed 24 hours after the final injection and the colons were harvested, pooled (by group), and prepped for digestion. A single cell suspension was established from the fresh tissue and Sca-1+ cells were isolated through magnetic bead separation. Cells were plated and kept in complex media. At passage 1, $2.5x10^5$ cells/well (3 wells per group) were plated and the following day were subjected to a serum deprivation of .5%FBS for 5 hours before cellular monolayers were collected with Trizol. Rna was isolated, cDNA synthesized, and genes of interest were measured in duplicate by qPCR. Bars represent the mean ± SEM of transcripts of interest expressed relative to the housekeeping gene HPRT, where n=2. $^{**}P < .01$, as calculated by the student's T test.

3.2d *T cruzi* invades and infects colonic Sca-1+ cells

During acute infection, *T cruzi* can invade and infect virtually any nucleated cell in every tissue throughout the body (Longo & Bern, 2015). Published reports indicate the ability of *T cruzi* to signal and invade cells through interactions between numerous extracellular proteins of the parasite and a number of various host cell receptors including TLR2 (Fabiana S. Machado et al., 2012). Our lab has previously demonstrated the ability of sPDNF to enhance attachment to host cell, as well as signal through the neurotrophic receptors TrkA and TrkC (de Melo-Jorge & PereiraPerrin, 2007; Weinkauf et al., 2011). Given the transcriptional expression of neurotrophic receptors in CSCs (**Table 3.1**), we wanted to investigate whether the *T cruzi* parasite can invade our newly identified cell type.

Early passage CSCs were plated and subsequently incubated with Tulahuen parasites for 3 hours and the infection was allowed to proceed for for 2 or 3 days before being differentially stained for RNA and DNA to allow for detection and enumeration of intracellular amastigotes. Intracellular invasion and infection of CSCs by *T cruzi* was detectable at two days post infection and percent infection continued to rise three days post infection (**Fig. 3.8**). Interestingly, non infected cells in the infected monolayer displayed altered morphology in the form of increased cell size (**Fig. 3.9**).

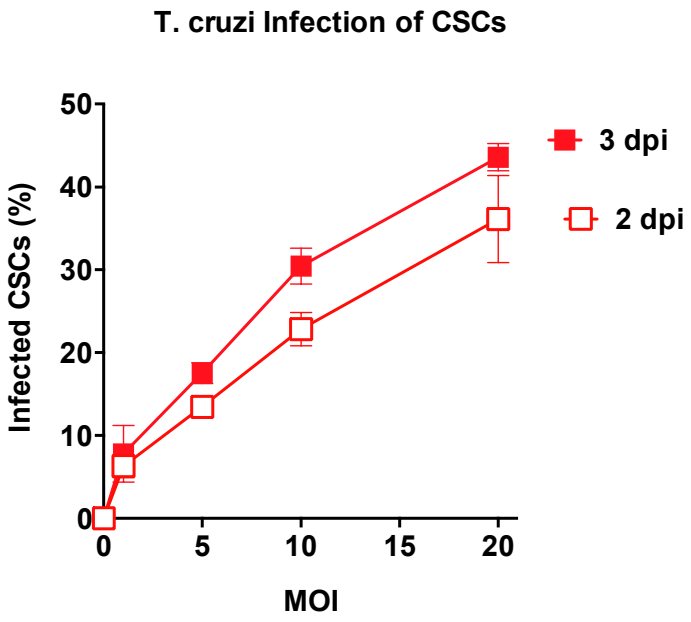

Fig. 3.8. Colonic Sca-1+ cells (CSCs) are permissive to *T. cruzi*
Colonic Sca-1+ cells were isolated from naive C57/BL6 mice (female) by affinity chromatography on immunomagnetic beads, plated in collagen-coated 96-well plates (2000 cells per well) and, after 14-18 h, incubated with *T. cruz*i (Tulahuen strain) or vehicle (0.1% BSA, MOI = 0) for 3 h, washed to remove uninvaded parasites, and further incubated for 2, 3. Cells were stained with Diff-Quick and the number of infected cells were counted using an Olympus IX70 microscope. Points represent the mean ± SEM percentage of infected cells counted from three individual wells. At least 300 cells were counted per well. Graph represents 2 independent experiments per time point.

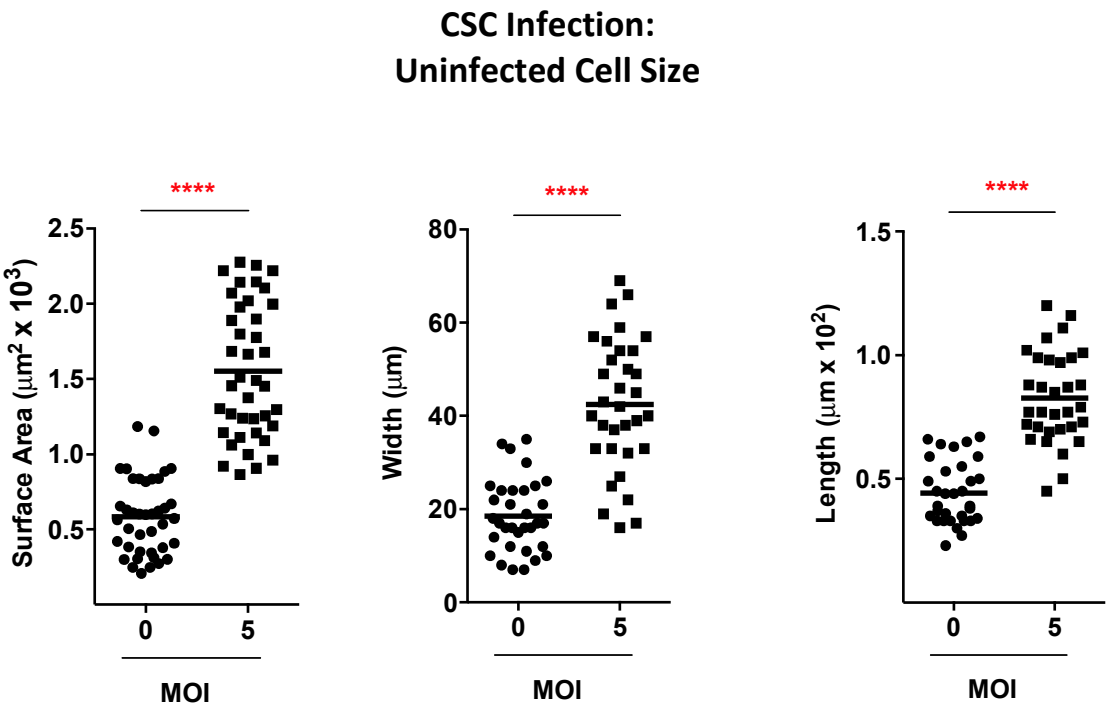

Fig. 3.9. T. cruzi triggers an increase in the size of uninvaded colonic Sca-1+ cells. Colonic Sca-1+ cells were isolated from naive C57/BL6 mice (female) by affinity chromatography on immunomagnetic beads, plated in collagen-coated 96-well plates (1500 cells per well) and, after 14-18 h, incubated with *T. cruz*i (Tulahuen strain, MOI =5) or vehicle (0.1% BSA, MOI = 0) for 3 h, washed and further incubated for 4 d. Cells were stained with Diff-Quick and size (area, lenght and width) of uninfected cells was computed using an Olympus IX70 microscope and SPOT software; only 3.8% of cells were invaded by *T. cruzi*. Each point (about 35 per group) represents an individually measured uninfected cell and the horizontal bar represents the mean measurement of each group. The graph represents 4 individual experiments. ****$P < 0.0001$.

3.3 Summary

In this thesis, we present a preliminary characterization of a novel population of stem/progenitor -like cells expressing Sca-1+ in the naïve murine colon, termed CSCs. Morphologically, these CSCs are a heterogenous mixture of fibroblast-like cells, nearly identical visually to the widely studied CPCs. A profile of the mRNA levels of surface markers present basally in cultured CSCs revealed a lack of any lineage-specific markers and varying levels of MSC markers, ISC markers, and Trk receptors. In accordance with the abundance of stem cell markers present, we demonstrated that CSCs possess the delineating stem cell property of self renewal. Until the potency of these cells are determined through differentiation potential, the most appropriate description of CSCs would be stem cell-like.

Additionally, we investigated the interaction of *T cruzi* PDNF with CSCs. sPDNF enhances proliferation of CSCs both *in vitro* and *in vivo*, while *in vitro* and *ex vivo* studies demonstrated CSC activation through upregulation of immunomodulatory cytokines. In culture, *T cruzi* efficiently invaded CSCs and established robust infection within the monolayers. Future investigations into the role of CSCs within disease models of Chagas disease should prove interesting.

Chapter 4. Discussion

4.1 Colonic Sca-1+ cells as stem/progenitor cells

The capacity for self renewal and maintenance of an undifferentiated population is the discriminating characteristic between stem cells and all other types of further differentiated cells (Fuchs & Chen, 2012). The newly identified colonic Sca-1+ cells, described and characterized in this thesis, do indeed demonstrate the property of self renewal (**Fig. 3.3**), albeit with a seemingly limited capacity in our *in vitro* system. It is quite plausible, and almost likely, that in the appropriate culturing conditions these cells would maintain an undifferentiated population indefinitely. Attempts were made through literature searches to identify a factor that would allow for long-term proliferation in an *in vitro* culture system. Both the Notch and Wnt signaling pathways have been identified as the mechanism through which stem cells are able to self renew (Fujimaki, Wakabayashi, Takemasa, Asashima, & Kuwabara, 2015; Sancho et al., 2015). However, in our system, R-spondin, Noggin, NGF, NT-3, and combinations of all failed to extend the life of the cultures past the average of six passages obtained with the CPC media developed in our lab (data not shown).

Analyzing the profile of CSC surface markers surveyed in this thesis (**Table 3.1**) provides more evidence for these cells as stem/progenitors in nature. For classification purposes, these transcripts represent published markers of both mesenchymal stem cells (CD29, CD34, CD44, CD90/Thy1, CD105, CD117/cKit,

CD127, CD133, and CD271/p75) (Álvarez-Viejo et al., 2015; Deans & Moseley, 2000; Hittinger et al., 2013; Kobolak et al., 2015; Sidney et al., 2014) and intestinal crypt stem cells (Lgr5, Bmi1, Lrig1)(Yan et al., 2012; Zhang & Huang, 2013). Since transcript levels are not directly proportional to protein expression, more sensitive immunologic techniques such as FACS analysis that are capable of identifying proteins on an individual cellular level will help definitively classify subsets within our CSC population and define stem cell type. However, between mesenchymal or intestinal stem cells, morphological observations points towards a mesenchymal-like subset for CSCs given their distinct fibroblast-like appearance.

As mentioned earlier, there is a high propensity for overlap within cellular markers and therefore a portion of these markers are often used in identification of hematopoietic stem cells, as well as non stem cells. For example, Sca1 and CD133 were initially discovered in hematopoietic precursors, while CD90/Thy1 can also be found in innate lymphoid cell populations (ILCs). However, solely based on visual observations, it is clear that our CSCs represent a heterogeneous population (**Fig 3.1**) and therefore stands to reason that different cellular subsets could be present within the *in vitro* cultures. In addition to the capacity for self-renewal, a true stem cell must have multidifferentiative potential and the resulting potency is key for classification (Visweswaran et al., 2015). Until one shows that CSCs differentiate into various phenotypes (osteocytes,

myocytes, endothelial cells, and so on), CSCs are most appropriately considered to be stem cell-like.

4.2 Sca-1 as a marker of intestinal stem cell population

Current understanding of intestinal stem cells is limited to two main subsets identified by the markers Lgr5 and Bmi1, with the former being more abundant active type, while the later is considered quiescent (Yan et al., 2012). These intestinal stem cells are epithelial precursors and can clonogenically form fully functional crypts in an organoid environment, earning the moniker crypt-forming stem cells. Recently, a group successfully isolated a high purity single-cell fraction of Lgr5+ ISCs that were able to propagate in classical media once the necessary unique growth factors were identified (Scott et al., 2016). In fact, this field is so focused on these two subsets that nearly all literature on ISCs are founded on isolation with with one of these markers.

In this thesis, we report a novel population of Sca-1+ cells in the murine colon with stem cell-like properties, potentially expanding known subsets of stem cells in the intestines. Sca1 is currently considered a putative marker of adult tissue resident stem cells in the heart, kidney, and lungs, with these subsets possessing tissue regenerative properties (Hishikawa et al., 2015; Hittinger et al., 2013; Valente et al., 2014). Moreover, Lgr5+ or Bmi1+ intestinal stem cells possess a potency limited to differentiation into epithelial cell lineages (Yan et al., 2012). However, other tissues types exist within the intestines, and it is therefore

reasonable to hypothesize that the colon would harbor tissue specific stem/progenitor cell subsets in addition to the widely studied Lgr5+/Bmi1+ crypt-forming stem cells. We propose that Sca1 marks a previously unappreciated stem/progenitor subset within the colon of naïve mice.

4.3 sPDNF as a mitogen and activator of CSCs

The addition of exogenous sPDNF to *in vitro* CSC cultures significantly enhanced cellular growth, and intravenous administration resulted in upregulated Sca1 transcripts in the colon, which suggests an *in vivo* population expansion (**Figs. 3.4 and 3.6**). Together, this implicates sPDNF as a mitogen of CSCs, both *in vitro* and *in vivo*. We have also presented that both *in vitro* stimulation and *in vivo* administration of sPDNF propagates transcriptional upregulation of various immunomodulatory cytokines in CSCs including TSG-6 and IL-10 (**Figs. 3.5 and 3.7**), which implies activation of a cellular signaling pathway important in disease pathogenesis.

Previous work in our lab on the interactions between *T cruzi* and CPCs demonstrated the ability of sPDNF to stimulate enhanced proliferation of these progenitor cells and induce activation of tissue regenerative pathways that lead to amelioration of fibrosis in CCC (Salvador, 2014). With the knowledge that CSCs possess stem cell properties, the results presented here provide additional supporting evidence for sPDNF as a mitogen and activator of stem cells with immunomodulatory properties.

4.4 *T cruzi* interaction with host cells

T cruzi possesses the ability to infect virtually every cell and tissue type of the human body. To accomplish this feat, *T cruzi* expresses myriad outer membrane proteins that are able to interact with a variety of host cell receptors (Fabiana S. Machado et al., 2012). In addition to notorious pattern recognition receptors like TLRs (Kayama & Takeda, 2010), *T cruzi* is able to invade and signal through Trk family receptors, which are found in abundance on neuronal cells, cardiomyocytes, and other cell types and are important receptors for the development and maintenance of the nervous system(Marina V. Chuenkova & Pereiraperrin, 2011). Previously, our lab demonstrated *T cruzi*-PDNF as a ligand for NGF and NT-3 receptors TrkA and TrkC, respectively, which leads to parasite entry of host cells and activation of pro-survival and protective events (Aridgides et al., 2013a, 2013b; Marina V. Chuenkova & PereiraPerrin, 2004; de Melo-Jorge & PereiraPerrin, 2007; Weinkauf et al., 2011).

Data presented within this thesis provides evidence of T cruzi invasion and infection of CSCs *in vitro* (**Fig. 3.8**). According to our surface marker profile (**Table 3.1**), CSCs express varying transcriptional levels of the neurotrophin receptors TrkA and TrkC, but not TrkB, mirroring the exact Trk receptors known to be triggered by T cruzi PDNF. In line with its high expression within the GI tract, TrkC expression levels were higher in comparison to TrkA (**Fig. 3.2**). Therefore, TrkC provides a plausible mechanism for invasion and infection of CSCs.

Interestingly, CD271 is routinely used in combinatorial expression as a positive selection marker for mesenchymal stem cells (Álvarez-Viejo et al., 2015, p. 271), but functionally is a member of the Trk family known as p75 and promiscuously binds all neurotrophins (NGF, NT-3, BDNF) with equal affinities. Reports published over ten years ago failed to establish evidence of an interaction between T cruzi-PDNF and p75, despite the fact that PDNF is an NGF mimic(Marina V. Chuenkova & PereiraPerrin, 2004). However, this has been called into question by a structural clue that revealed the NGF contact domain with p75 has a very high similarity to the PDNF amino acid sequence (de Melo-Jorge, 2008). Subsequent preliminary experiments used whole-cell pull-down assays to seemingly demonstrate live T cruzi binding to the ectodomain of p75, however this still needs to be further investigated. Therefore, there is a potential for T cruzi PDNF to invade and infect various subsets of mesenchymal stem cells through p75.

4.5 Conclusion

This thesis marks the first time a population of Sca-1+ cells with stem/progenitor-like properties (CSCs) was identified and characterized in the murine colon. These CSCs had the capacity for self renewal upwards of six passages in culture and displayed putative markers for stem cells on their surface. However, true classification of this cell type cannot be complete until determination of the multidifferential potential of the cells, which will provide great insight into CSC

functionality and importance *in vivo.* For example, differentiation into adipocytes, chondrocytes, or osteoblasts would be indicative of an early precursor stem cell population, like MSCs. However, if CSCs differentiate into cells solely of the colonic lineage, meaning multi or oligopotent, they would be considered a “later stage” progenitor cell population.

Visual observations alone suffice in identifying this novel population of cells as a heterogeneous mixture. With this knowledge, mRNA transcript expression becomes limited in scope, since it is unknown how much of the marker each cell produces. Therefore, more sensitive immunological techniques such as FACS analysis will allow for a more complete overview of the cellular subpopulations present in our culture based on surface marker profile.

We have demonstrated here that CSCs interact *in vitro* and *in vivo* with *T cruzi*, and more specifically PDNF. Administration of sPDNF was able to enhance proliferation and trigger cellular activation though upregulation of mRNA transcripts. Future studies will focus on protein secretion from these cells to prove the biological relevance of increased transcript expression. Production of anti-inflammatory proteins such as IL-10 and IL-1Ra could easily create an altered local environment, leading to immunomodulation and potential tissue regeneration and repair. Given the high rate of infection of CSCs by *T cruzi*, it stands to reason that these stem cells might play a role in disease pathogenesis.

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