CHARACTERIZATION OF IMMUNOLOGICAL RESPONSES IN AN EXPERIMENTAL MURINE MODEL TO INFECTION WITH DISTINCT UNITED STATES *TRYPANOSOMA CRUZI* ISOLATES

by

JESSICA EDWARDS

(Under the Direction of Michael Yabsley)

ABSTRACT

*Trypanosoma cruzi* strains are genetically and biologically diverse. *T. cruzi* is divided into six discrete typing units which have specific geographical and ecological associations. In the United States (US), TcI and IV have been isolated from various hosts. Although human cases of Chagas' disease are rare within the US, the prevalence of *T. cruzi* in sylvatic cycles is high. Experimental murine models closely mimic various aspects of Chagas' disease, including immune mechanisms and histopathological implications of infection with different *T. cruzi* strains. Currently, little characterization work has been conducted on US strains. The goal of this thesis was to characterize the cytokine production of mice experimentally inoculated with diverse *T. cruzi* strains from the United States and South America and to determine changes in cytokine production and pathology in mice previously exposed to a US *T. cruzi* strain and subsequently challenged with a South American *T. cruzi* strain.

INDEX WORDS: *Trypanosoma cruzi*, Chagas' disease, United States, wildlife, immunology, cytokines, DTU's, acute phase, challenge study

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DEDICATION

To the most important people in my life: Marianne, Mark, April, and Ken. Thank you for all of your love and support. I would not be here if it weren't for you!!

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# CHAPTER 1

## INTRODUCTION

*Trypanosoma cruzi* is a highly diverse species, biologically and genetically, and infects a wide range of wildlife reservoirs over large geographic regions. *T. cruzi*, the cause of Chagas disease, is a significant cause of morbidity and mortality in many regions of Latin America. An estimate of 8-10 million people are infected with *T. cruzi* and approximately 30% of these infections result in the potentially fatal, chronic cardiomyopathy form of the disease (Prata, 2001). *T. cruzi* is found from southern South America to as far north as the United States (US) and circulates in a complex cycle that involves numerous species of vectors and wildlife reservoirs (e.g., opossums, armadillos, raccoons, and various rodents), domestic dogs, and humans (Clark and Pung, 1994; Yabsley et al., 2001). In the US, there have been seven autochthonous human cases confirmed, yet recent serologic testing of targeted groups and of blood donations indicate a large number of individuals living in the US are infected, some of which are likely undiagnosed autochthonous cases (Shulman, 1999; Herwaldt et al., 2000; Beard et al., 2003; Dorn et al., 2007; Bern et al., 2008; Leiby et al., 2008).

Recent field and molecular studies of *T. cruzi* in the US have revealed that there is a diverse host range across the southern US (Yabsley et al., 2001; Roellig et al., 2008; Brown et al., 2010). Currently *T. cruzi* is divided into six discrete typing units (DTU) (TcI –VI) (Zingales et al., 2009), . In the US, all isolates from wildlife, dogs, vectors, and humans have been classified as TcI and TcIV, with TcI strains typically isolates from opossums and humans, TcIV

strains typically associated with raccoons, and both genotypes have been detected in dogs, woodrats (*Neotoma micropus*), and various vectors (Roellig et al., 2008; Charles et al., unpublished data) . To date, few studies have investigated the biological characteristics of these US TcI and TcIV strains (Yabsley and Noblet, 2002; Hall et al., 2010; Roellig et al., 2010; Roellig and Yabsley, 2010).

Considerable work has been conducted on the host-parasite interaction and immunological responses during the acute and chronic phase of Chagas disease in laboratory mice models (reviewed in Tarleton, 2007; Junqueira et al., 2010; Kayama and Takeda, 2010). The majority of these studies have been conducted with a limited number of *T. cruzi* strains and primarily with strains isolated from humans from South America. Experimental studies characterizing the immune responses of the host due to infection with different strains of *T. cruzi* may illuminate our understanding of the complex host-parasite interaction and apparent virulence of particular strains. Therefore, this thesis research had two main aims:

1. To characterize cytokine profile production in mice experimentally inoculated with diverse *T. cruzi* strains from the United States and South America.
2. To determine changes in cytokine production and pathology in mice previously exposed to a United States *T. cruzi* strain and subsequently challenged with a South American *T. cruzi* strain.

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# CHAPTER 2

# LITERATURE REVIEW

## History of *Trypanosoma cruzi*

In 1909, Carlos Chagas, a young physician identified and characterized one of the most important parasitic diseases in South America, now known as Chagas Disease or American trypanosomiasis. Dr. Chagas detected small trypanosomes in patients exhibiting malarial-like symptoms, which he later named *Trypanosoma cruzi*. His patients typically came from rural, poverty-stricken backgrounds, living in mud-thatched huts infested with bugs. These bugs, (known to the locals as "barber bug" or "kissing bug"; Hemiptera:Reduviidae) were found to harbor and transmit *T. cruzi*, often feeding on people as they slept (Bastien, 1998). Discovering the link between bug-infested dwellings and disease was an important step for advocating better housing and living conditions in South America. While, control of natural transmission of Chagas disease has been achieved in some countries by extensive control efforts targeting the bugs with insecticide (Coura and Borges-Pereira, 2010), the pathogenesis of *T. cruzi* is largely unknown and there are still no effective vaccines or drugs to prevent or treat chronic infection (Khaw and Panosian, 1995; Urbina, 2010).

## Description and Life Cycle

*Trypanosoma cruzi* is a flagellated, kinetoplastid, protozoan parasite. The life cycle of *T. cruzi* includes three distinct developmental stages: the trypomastigote, epimastigote, and

amastigote (Figure 2.1). The trypomastigote is the non-replicative extracellular stage found within the vertebrate host and the amastigote is the intracellular replicative (binary fission) form. When trypomastigotes are ingested by a vector, they transform into replicating epimastigotes in the midgut of the reduviid vector.

Standard vector-borne transmission *T. cruzi* begins when infective trypomastigotes are deposited in the feces of reduviid vectors during feeding and subsequent entrance in the host occurs through an open wound (e.g., bug bite) or mucus membrane. The infective metacyclic trypomastigotes enter vertebrate host cells by active penetration or induction of phagocytosis. Once inside the cell, the trypomastigote recruits host endosomes to form a parasitophorous vacuole. Within 24 hours after cell entry, the trypomastigote escapes into the host cytoplasm and transforms into the amastigote form (de Souza et al., 2010). Amastigotes replicate and then either transform into infective trypomastigotes or remain in the amastigote form to form a pseudocyst. Released trypomastigotes circulate through the blood (parasitemia) and eventually enter other tissue cells to transform into and replicate as amastigotes. Trypomastigotes in the blood can also be ingested by reduviid vectors during a blood meal. Once ingested, trypomastigotes travel to the midgut of the reduviid and transform to epimastigotes, which proliferate and transform back into infective trypomastigotes in the hindgut.

In addition to standard vector-borne transmission, *T. cruzi* can be transmitted by several alternative routes. An individual can become infected via the bug vector by either the feces entering a wound, or oral ingestion of the feces or reduviid (Ianni and Mady, 2005). Infected donors can also transmit *T. cruzi* through blood or organ donations (Bern et al., 2008; Leiby et al., 2008). Since the 1970's and 1980's, South American populations transitioning from rural to urban environments has increased contamination of blood banks (Luquetti and Schmunis, 2010).

Most blood banks in Latin America now perform compulsory screens for infected blood. In January 2007, widespread blood-bank screening was initiated by the American Red Cross, which covers 75-90% of the US blood supply (CDC, 2007). *T. cruzi* can also be transmitted from mother to child and has been progressively recognized as a major contributor to the epidemiology of Chagas disease (Andrade and Gontijo, 2008; Buekens et al., 2008; Hall et al., 2010). These alternative routes of transmission, especially congenital and blood transfusion, has led to increase in diagnosis of Chagas disease cases in areas that lack endemic transmission of *T. cruzi* (e.g., Europe) (Schmunis, 2007; Schmunis and Yadon, 2010).

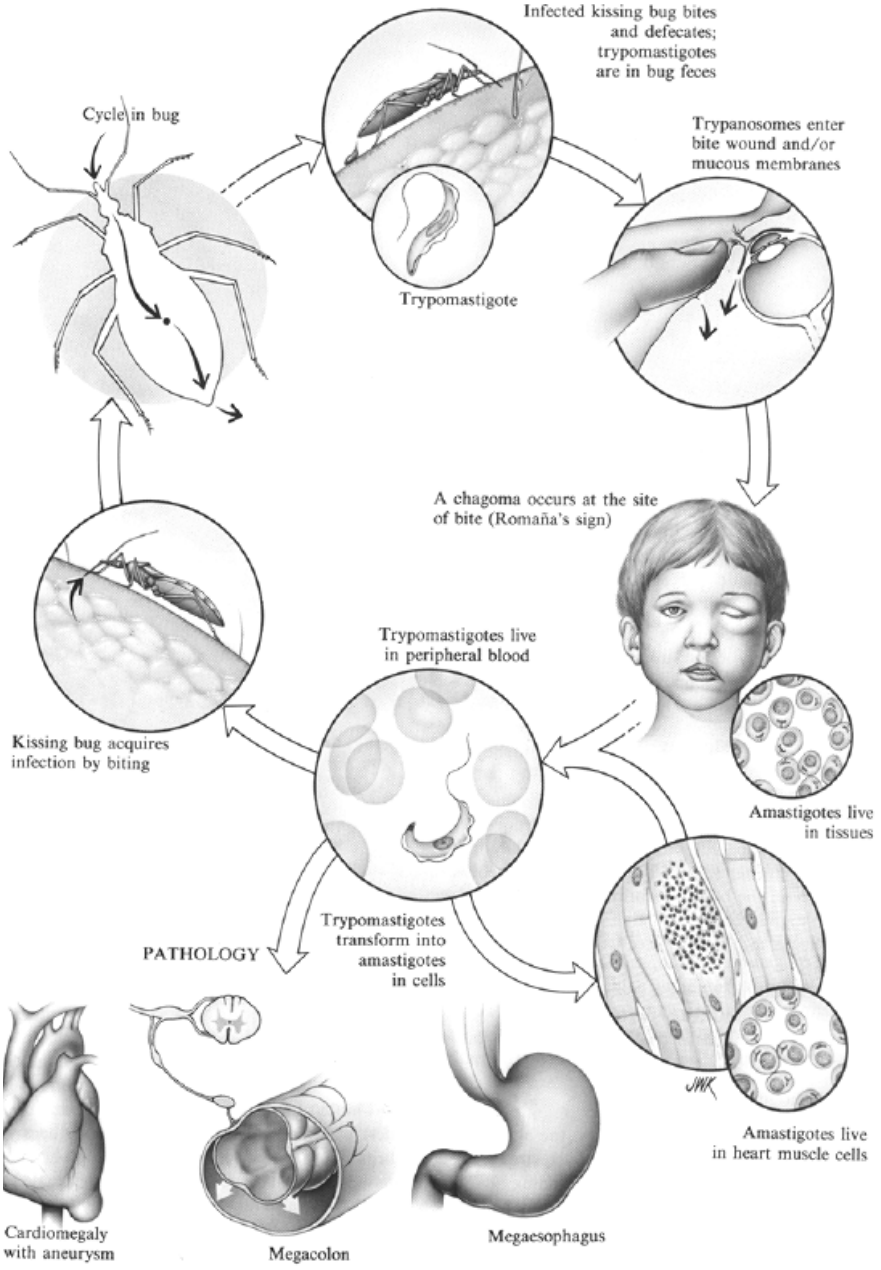


Figure 2.1 The life cycle of *Trypanosoma cruz*

Chagas Disease

Chagas disease can manifest in an acute and chronic phase. The acute phase is normally asymptomatic, with a few individuals developing nonspecific febrile disease. The chronic phase is characterized by two possible disease presentations: cardiac and digestive (megaesophagus and megacolon respectively) (Rassi et al., 2010). Importantly, not all infected individuals develop clinical chronic Chagas disease, and can remain asymptomatic throughout life.

The acute phase lasts from onset of infection upwards to two months and is characterized by a high number of circulating parasites and widespread tissue parasitism. The acute phase is rarely lethal, and is typically asymptomatic. In some patients, a chagoma (painless swelling) will form at the site of parasite entry into the skin. When entry occurs near the eye, the swelling is known as Romaña's sign. Other symptoms of the acute phase are fever, malaise, weakness, anorexia, and headaches. Chagas disease is diagnosed in the acute phase based on the demonstration of the parasite in peripheral blood or seroconversion. Other methods of diagnosis include skin biopsy of suspected chagoma or xenodiagnosis (Rassi et al., 2010). A low percentage of patients (~5-10%) will die during the acute phase due to acute heart disease or encephalitis.

The chronic phase begins directly after the acute phase, during which time parasitemia levels fall to virtually undetectable levels. Approximately 50% of infected people will enter an indeterminate state without any clinical signs or disease; however, they remain positive in serological and/or parasitological tests. Indeterminate patients also do not exhibit any abnormal ECGs and have normal chest, esophagus, and colon radiographs (Rassi et al., 2010). The remaining 25-40% of infected individuals will develop either cardiac or digestive disease. The cardiac form is the most serious and affects 20-30% of all infected individuals. It manifests as

three major syndromes: arrhythmia, heart failure, and thromboembolism (Rassi et al., 2000). The digestive form affects 5-10% of individuals infected, though this determinate manifestation is thought to be regional (western Brazil), possibly due to certain *T. cruzi* strains in the region or a predilection of people in that region to develop this syndrome (Rezende and Luquetti, 1994; Rezende, 1976). Rarely, individuals can develop a cardiodigestive form with both heart disease and a megasyndrome (either megaesophagus or megacolon) (Rassi et al., 2010).

Most non-human infections with *T. cruzi* result in little to no disease as many of these wildlife (rodents, opossums, armadillos) are natural hosts (Packchanian, 1942). However, some domestic animals, such as dogs and cats, do develop clinical disease (Kjos et al., 2008). Domestic dogs can develop both acute and chronic disease. During the acute stage, dogs may be lethargic and may develop pale mucous membranes, generalized lymphadenopathy, and hepatosplenomegaly (Barr, Schmidt, et al., 1991). During the chronic stage, dogs may die unexpectedly or display exercise intolerance and can develop ascites, pleural effusion, distended jugular veins, and/or hepatomegaly (Barr, Schmidt, et al., 1991; Meurs et al., 1998). In the US, canine Chagas disease is a significant veterinary problem in Texas, Louisiana, and some other southern states.

Epidemiology

*T. cruzi* is a zoonotic parasite that is maintained in nature by a large number of wild and domestic mammals and numerous species of reduviid bugs. In Latin America, where the majority of endemic transmission occurs, an estimate of 8-10 million people are infected with *T. cruzi* and nearly 50,000 deaths occur each year due to the disease (WHO, 2010). Approximately

25 million are at risk of infection due to environmental and socio-economic factors which may favor transmission, as well as inadequate vector-control (WHO, 2010).

In the United States, there have been seven autochthonous cases reported in humans (Herwaldt et al., 2000; Beard et al., 2003; Dorn et al., 2007). Although autochthonous cases in the United States are rare, historical serologic surveys indicate many cases have gone undiagnosed (Dorn et al., 2007). The first human case in the United States was reported in a 10 month old infant in Corpus Christi, Texas in 1909 (Woody and Woody, 1955). A subsequent study of 500 children in rural southern Texas detected seven children that were seropositive (Woody et al., 1961). *T. gerstaeckeri* is a common vector in southern Texas and was implicated in biting these seropositive children. Since the initial discovery of the first human infection in the US, six additional human cases have been documented in California, Texas, Tennessee, and Louisiana. The majority of these cases have occurred in young children (Woody et al., 1961; Schiffler et al., 1984; Ochs et al., 1996; Herwaldt et al., 2000; Dorn et al., 2007; Kjos et al., 2009).

*T. cruzi* persists in the US in a primarily sylvatic transmission cycle that involves numerous wildlife reservoirs and triatomine vectors. In the US, *T. cruzi* was first discovered in a triatomine vector, *Triatoma protracta* and was first isolated from a vertebrate reservoir, the dusky-footed woodrat (*Neotoma fuscipes*) in 1936 (Kofoid and Whitaker, 1936). Since then, 11 potential *T. cruzi* vector species have been reported in the US including: *T. protracta, T. sanguisuga, T. lenticularia, T. neotomae, T. recurva, T. rubida, T. gerstaeckeri, T. indictiva, T. incrassate, T. rubrofasciata, and Paratriatoma hirsuta* (Lent and Wygodzinsky, 1979; Ikenga and Richerdson, 1984). In the US, evidence of infection has been reported from at least 18 species of mammals, including raccoons, opossums, armadillos, foxes, skunks, dogs, ground

squirrels, nonhuman primates, and various rodents (primarily woodrats) (John and Hoppe, 1986; Yaeger, 1988; Barr et al., 1991; Brown et al., 2010).

*T. cruzi* Genotypes in the US

As a species, *T. cruzi* is very genetically and biologically diverse. Initially, this parasite was divided into two groups based on iso-enzyme analysis (Miles et al., 1981). Numerous subsequent studies based on several molecular techniques have supported the separation of *T. cruzi* into two major lineages (TcI and TcII), with TcII divided into five subgroups (TcIIa-TcIIe) (Souto et al., 1996; Westenberger et al., 2005; Miles et al., 2009). Recently, these six discrete typing units (DTU) have been reclassified as TcI to TcVI (Zingales et al., 2009). TcI and TcII are recognized as ancestral lineages while TcV (old TcIId) and TcVI (old TcIIe) are hybrid lineages. Currently the origins of TcIII and TcIV are not fully understood (Westenberger et al., 2005; Zingales et al., 2009).

The geographical distributions and ecological associations of the various Tc DTUs are distinctive, yet partially overlap (Lewis et al., 2010). *T. cruzi* I has been reported as the main agent of Chagas disease in endemic regions north of the Amazon, and is relatively diverse and widespread (Añez et al., 2004; Black et al., 2007; Mejía-Jaramillo et al., 2009). DTUs TcII, TcV, and TcVI are almost entirely found in domestic transmission cycles in the Southern Cone region of South America, whereas TcIII and TcIV are associated with sylvatic hosts and vectors (but can cause sporadic human infections) (Lewis et al., 2010).

Of these six DTUs, *T. cruzi* I and IV have been isolated within the US (Roellig et al., 2008). Field and experimental-infection trials indicate that certain genotypes of *T. cruzi* are associated with particular wildlife reservoirs (Roellig et al., 2008; Roellig et al., 2009, 2010).

TcI genotypes are typically associated with opossums, while TcIV genotypes are associated with raccoons (Barnabé et al., 2001; Roellig et al., 2010). Some hosts such as skunks, armadillos, and woodrats are infected with both strains of *T. cruzi*. The autochthonous human cases in the US have all been due to the TcI genotype, which may suggest opossums to be the more important reservoirs of *T. cruzi* in the US (Dorn et al., 2007).

Experimental Models of Chagas Disease

Experimental animal models are important in studying the various facets of Chagas disease. Experimental infection of several animal species, including dogs, cats, monkeys, rabbits, guinea pigs, and rodents, have been conducted by Oswaldo Cruz and colleagues as they tried to understand the epidemiology and pathogenesis of *T. cruzi* (Bastien, 1998). Dogs are frequently used as an animal model for Chagas disease due to their similarity of cardiac morphology with humans (Lumb et al., 1959).

Experimental murine models have been shown to closely mimic various aspects of Chagas disease, including immune mechanisms and histopathological implications of infection with different *T. cruzi* strains (Andersson et al., 2003). The acute phase is easily reproduced in mice and can develop with symptoms including anorexia, weight loss, patent parasitemia, edema, and mortality (Federici et al., 1964). Histopathological and gross lesions during the acute phase may include diffuse myocarditis, myositis, lymphadenopathy, and hepatosplenomegaly (Desquesnes and Lana, 2010). Several pathological phenomena were first studied in murine models such as cellular damage, inflammation, and fibrosis. Importantly, different mice lineages (C57BL/10/6, CBA, AKR, C3H, and BALB/c) have varying levels of resistance and susceptibility to *T. cruzi* infection and risk of developing clinical disease (Trischmann et al.,

1978). C3H were found to be the most susceptible, BALB/c of intermediary susceptibility, and C57BL/10 or /6 was the most resistant to *T. cruzi* infection. Resistance in C57BL/6 mice is due to early increased production of interferon-gamma (IFN-γ) during the infection (Hoft et al., 1993). Use of knockout mice has allowed for the elucidation of the importance of certain humoral and cellular immunological processes (Tarleton et al., 1996; Campos et al., 2004; Da Silva et al., 2003; Gao et al., 2002; Holscher et al., 2000; Miyazaki et al., 2010). One study showed that during the acute stage in C57BL/6 mice, there was an intense and polyclonal lymphocyte activation with a majority of the lymphocytes being non-*T. cruzi* specific (Minoprio et al., 1989).

Many experimental *T. cruzi* studies have focused predominately on the factors that alter the parasite-host interaction. Important aspects to consider for these studies are: the strain of *T. cruzi*, the developmental stage of the parasite used for inoculation, the route of exposure, and the host genetics (de Souza et al., 2010). Different strains of *T. cruzi* express different proteins or different levels of certain proteins on their surface, which can alter the intricate interplay of signaling cascades that involve both parasitic and cellular components and seem to regulate the infection process (Yoshida, 2006; Mortara et al., 2008). Different strains may also be more or less virulent for a particular host, such as humans in which the TcI types typically cause a milder form of Chagas disease than TcVI types (Souto et al., 1996; Fernandes et al., 1999; Miles et al., 2003). Additionally, some strains may have differential tissue distribution which may be important for development of tissue damage leading to chronic disease (Andrade et al., 1999, 2010). During chronic infections of mice with TcII and TcVI lineages, Andrade et al. (1999) found that parasites from either strain were detected in mouse hearts during single infections, but during co-infections with both strains, the heart tissue was parasitized exclusively by the TcVI

parasites. A similar co-infection study by Botero et al. (2007), demonstrated that a virulent Columbian strain had a preferential tissue tropism in the heart, rectum, and skeletal muscle. A previous study with US *T. cruzi* strains inoculated into outbred mice showed that no morbidity or mortality developed, but chronic infections were detected (Roellig and Yabsley, 2010).

## Immunology of *T. cruzi* Infection

The outcome and course of *T. cruzi* infection in a host is largely due to the immunological responses of the host and the immune evasion mechanisms of the parasite. The initial infection activates the innate immune system, which has a fundamental role in the control of parasite replication and the inflammatory reaction in host tissue. Innate cells such as natural killer (NK) cells, dendritic cells, and macrophages are key elements in the control of *T. cruzi* replication. These innate cells also produce cytokines, such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin (IL)-12, which can alter the entire subsequent adaptive immune response in the host, and also may infer increased susceptibility or resistance for the host (Brener and Gazzinelli, 1997; Golgher and Gazzinelli, 2004). The adaptive response is characterized by lymphocyte activation and proliferation by antigen presentation, cytokine signaling, and/or immunological memory. Adaptive cells include CD4+ T cells (helper T cells), CD8+ T cells (cytotoxic T cells [CTLs]), and B cells. CTLs are a necessary T cell subset for *T. cruzi* control, as mice deficient in β2-microglobulin are unable to survive a parasite challenge (Tarleton, 1990; Tarleton et al., 1992).

### *Innate Immune Response*

The innate immune response occurs within hours of the initiation of a *T. cruzi* infection. Upon entering the host, extracellular trypomastigotes encounter the complement system,

antibodies, and antimicrobial peptides. Although epimastigotes are susceptible to complement lysis, trypomastigoes actively express various complement system inhibitors (Tambourgi et al., 1993; Norris, 1998; Atayde et al., 2004), however the levels of the functionality and expression of these molecules could be different among *T. cruzi* strains, due to observed variable levels of resistance to complement killing (Krettli et al., 1979; Cestari et al., 2008).

Trypomastigotes and amastigotes infect a variety of phagocytic and non-phagocytic host cells including, macrophages, dendritic cells (DC), fibroblasts, endothelial cells, and cardiomyocytes (Huang et al., 1999; Petersen and Burleigh, 2003; Sibley, 2011). Host cells are capable of recognizing particular microbial peptide motifs in the activation of the innate immune system. Pattern recognition receptors (PRRs) recognize conserved molecular motifs called pathogen-associated molecular patterns (PAMPs). Important PRRs include Toll-like receptors (TLRs) which are localized on the cell surface or within endosomes. The TLRs undergo conformational changes when recognizing a general ligand and activate various signaling pathways and transcription factors. To date, TLR2, TLR4, and TLR9 have all been implicated in the recognition of *T. cruzi* derived components (Gazzinelli and Denkers, 2006; Tarleton, 2007). TLR2 recognizes glycosylphosphatidylinositol (GPI) anchors of blood trypomastigotes and induce an inflammatory response (Campos et al., 2001). TLR4 recognizes ceramide-containing GPI anchors that are expressed on the parasite surface, though abundant on the surface of epimastigotes, these anchors are rare on trypomastigotes, which may have little relevance in *in-vivo* infection (Pereira-Chioccola et al., 2000). However, a recent study showed that the *T. cruzi* enzyme trans-sialidase (TS) is able to directly induce dimerization of the TLR4, activating the downstream signaling independent of PAMP recognition (Amith et al., 2010). TLR9 recognizes unmethylated CpG motifs in *T. cruzi* DNA. These CpG motifs are immunostimulatory, yet as

studies have shown, are only available once the parasites are destroyed in the lysosome-fused vacuoles (Bafica et al., 2006; Bartholomeu et al., 2008). *T. cruzi* can also activate the innate immune response independently from TLRs. *T. cruzi* is able to directly induce interferon-beta (IFN-β), which plays an important role in the control of the parasite (Chessler et al., 2008). Kayama et al. (2009) found that cells infected with *T. cruzi* displayed elevated calcium levels which activated the pathways mediated by NFATC1, leading to IFN-γ production.

Macrophages, Greek for "big eaters", respond to a variety of nonspecific stimuli and are integral in helping initiate the adaptive immune response. The primary role of macrophages is to phagocytose cellular debris and pathogens. Activation of macrophages can include stimulation by microbial products, classical activation by IFN-γ produced by T helper 1 cells (Th1), alternative activation by interleukin-4 (IL-4) and interleukin-13 (IL-13) produced by T helper 2 cells (Th2), or macrophage deactivation by interleukin-10 (IL-10) or tumor growth factor beta (TGF-β) (Fiorentino et al., 1991; Anderson et al., 2002). In a recent microarray study, *T. cruzi*-infected macrophages produced transcriptional signatures closely related to signatures of alternative macrophage activation and macrophage deactivation (Zhang et al., 2010). Macrophage trypanocidal properties involve the production of inducible nitric oxide (NO) synthase which is stimulated by IFN-γ and TNF-α (Golden and Tarleton, 1991; Muñoz-Fernández et al., 1992; Reed, 1998). High amounts of NO with reactive oxygen species (ROS) mediates the intraphagosomal peroxynitrite-dependent killing of the parasites before they escape from the parasitophorous vacuole within the macrophage (Piacenza et al., 2009). Extracellular NO may also kill extracellular trypomastigotes, as well as inhibit cruzipain, a major *T. cruzi* cysteine protease which allows the parasite to penetrate into host cells (Vespa et al., 1994; Venturini et al., 2000). Immunization of BALB/c mice with cruzipain induces alternatively

activated macrophages leading to a dominate Th2 response, whereas immunization of C57BL/6 mice with this protein induces classically activated macrophages, a dominate Th1 response (Guiñazú et al., 2004).

Natural killer (NK) cells are essential effector cells of innate immunity. NK cells are able to directly recognize infected cells before sensitization, as well as interact with a variety of immune cells (DCs, macrophages, T cells) and release potent cytokines (primarily IFN-γ). They are also able to become cytotoxic cells that can remove infected target cells by employing the use of perforin, granzymes, and Fas ligand (Korbel et al., 2004). During *T. cruzi* infection, both cytotoxic and IFN-γ producing NK cells are quickly recruited and activated within the first few days (Hatcher et al., 1981; Hamano et al., 2003). Though an absence of NK cells does not severely impair a Th1 response (Une et al., 2000), NK cells are necessary to activate a Th1 CD4+ T cell-mediated protective mechanism related to expression of HSP65 in macrophages (Sakai et al., 1999).

*Adaptive Immune Response*

The function of the adaptive immune system is specificity and memory of pathogens, which generates stronger and more effective immune responses during subsequent exposures or infections. The adaptive immune system is primarily comprised of T and B cells. T cells are vital for control of *T. cruzi* infection. Genetically modified knockout mice that do not express MHC class I or II, CD4+ or CD8+ antigens, or mice depleted of T cells are highly susceptible to infection and have earlier and higher mortality compared with wild-type mice (Tarleton et al., 1996; Padilla et al., 2009). A mixed Th1/Th2 response has been implicated as the foremost reason for *T. cruzi* persistence in the host (Une et al., 2000; Kumar and Tarleton, 2001). B cells also play an important role in the host susceptibility to *T. cruzi*. Typically, *T. cruzi* infection

induces nonspecific polyclonal B cell activation, which result in a large production of immunoglobulins (Ig) that lack parasite specificity (Ortiz-Ortiz et al., 1980).

T cells are primarily composed of CD4+ and CD8+ T cells. CD8+ T cells target and become cytotoxic to *T. cruzi*-infected cells (Tarleton et al., 1992). In the chronic phase, CD8+ T cells become parasite specific and present an effector phenotype that will suppress the number of infected cells in tissues (Tarleton et al., 1994). Though CD8+ T cells have been shown to be protective, infection is rarely, if ever, completely cured in humans or experimentally infected animals (Bustamante et al., 2008). CD4+ T cells are labeled as helper cells and assist in producing cytokines that enhance immunological functioning. CD4+ T cells are primed by cytokines and co-stimulation of antigen presenting cells (APCs) and develop into effector cells producing distinct cytokine profiles. Interleukin-12 (IL-12), produced by macrophages, transforms CD4+ into Th1 cells, while IL-4 exposure induces a Th2 response. As the infection progresses, CD4+ T cells are the primary producers of IFN-γ, which has been identified as a controlling factor in *T. cruzi* infection (McCabe et al., 1991; Torrico et al., 1991; Hoft et al., 2000). Tarleton et al. (2000) showed that Th1 CD4+ T cells are significant in controlling *T. cruzi* infection, while Th2 cells contribute to parasite persistence.

Humoral immunity during *T. cruzi* infection is characterized by the production of both parasite-specific and nonspecifc antibodies from polyclonal B-cell stimulation (Brener, 1980; Paola, 2001). B cells are lymphocytes that produce Abs and perform the role of APCs to T cells. B cells recognize antigens in their native form and produce a complementary immunoglobulin, with the help of external cues from T cells and cytokines. All B cells are clones, though they can hypermutate their immunoglobulin gene and undergo class switching, which is induced by cytokines. Though the majority of B cells are not parasite-specific early in the infection,

adoptively transferred Abs from chronically infected mice to naïve mice actively clear the parasite from circulation (Minoprio et al., 1988; Brodskyn et al., 1989; Bermejo et al., 2011). Surprisingly, Bryan et al (2010) found resistant C57Bl/6 mice had improved parasite-specific humoral responses associated with a decrease in polyclonal B cell activation, though the infection caused a skewed Th1 response. Typically, a Th2 cytokine profile induces improved antibody response, yet *T. cruzi* infection induces an amplified polyclonal B cell activation and a weakened specific humoral immunity in susceptible hosts. In a study by Cardillo et al. (2007), knockout mice lacking B cells produced decreased amounts of inflammatory cytokines and fewer central and memory T cells compared to wild-type mice during *T. cruzi* infection.

*Chemokines*

Chemokines are small cytokines secreted by cells that act as an attractant to guide the migration of specific cells. There are four groups of chemokines with various roles in the immune system: CC chemokine (β-chemokine), CXC chemokine (α-chemokine), C chemokine (γ-chemokine), and $CX_3C$ chemokine (d-chemokine). Specific chemokines are associated with different Th responses, such as CCR5 and CXCR3 are characteristically coupled with the Th1 response (Bromley et al., 2008). Th1 chemokine expression was associated with the development of chronic cardiac pathology in dogs experimentally infected with *T. cruzi* (Guedes et al., 2010). Chemokines are also able to influence macrophage activation pathways, such as CCL5/RANTES is able to induce NO production to kill *T. cruzi* (Villalta et al., 1998).

*Innate Cytokines*

Cytokines released from innate immune cells play key roles in the regulation of the immune response of *T. cruzi* infection. During the acute phase, production and release of cytokines from innate cells are critical in the inflammation and control of infection. These

cytokines that are released from NKs, macrophages, DCs, and neutrophils include: TNF-α, IFN-γ, IL (-1β, -4, -6, -10, -12, and -18), CCL4/RANTES, and TGF-β (Lacy and Stow, 2011). Pro-inflammatory cytokines serve to recruit and activate T cells for the adaptive immune response (Iijima et al., 2011). TNF-α is a systemic pro-inflammatory cytokine that mediates acute inflammation and contributes to both control of the parasite and host tissue injury (Tarleton et al., 1988; Montalvão et al., 2010). TNF-α also induces apoptosis in cells (Hernandez-Caselles and Stutman, 1993). Yet high levels of TNF-α are associated with toxemia symptoms such as anorexia, lethargy, and cachexia, and can produce toxic shock in the host (Truyens et al., 1995; Holscher et al., 2000). IFN-γ and TNF-α are also important cytokines during the acute phase of *T. cruzi* infection because they induce NO synthesis (Muñoz-Fernández et al., 1992; Arantes et al., 2004). IL-6 is a pleiotropic cytokine that coordinates the transition from innate inflammatory response to adaptive immunity. IL-6 in serum during *T. cruzi* infection is required for parasite-specific responses and host resistance (Truyens et al., 1994; Chandrasekar et al., 1996; Saavedra et al., 1999). Overall, the main functions of cytokines produced by innate cells are to quickly rid the host of the pathogen, and if unsuccessful, initiate the adaptive immune response.

*Adaptive Cytokines*

The direction of the adaptive immune response is driven by the type of pathogen, the innate cells activated, and primarily by the production of cytokines. The Th1 response is normally induced by intracellular pathogens, while the Th2 response is induced by extracellular pathogens, helminthes, and allergens. Adaptive cytokines are mostly produced by T cells that have recognized an antigen from an APC. Proliferation and differentiation of T and B cells are driven by these cytokines which include: IL (-2, -4, -5, -13, -17), IFN-γ, TGF-β, TNF-β, and lymphotoxin (LT). Particular cytokines are characterized within different Th responses. Th1

cells induce production of IL-2, IFN-γ, TNF-β, and LT, and maximize the killing efficacy of macrophages. Th2 cells produce IL-4, IL-5, IL-10, and IL-13 which stimulate the humoral immune response and B cell proliferation. Th17 cells produce IL-17 which in turn stimulates stromal cells to produce IL-6 and IL-8, as well as TNF-α and IL-1β in macrophages and are mostly implicated in triggering excessive inflammatory reactions (Jovanovic et al., 1998).

A mixed Th1/Th2 immune reaction during infection has been implicated in the persistence of *T. cruzi*, though a Th1 skewed response is typically associated with protection. Th1 cells are able to induce NO production and inhibit intracellular parasite replication in *T. cruzi* infected macrophages (Hoft et al., 2000). Recombinant IFN-γ administered to mice increases their resistance to *T. cruzi* (Torrico et al., 1991). Antigen-specific T cells in the resistant C57Bl/6 mice have been shown to produce high levels of IFN-γ after stimulation with *T. cruzi* antigens, whereas no IFN-γ production was seen in the susceptible BALB/c model (Hoft et al., 1993). IL-10 is another important cytokine produced by Th2 cells that implicates susceptibility to the host. IL-10 has antagonistic effects on IFN-γ and can actually suppress the Th1 response. IL-10 has been shown to be elevated in susceptible mouse strains, compared to resistant strains (Silva et al., 1992; Reed et al., 1994). However, there is a large amount of literature that does not ascribe IL-10 with susceptibility (Zhang and Tarleton, 1996; D'Avila et al., 2009; Guedes et al., 2009). An increase in IL-10 has been implicated in enabling parasite persistence but also balancing the host immune response in prolonging the indeterminate form of Chagas disease (Rodrigues et al., 2010; Abrahamsohn and Coffman, 1996; D'Avila et al., 2009). An experimental canine study found high levels of IFN-γ and TNF accompanied by low IL-10 during the acute phase correlated with the development of chronic cardiomyopathy (Guedes et al., 2009).

In conclusion, the literature of experimental and field studies of *T. cruzi* have characterized this pathogen as extraordinarily diverse in host preference, infection dynamics, parasite genetics and evolution. Little is known about *T. cruzi* strains in the US in comparison with the extensive studies of SA strains. Characterization of the biological properties of these strains can provide the US with an assessment of the overall risks these *T. cruzi* strains pose for Americans.

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# CHAPTER 3

# DIVERGING IMMUNE RESPONSES OF BALB/C MICE TO INFECTION WITH DISTINCT *TRYPANOSOMA CRUZI* STRAINS FROM THE UNITED STATES AND SOUTH AMERICA[1]

[1]Edwards, Jessica F., Michael J. Yabsley, Angela E. Ellis, and Wendy T. Watford. To be submitted to PLOS Neglected Tropical Diseases

Abstract

*Trypanosoma cruzi*, the causative agent of Chagas disease, is divided by molecular typing into six groups (Type I-VI), with only TcI and TcIV being found in the United States (US). Infection and virulence of different *T. cruzi* isolates is dependent on numerous factors including parasite and host genetics. Previous studies have indicated that US isolates are less virulent than South America (SA) *T. cruzi* isolates for mice. The aim of the current study was to evaluate the immune response of BALB/c mice during the acute phase of infection with thirteen US and four SA genetically distinct *T. cruzi* isolates. The US strains were isolated from a variety of hosts in different geographical regions including TcI and TcIV genotypes. SA isolates included TcI, TcII, and TcVI. Groups of six mice were intraperitoneally inoculated and cytokine levels were assessed at 0, 1, 3, 5, 7, 10, and 14 days post inoculation (DPI). Mice were sacrificed at 21 and 28 DPI and inflammatory lesion scores were determined for heart and quadriceps tissue. Generally, SA strains induced higher levels of pro-inflammatory cytokines (IFN-γ and TNF). SA Tulahuen (TcVI) infected mice developed exacerbated TNF levels accompanying cachexia through infection. Levels of IFN-γ, TNF, and IL-10 for US TcIV strains were lower than SA and US TcI strains, which corresponded to mild to normal tissue lesions. A US TcI opossum strain (USAOPO) induced all cytokines (except IFN-γ) at 7 DPI, indicating a balanced immune response, yet tissue lesions were comparable with Tulahuen inoculated mice. Overall, results indicate that infection with different *T. cruzi* strains elicit diverging immune responses in mice, with SA strains eliciting a higher pro-inflammatory profile.

Introduction

*Trypanosoma cruzi*, the causative agent of Chagas disease, is a significant cause of morbidity and mortality in Latin America. Approximately 30% of *T. cruzi* infections results in the potentially fatal, chronic cardiomyopathy (Prata, 2001). *T. cruzi* is found from southern South America to as far north as the United States (US) circulating in numerous species of vectors and wildlife reservoirs (e.g., opossums, armadillos, raccoons, and various rodents), domestic dogs, and humans (Clark and Pung, 1994; Yabsley et al., 2001). In the US, only seven autochthonous cases have been confirmed, but recent serologic testing of selected groups and blood donations indicate that a large number of individuals living in the US are infected, some of which are likely undiagnosed autochthonous cases (Herwaldt et al., 2000; Beard et al., 2003; CDC, 2007; Dorn et al., 2007; Leiby et al., 2008). Although human cases are rare, infections in domestic dogs in some areas, such as Texas, are not uncommon and infections in certain wildlife species throughout the southern US are very common (Meurs et al., 1998; Kjos et al., 2008).

*T. cruzi* is a genetically and biologically diverse species and has historically been divided into two main lineages, *T. cruzi* I and *T. cruzi* II, with *T. cruzi* II being subdivided into 5 subtypes (IIa-e). Recently, these divisions have been reclassified as discrete typing units (DTU) (Tc.I – VI) (Zingales et al., 2009). TcI strains are generally regarded as highly variable genetically and present in both the sylvatic and domestic cycles from Mexico down into northern South America (Miles et al., 2009) while TcII, V, and VI predominate in domestic cycles in South America. The TcIV strains are poorly understood and predominately found in sylvatic cycles (Coura et al., 2002). In the US, all isolates from wildlife, dogs, vectors, and humans have been classified as TcI and IV with TcI strains typically associated with opossums and humans, TcIV strains typically associated with raccoons, and both genotypes have been detected in

domestic dogs, woodrats (*Neotoma micropus*), and vectors (Roellig, 2008; Brown et al., 2010; Bern et al., 2011). To date, few studies have investigated biologic characteristics of these US TcI and TcIV strains (Yabsley and Noblet, 2002; Roellig and Yabsley, 2010).

Considerable work has been conducted on the immunological response during the acute and chronic phases of Chagas disease in laboratory mice models (reviewed in Tarleton, 2007; Junqueira et al., 2010). To date, the majority of these studies have been conducted with a limited number of *T. cruzi* strains or inbred mouse strains (Federici et al., 1964; Thomas M., 1986; Andersson et al., 2003; Desquesnes and Lana, 2010). However, because *T. cruzi* is such as diverse species, characterizing the immune responses of the host due to infection with different strains of *T. cruzi* is important to increase our understanding of host-parasite interactions. During the acute phase of infection, IFN-γ, TNF, and IL-10 have been identified as important cytokines of the immune response to *T. cruzi* infection (Guedes, 2009; Savino 2007). IFN-γ and TNF synergistically initiate nitric oxide (NO) (and reactive oxygen species (ROS)) production that represents the first line of defense against *T. cruzi* infection by enhancing macrophage-mediated killing of parasites (Minoprio et al., 1993; Antúnez and Cardoni, 2000; Une et al., 2000). Activation of these cytokines serves key roles in skewing the adaptive immune response to a Th1 favor. Studies have attributed skewing towards a Th1 response to be a hallmark for host resistance to Chagas disease, while skewing towards a Th2 response drives host susceptibility (Kumar and Tarleton, 2001; Hoft et al., 2000; Galvão da Silva et al., 2003). Though IFN-γ and TNF have shown to be protective experimentally, the presence of these cytokines in high levels could promote tissue pathology (Truyens et al., 1995; Roggero et al., 2002; Guedes et al., 2009). Induction of high levels of TNF during *T. cruzi* infection is associated with host tissue damage and cachexia (Truyens et al, 1995); therefore, regulatory cytokines (e.g., IL-10) are induced to

limit exacerbated pro-inflammation. An increase in IL-10 has been implicated in enabling parasite persistence but also balancing the host immune response in prolonging the indeterminate form of Chagas disease (Rodrigues et al., 2010; Abrahamsohn and Coffman, 1996; D'Avila et al., 2009). The roles of IL-2, IL-4, IL-6, and IL-17a during *T. cruzi* infection have also been extensively studied and their potential roles in host resistance or susceptibility vary. For example, although some studies suggest that increased production of the Th2 signature cytokines IL-4 and IL-10 lead to increased host susceptibility (Barbosa de Oliveira et al., 1996; Hiyama et al., 2001); other studies fail to find an association of IL-4 and IL-10 levels with susceptibility (Zhang and Tarleton, 1996; D'Avila et al., 2009; Guedes et al., 2009). Although IL-6 is a potent pro-inflammatory cytokine, it has not been shown to affect the disease outcome of *T. cruzi* infection (Truyens et al., 1994; Truyens et al., 1995). Recently, IL-17a has been demonstrated to be necessary for host protection against acute-phase *T. cruzi* infection (Miyazaki et al., 2010).

The acute phase of *T. cruzi* infection is usually asymptomatic and is characterized as having a high parasitemia and low mortality rate (~5-10% of patients develop encephalitis or acute myocarditis (Prata, 2001). Numerous studies have characterized the immune response of mice and other hosts to *T. cruzi* during the acute phase (Brener, 1980; Roggero et al., 2002); however, relatively few strains have been studied, and most are derived from humans from South America. In recent years, several experimental studies on US *T. cruzi* strains have have shown that they are avirulent for laboratory mice, despite causing persistent infections in wildlife species (Roellig et al., 2010; Roellig et al., 2009; Roellig et al., 2010). Furthermore, molecular characterization of US isolates suggests that they are not as genetically diverse as South American strains and that they may utilize alternative transmission schemes (e.g., vertical transmission) ( Roellig et al, 2008; Hall et al., 2010; Roellig et al., 2011). Although murine

mouse models are routinely used to study the biological characteristics of *T. cruzi*, inoculation of laboratory mice with US *T. cruzi* strains rarely result in detectable parasitemias or pathology, although infections in mice are documented by PCR, culture, or serology (Yabsley and Noblet, 2002; Hall et al., 2010; Roellig and Yabsley, 2010). To date, no work has been conducted on the immune reaction of mice in response to inoculation with US strains of *T. cruzi;* therefore, the primary aim of the current study was to characterize and compare the cytokine profiles produced by BALB/c mice inoculated with genetically and geographically distinct *T. cruzi* strains from the US and SA.

## Materials and Methods

### *Parasites*

A total of 13 *T. cruzi* isolates from the US and four SA isolates were used in this study (Roellig, 2008; Zingales, 2009) (Table 3.1). All strains were previously stored in liquid nitrogen and were rapidly thawed and established in DH82 canine macrophage monolayers to yield the infective culture-derived trypomastigotes as described (Roellig et al., 2009).

### *Mice*

Six-to-eight-week-old female BALB/c mice (n=114) (Harlan Sprague-Dawley, Inc, Indianapolis, Ind.) were housed in microisolator cages in climate-controlled animal facilities at the College of Veterinary Medicine, University of Georgia (Athens, GA). All methods were approved by the Institutional Animal Care and Use Committee at the University of Georgia (A2009 3-066).

*Experimental design*

Groups of six randomly selected mice were inoculated intraperitoneally with 5 x $10^5$ culture-derived trypomastigotes of one of 17 isolates, four from South America and 13 from the US (Table 3.1). Negative controls (n=12) were similarly inoculated with an equivalent volume of culture medium. All mice were observed daily for any physical or behavioral changes indicative of Chagas disease, such as lethargy, hind limb paralysis, weight loss, or ruffed coat. Three mice from each group were bled via the tail vein on 0, 1, 3, 5, 7, 10, 12, & 14 days post inoculation (DPI). Plasma was separated from blood and frozen until cytokine analysis. At days 21 & 28 DPI, three mice from each group were humanely euthanized and ~0.75 mL of whole blood was collected by cardiocentesis into ethylenediaminetetraacentic acid (EDTA) tubes. A necropsy was conducted and ~50 mg of heart and quadriceps tissue was collected. One half of each tissue sample was fixed in formalin for histological evaluation and the remaining half was frozen at -20C for future PCR analysis. Groups of two age-matched control mice were sacrificed at the same time points and sampled as noted above.

*Histopathological Evaluation*

Formalin-fixed tissues were routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Slides were examined by light microscopy and scored blindly by a pathologist. Based on the severity of tissue damage and inflammation, each tissue was given a score of normal (0), mild (1), moderate (2), or severe (3). Presence of amastigote nests were also noted in tissues after scanning 40 fields at 400X magnification.

*Quantification of Murine Cytokines*

Levels of IL-2, IL-4, IL-6, IL-10, IL-17a, IFN-γ, and TNF cytokines in the plasma of mice were quantified using the Mouse Th1/Th2/Th17 Cytokine Kit from BD™ (Cytometric

Bead Array- CBA) (BD Biosciences, San Jose, CA). The assay was performed according to the manufacturer's instructions, with modifications. Briefly, capture beads (25 µl) were added to a polystyrene round-bottom tube (Becton and Dickinson Inc, Franklin Lakes, NJ) combined with either 25 µl of cytokine standard (range of 0 to 5,000 pg/ml), or with 25 µl of plasma samples. Phycoerythrin (PE) -conjugated detection reagent (25 µl) was added to each tube and incubated for 2 h at room temperature in the dark. CBA wash buffer was added and tubes were centrifuged at 400 x g for 10 min. Supernatants were removed, and bead pellets resuspended in 200 µl of CBA wash buffer. The fluorescence intensity of the CBA beads was measure on a BD$^{TM}$ LSRII flow cytometer using the FACSDiva software (BD Biosciences, San Jose, CA) and analyzed using FCAP Array software (Soft Flow Inc., Burnsville, MN).

*Statistical Analyses*

The data were analyzed using analysis of variance to compare between more than two groups. For post-hoc analysis, Fisher's least significant difference method was used and p-values of $<0.05$ were considered significant. Results in Figures 3.1 and 3.2 are expressed as means ± SE.

## Results

*General characteristics of infections*

No mice during the pilot or main trials inoculated with US strains of *T. cruzi* developed morbidity or mortality. On 12 DPI, mice infected with the SA Tulahuen strain became lethargic and developed mild cachexia and diarrhea. By 14 DPI, the Tulahuen-infected mice weighed significantly less than control mice (data not shown) and due to increased severity of clinical

signs, they were euthanized. Weights of mice inoculated with all other strains were similar to weights of control mice.

*IFN-γ, TNF, and IL-10 (Figure 3.1)*

IFN-γ levels between strains were significant from 1 DPI through 28 DPI. Generally SA and US TcI strains induced higher levels of IFN-γ than the US TcIV strains, specifically Tulahuen and LC induced 10-fold greater levels of IFN-γ compared with the all of the US TcIV strains. The strains that induced the highest levels during the experiment were LC, Tulahuen, TXWR22, and Brazil *($p<0.05$)*. Mice inoculated with the SA Tulahuen (TcVI) strain produced significantly more IFN-γ at 3 and 7 DPI compared with all other strains (*$p<0.05$*). LC induced the highest IFN-γ at 1, 5, and 10 DPI of all the strains ($p<0.05$). At 21 DPI, three out of the four opossum strains (GAOPO75, USAOPO, and GAOPO43) had significantly higher levels than all other strains excluding TXWR22 and Ca-1. By day 28 post inoculation, mice inoculated with the Brazil strain displayed the highest IFN-γ levels ($p<0.05$). Interestingly, RTL Meg induced the least amount IFN-γ over the time-course, with an average level of 2.95 pg/ml, which was not statistically different from the control.

Levels of TNF began to increase for all strains by 5 DPI and for most strains, slowly rose, peaked, and began to decrease by DPI 28, indicating systemic inflammation later in the acute phase. However, there was individual variation in time and intensity of peak TNF levels. One exception was the Tulahuen (TcVI) strain, which induced significantly higher levels of TNF from 10-14 DPI, that continued to increase until they were euthanized on 14 DPI due to morbidity (*$p<0.05$*.). Interestingly, USAOPO (TcI) induced significantly greater levels of TNF on 5 DPI than all other strains, except for Tulahuen, Y Strain, FLRac9, Ca-1, TXWR22, and TXTG2, yet exhibited similar levels of TNF to other strains throughout the rest of the experiment

(*p<0.05*). Of the US TcIV strains, only FL Rac9 induced significantly higher TNF at 5 and 7 DPI ($p<0.05$). At 21 DPI, TXWR22 induced the greatest levels of TNF that were similar to Ca-1 and USAOPO, yet significantly different from all other strains ($p<0.05$). Brazil, USAOPO, and LC were all significantly higher at 28 DPI than other strains ($p<0.05$).

Although the overall average levels of IL-10 produced by SA strains were generally higher than US strains, TXWR30, TXWR22, FL Rac9, FLOPO3, and TXTG2 induced higher IL-10 levels at 5 DPI than all other strains (*p<0.05*). IL-10 levels did not always correspond with increased IFN-γ or TNF levels for most strains to maintain immune homeostasis, even in mice inoculated with US strains, and was relatively absent after day 7 with the most virulent strain, Tulahuen (TcVI). FL Rac9 and TXWR22 mice showed correlation of the pro-inflammatory cytokines (IFN-γ and TNF) with the anti-inflammatory cytokine, IL-10 at 5 DPI. Brazil also had significantly higher levels of IL-10 at 28 DPI, which correlated with IFN-γ and TNF levels at that time ($p<0.05$). Considerable variation in peak IL-10 levels were also noted within US strains, specifically between 3-7DPI.

Interestingly, mice inoculated with US TcI strains developed considerable variation in levels of IFN-γ, IL-10, and TNF compared with the US TcIV strains. Within the US TcI strains, variation was noted by cytokine, strain, and day. For example, USAOPO (TcI) strain induced the highest TNF levels at 7 DPI (*p<0.05*), yet had similar IFN-γ and IL-10 levels to other strains that same day. US TcI strains induced significantly higher IFN-γ and TNF than the US TcIV strains at 3-5 DPI and 10-28 DPI (*p<0.05*). Unexpectedly, mice inoculated with some US TcIV strains induced very low IFN-γ and TNF levels that were similar to control mice (RTL and GA Arm20) (*p<0.05*). FLRac9 induced significantly higher levels of TNF compared with the other TcIV strains (*p<0.05*). Among the TcIV strains, similar levels of IFN-γ, TNF, and IL-10 levels

were induced, with exceptions of strains FLRac9 and TXWR30 which induced relatively higher levels of these cytokines.

*IL-2, IL-4, IL-6, and IL-17a (Figure 3.2)*

Generally, levels of IL-4 and IL-6 were similar between strains from SA and US across time points. However there was significant difference in IL-6 levels on 7, 10, 14, and 28 DPI, with the USAOPO, Tulahuen and Brazil strain inducing the highest levels ($p<0.05$). Brazil and USAOPO mice were also significantly higher in IL-4 levels on those days, as well as TXWR22 at 10 DPI ($p<0.05$). IL-2 levels appeared to be higher in SA strains compared with the US strains, yet GASK1, TXWR22, and USAOPO actually induced the highest levels at 3, 10, and 21 DPI, respectively ($p<0.05$). SA strains generally induced higher IL-17a levels than the US strains, and within the US strains, IL-17a levels were comparable. Yet the Brazil strain was the only strain that induced significantly more IL-17a at 28 DPI ($p<0.05$).

*Cytokine levels by host and geographical regions*

Among the TcI strains isolated from human and woodrats, IFN-γ and TNF levels were significantly higher than TcI strains isolated from other hosts ($p<0.05$, Figure 3.1). Among the sylvatic hosts, woodrat strains induced the highest levels of IFN-γ, TNF, IL-6, and IL-10 in mice compared with the other US strains ($p<0.05$, Figures 3.1 and 3.2). Interestingly, the TcIV isolate from the ring-tailed lemur (RTL Meg) induced relatively no immune response in mice. Strains derived from opossums and raccoons induced similar cytokine profiles, except opossum strains generally induced greater IFN-γ levels (Figure 3.1).

When cytokine levels among regions were compared (both genotypes combined) strains from Georgia and Florida induced significantly lower levels of pro-inflammatory cytokines (IFN-γ, TNF, and IL-6) compared with the levels induced by SA strains ($p<0.05$, Figures 3.1 and

3.2). Strains (both genotypes combined) from Louisiana and Texas induced similar pro-inflammatory cytokine levels to SA strains ($p< 0.05$, Figure 3.1 and 3.2). When cytokine levels from sylvatic strains from Texas and Georgia (2 TcI and 2 TcIV from each state) were compared, significant differences were only noted for IL-10 levels, with significantly higher levels observed for the two Texas strains ($p<0.05$, Figure 3.1).

*Histopathological Lesions*

Quadriceps and heart tissues were scored normal, mild, moderate, or severe for inflammation and lesions. In Tulahuen inoculated mice, tissues were severely inflamed and amastigote nests were seen in both the heart and quadriceps. Surprisingly, TXWR30 (TcIV) mice had relatively severe inflammation in the heart, yet had normal scores in the quadriceps tissues, suggesting a tissue tropism for this strain. Tulahuen, Brazil, and USAOPO mice developed more severe lesions in both the heart and the quadriceps compared with the other strains. Generally, TcIV strains did not induce tissue damage in either the heart or quadriceps.

## Discussion

This represents the first study to characterize the cytokine profiles induced by a diverse group of *T. cruzi* strains from the US. In general, mice inoculated with *T. cruzi* responded with a pro-inflammatory response which is consistent with other studies (Rodrigues et al., 2010; Cummings and Tarleton, 2003; Kumar and Tarleton, 2001). However, each of the 17 strains included in this study exhibited its own unique cytokine profile, which is most likely attributable to biological variability (e.g., cell preference, replication rates and/or parasite induced immunological modulation). In similar studies, US isolates have displayed a reduced reproductive rate *in vivo* and *in vitro*, compared with SA isolates (Yabsley and Noblet, 2002;

Hall et al., 2010; Roellig et al., unpublished data). This reduced parasite reproductive rate likely results in the lower levels of pro-inflammatory cytokines, and subsequent tissue inflammation. Despite the variability, some trends were noted. For example, the six TcIV strains generally induced lower levels of cytokine production. The four SA strains, in general, induced greater IFN-γ, TNF, IL-6, and IL-10 than the 13 US strains. Interestingly, IL-10 levels dropped to low levels after 10 DPI and remained low until DPI 28. In a similar murine study using SA strains, IL-10 levels were also maintained close to basal levels from 7-21 DPI, while pro-inflammatory cytokine levels presented great variation between experimental groups in the acute phase (Rodrigues et al., 2010).

None of the US strains resulted in morbidity or mortality which is consistent with most experimental studies with laboratory mice (Yabsley and Noblet, 2002; Roellig et al., 2008; Hall et al., 2010). Though certain US strains (e.g., TXWR22) induced similarly high levels of pro-inflammatory cytokines as SA strains, US strains, in general, did not elicit as intense of an immune response as SA strains. In the current study, one SA strain (Tulahuen) caused morbidity which was associated with high TNF levels. This strain was known to be virulent and previous studies have shown that the wasting syndrome associated with Tulahuen infection in mice is a result of high TNF levels, not of high IFN-γ or IL-6 levels (Truyens et al., 1995; Holscher et al., 2000). Furthermore, Tulahuen also induced the earliest IL-10 peak at 3 DPI after which IL-10 was fairly absent. In a similar experiment, IL-10 $^{-/-}$ mice inoculated with the Tulahuen strain experienced intense TNF-mediated toxic shock syndrome and death within 14 DPI, despite presenting low parasitemia levels and high systemic pro-inflammatory cytokines (IFN-γ, IL-12, and TNF) during the acute phase of infection (Abrahamsohn and Coffman, 1996; Holscher et al., 2000). The other three SA strains (Brazil, Ca-1, and Y strains) induced lower inflammatory

cytokine levels compared with the Tulahuen strain and induced IL-10 peaks at later DPIs. These later IL-10 levels may have provided some protection to the mice making these strains relatively less virulent in the acute phase compared with the Tulahuen strain. Also, high levels of IL-10 can enhance parasite persistence and in some studies, susceptibility to infection (Reed et al., 1994). These results are similar to previous studies on cytokine profiles induced by these three strains (Une et al., 2000; Cummings and Tarleton, 2004; Rodrigues et al., 2010).

The cytokine profiles induced by the US *T. cruzi* strains were highly variable, regardless of DTU or host origin. Only two of the four TcI opossum strains from the US exhibited similar cytokine profiles (GAOPO75 & GAOPO43), with moderate IFN-γ levels and little to no TNF or IL-10 production. Interestingly, the cytokine profiles of US TcIV strains were less variable than US TcI strains; although some TcIV strains, such as FL Rac9 and TXWR30 induced moderately higher levels of IFN-γ and IL-10, respectively. Recently, genetic characterization of US TcIV strains indicated that they are in a separate clade from SA TcIV reference strains (Yeo et al., 2011; Roellig et al, unpublished). Furthermore, US TcIV strains have limited genetic variability which may explain their relative homogenous effects on the immune system compared to other strains. Similarly, tissue inflammation or damage in the heart and quadriceps was not observed with the TcIV isolates, which suggests that these strains are avirulent for mice during the acute phase. A recent study identified a raccoon US TcIV isolate that had an enhanced ability for vertical transmission compared with a SA TcI (Brazil) strain (Hall et al., 2010) which may have resulted in US TcIV isolates having adapted to induced lower levels of pro-inflammatory cytokines during acute phase. Future studies should investigate the virulence of US TcIV during the chronic phase of the infection.

Some studies have associated certain cytokine profiles with the development of Chagas disease. For example, high IFN-γ, TNF, and IL-10 with moderate IL-4 and IL-6 levels in the acute phase generally contributes to the indeterminate form of Chagas disease (Guedes et al., 2009). In chronic Chagasic patients, high IFN- γ and low IL-10 levels corresponded with the cardiac form of the disease (D'Avila et al., 2009). Based on these previous studies, none of the TcIV isolates are predicted to be virulent because cytokine levels were very low. Interestingly, considerable variation was noted among the US TcI isolates with some strains having high levels of certain pro-inflammatory cytokines but low levels of others that should be correspondingly high (e.g., LC strain had high IFN- γ but low IL-10 and TNF and USAOPO had low IFN- γ and high levels of IL-10 and TNF). LC may be initiating IFN-γ, yet inhibiting or neutralizing expression of TNF and IL-10. A *T. cruzi* mucin, AgC10, has been shown to inhibit TNF, IL-10, and cyclooxygenase-2 (COX-2) synthesis by macrophages activated with LPS plus IFN-γ. Consistent with previous studies, levels of IL-2, IL-4, and IL-17a were variable, yet was not a dominant factor in difference of immune responses between the strains (Truyens, 1994; Soares, 2003; Rodrigues, 2010). Only one US isolate (USAOPO) had a relatively high level of IL-4, which has been associated with parasite persistence, but we were not able to investigate persistence as this study only examined the acute phase of infection. USAOPO also notably initiated high levels of every cytokine (except for IFN-γ) at 7DPI, which suggests activation of a balanced and regulated cytokine response not seen in the other strain infections. Further investigations into the parasite components of USAOPO may uncover factors that contribute to such a robust immune response not regularly seen in *T. cruzi* infection. Additional studies on the infection dynamics and histopathologic lesions produced by US *T. cruzi* isolates during the chronic phase are needed to better understand the chronic disease potential of US strains.

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Table 3.1. Description of 13 *Trypanosoma cruzi* strains isolated in the United States and South America

| **Strain** | **Lineage*** | **Host** | **Origin** |
|---|---|---|---|
| FL Opo 3 | I | *Didelphis virginiana* | Wakulla County, FL |
| USA Opossum | I | *D. virginiana* | Orleans Parish, LA |
| GA Opo 75 | I | *D. virginiana* | Clarke County GA |
| GA Opo 43 | I | *D. virginiana* | Chatham County , GA |
| TxTg2 | I | *Triatoma gerstackeri* | TX |
| TX WR 22 | I † | *Neotoma micropus* | Uvalde County , TX |
| TX WR 30 | IV† | *N. micropus* | Uvalde County, TX |
| FL Rac 9 | IV | *Procyon lotor* | Liberty County , FL |
| TX08 Rac 5 | IV † | *P. lotor* | Uvalde County , TX |
| RTL Meg | IV | *Lemur catta* | Liberty County , GA |
| GA Sk1 | IV | *Mephitis mephitis* | Long County , GA |
| GA Arm 20 | IV | *Dasypus novemcinctus* | Chatham County , GA |
| LC | I | Human | Orleans Parish, LA |
| Brazil | I | Human | Brazil |
| Ca-1 | I | Human | Argentina |
| Y | II | Human | São Paulo, Brazil |
| Tulahuen | VI | Human | Tulahuen, Chile |

* Unless noted, lineage was determined by Roellig et al 2008.

† Lineage determined by Charles et al. (submitted) by methods of Brisse et al 2006.

Figure 3.1. Levels of IFN-γ (row 1), IL-10 (row 2), and TNF (row 3) at 0-28 DPI for South American strains (column A), United States TcI strains (column B), and United States TcIV strains (column C). NOTE: each graph has a different scale for the y-scale (pg/µl of each cytokine).

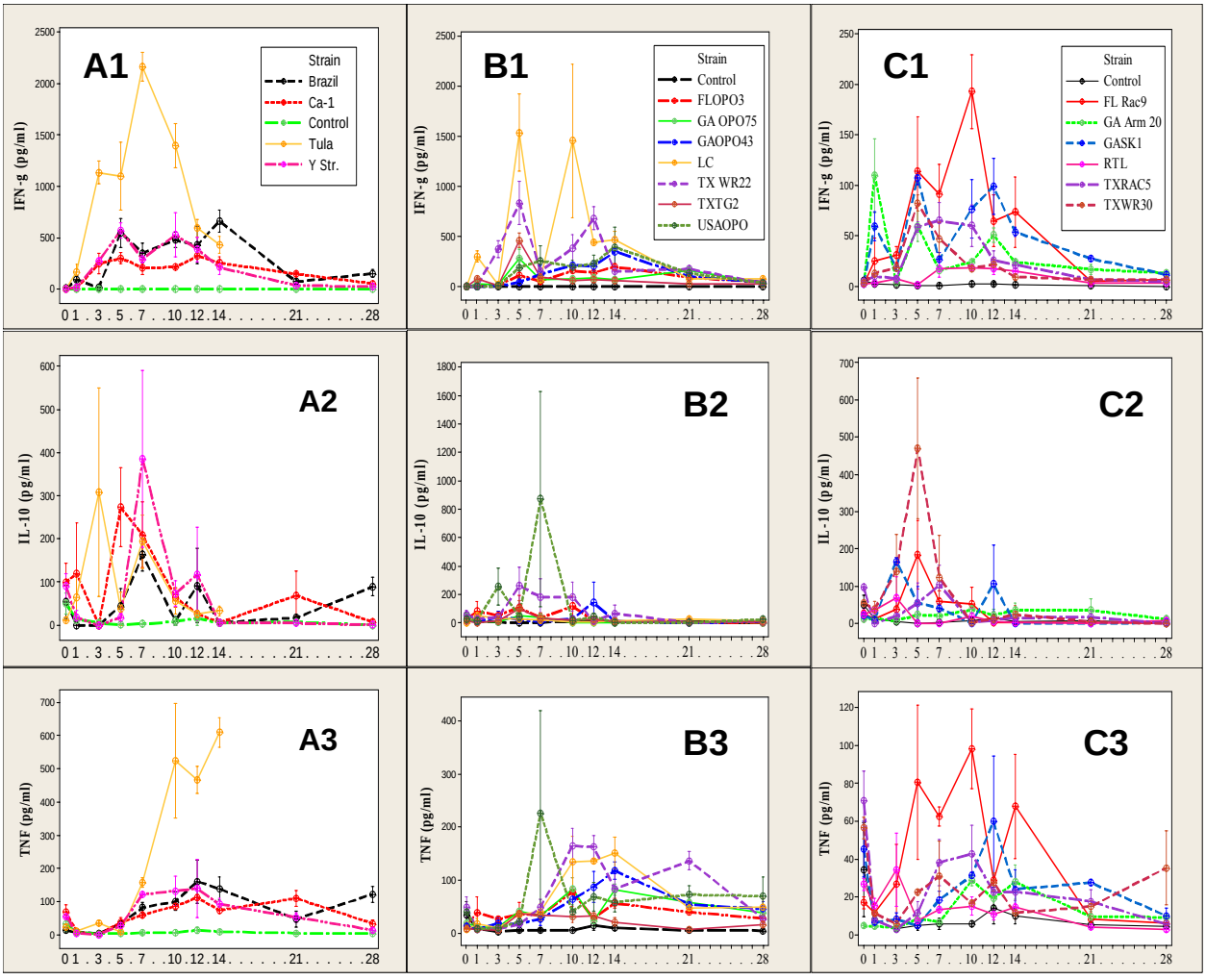
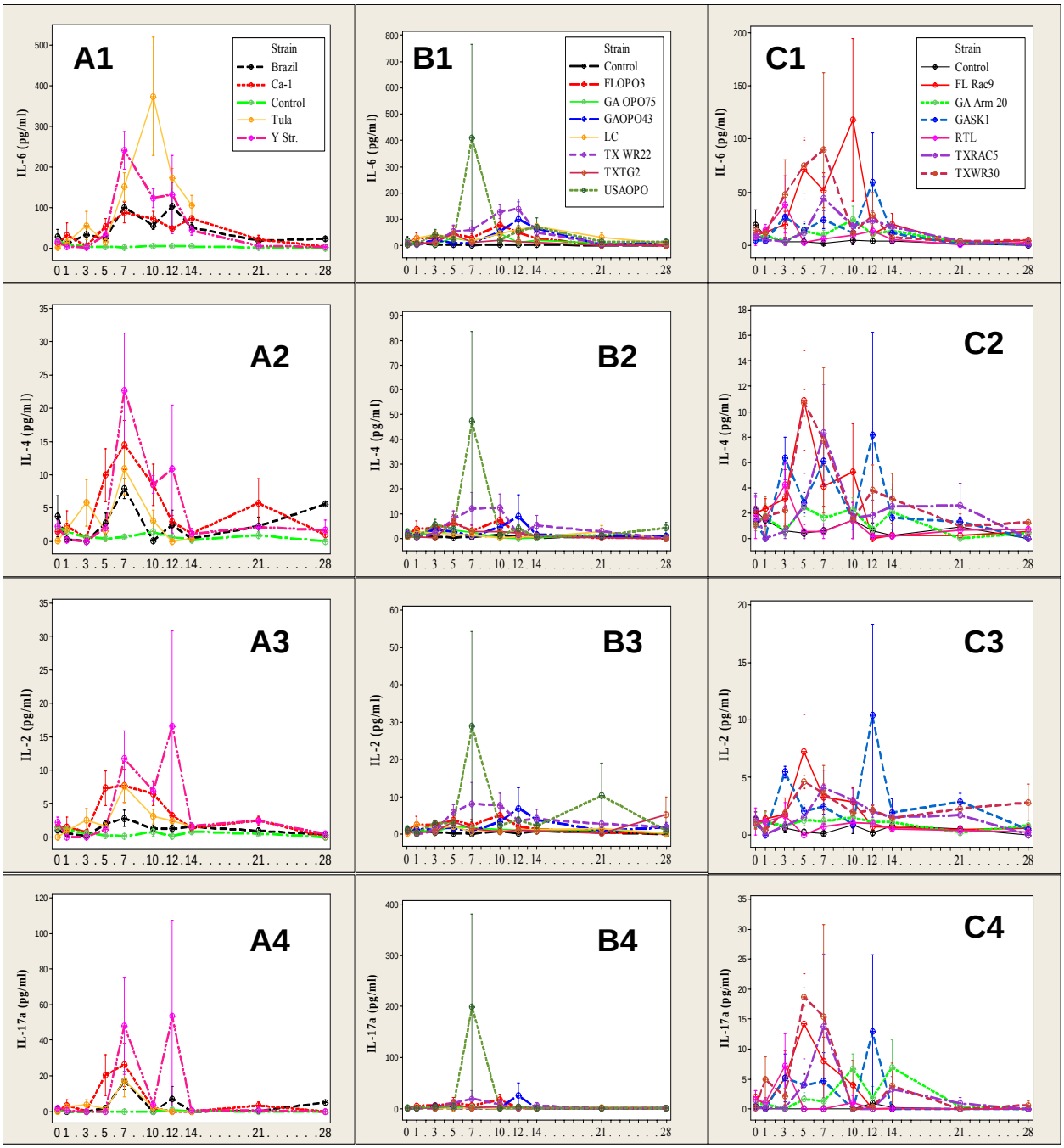


Figure 3.2. Levels of IL-6 (row 1), IL-4 (row 2), IL-2 (row 3), and IL-17a (row 4) at 0-28 DPI of South American strains (column A), United States TcI strains (column B), and United States TcIV strains (column C). NOTE: each graph has a different scale for the y-scale (pg/µl of each cytokine).



# CHAPTER 4

# PRIMARY INFECTION OF BALB/c MICE WITH A UNITED STATES *TRYPANOSOMA. CRUZI* ISOLATE RESULTS IN ALTERED IMMUNE RESPONSE DURING A CHALLENGE INOCULATION[2]

[2]Edwards, Jessica F., Wendy T. Watford, Angela E. Ellis, and Michael J. Yabsley. To be submitted to Journal of Parasitology.

Abstract

*Trypanosoma cruzi*, the causative agent of Chagas disease, is an important cause of morbidity and mortality for people and some animals. The current study was conducted to determine if previous inoculation of laboratory mice with a US *T. cruzi* isolate results in an altered immune response after a challenge inoculation. Groups of mice were inoculated with either a US strain (FLOPO3) or the Brazil strain. After 112 days, subsets of FLOPO3-inoculated mice were challenged with either Brazil or FLOPO3 and cytokine levels were determined at 0, 1, 7, 14, 21, and 28 days post infection/challenge (DPI/DPC). Mice were sacrificed at 28 DPI/DPC and inflammatory lesion scores determined for heart and quadriceps tissue. In general, IFN-γ, IL-10, and IL-6 levels were highest in Brazil-infected mice compared with FLOPO3-infected mice. Mice challenged with the FLOPO3 strain produced lower levels of IFN-γ and TNF and higher levels of IL-4 compared with primary infection mice while Brazil-challenged mice produced lower levels of IFN-γ, IL-6, IL-10, IL-17a, and TNF compared with primary infected mice. Tissue inflammation in challenge mice was mild. Overall, our results indicate that previous inoculation with FLOPO3 and subsequent challenge with the Brazil strain results in lower pro-inflammatory cytokine levels and milder inflammation.

Introduction

*Trypanosoma cruzi* is a zoonotic parasite that is maintained in nature in a large number of wild and domestic mammals and numerous species of reduviid bugs. In Latin America, where the majority of endemic transmission occurs, an estimate of 8-10 million people are infected with *T. cruzi* and nearly 50,000 deaths occur each year due to Chagas disease (Bern et al., 2011). In the United States (US), there have been seven autochthonous cases reported in humans

(Herwaldt et al., 2000; Beard et al., 2003; Dorn et al., 2007). *T. cruzi* mainly persists in the US in a primarily sylvatic transmission cycle that involves numerous wildlife reservoirs and triatomine vectors. *T. cruzi* is a very genetically and biologically diverse species and is divided into six discrete typing units (DTUs) (TcI – TcVI) (Zingales et al., 2009). TcI has been reported as the main agent of Chagas disease in endemic regions north of the Amazon, and is relatively diverse and widespread (Añez et al., 2004; Black et al., 2007; Mejía-Jaramillo et al., 2009). DTUs TcII, TcV, and TcVI are almost entirely found in domestic transmission cycles in the Southern Cone region of South America, whereas TcIII and TcIV are associated with sylvatic hosts and vectors (but cause sporadic human infections) (Lewis et al., 2010).

In the US, all isolates from wildlife, dogs, vectors, and humans have been classified as TcI or IV with TcI strains being most commonly isolated from opossums and humans, TcIV strains from raccoons, and both genotypes have been detected in domestic dogs, woodrats (*Neotoma micropus*), and vectors (Roellig et al., 2008; Brown et al., 2010; Bern et al., 2011). Several studies have indicated US *T. cruzi* isolates are less virulent compared with SA strains because laboratory mice inoculated with US strains have lower or absent parasitemias and don't develop morbidity and/or mortality (Hall et al., 2010; Yabsley and Noblet, 2002). One study also reported that mice inoculated with a US TcIV isolate from a Georgia raccoon (*Procyon lotor*) and subsequently inoculated with a virulent SA Brazil strain had lower parasitemia levels compared with mice only infected with the Brazil strain (Hall et al., 2010).

To better understand the complex host-parasite relationships, it is important to understand the immune responses and pathology due to different strains of *T. cruzi*,. Experimental murine models closely mimic various aspects of Chagas disease, including immune mechanisms and histopathological implications of infection (Andersson et al., 2003). Cytokines are an important

factor in the development of disease and control of infection and IFN-γ is one of the most important protective cytokines during *T. cruzi* infection. IFN-γ and TNF act synergistically on macrophages to induce nitric-oxide synthesis (NO), which is an essential trypanocidal factor in host resistance (Antúnez and Cadoni, 2000). However, induction of high levels of TNF during *T. cruzi* infection is associated with host tissue damage and cachexia (Truyens et al, 1995); therefore, regulatory cytokines (e.g., IL-10) are induced to limit exacerbated pro-inflammation. An increase in IL-10 has been implicated in enabling parasite persistence but also balancing the host immune response in prolonging the indeterminate form of Chagas disease (Rodrigues et al., 2010; Abrahamsohn and Coffman, 1996; D'Avila et al., 2009). In dogs, high levels of IFN-γ and TNF accompanied by low IL-10 during the acute phase correlates with the development of chronic cardiomyopathy (Guedes et al., 2009).

Studies on host-parasite interactions with US *T. cruzi* strains are limited; therefore, the goal of the current study was to investigate the virulence and protective immunologic response of a US TcI isolate. Specifically, we evaluated the immune responses of BALB/c mice to primary and challenge infection with an avirulent US TcI isolate or with a SA virulent TcI isolate. Our data indicated that prior infection with the avirulent US TcI isolate modulates the host immune response in a subsequent challenge.

## Materials and Methods

### *Parasites*

The Brazil strain of *T. cruzi* (TcI), originally isolated from a human, was obtained from Dr. Rick Tarleton (University of Georgia, Athens, GA) and the FLOPO3 *T. cruzi* strain (TcI) was obtained from a Virginia opossum (*Didelphis virginiana*) and characterized during

epizootiological studies of reservoir hosts in the southeastern US (Brown et al., 2010; Roellig et al., 2010). All strains were previously stored in liquid nitrogen and were rapidly thawed and established in DH82 canine macrophage monolayers to yield the infective culture-derived trypomastigotes as described (Roellig et al., 2009).

*Mice*

Six-to-eight-week-old female BALB/c mice (n=26) (Harlan Sprague-Dawley, Inc, Indianapolis, Ind.) were housed in microisolator cages in climate-controlled animal facilities at the College of Veterinary Medicine, University of Georgia (Athens, GA). All methods were approved by the Institutional Animal Care and Use Committee at the University of Georgia (A2009 3-066).

*Experimental Design*

A total of 18 mice were randomly divided into three groups of six mice. Group 1 was inoculated with 5 x $10^5$ trypomastiogotes of the Brazil strain, whereas Groups 2 and 3 were inoculated with 5 x $10^5$ trypomastiogotes of the FLOPO3 isolate. Four control mice were inoculated with an equivalent volume of media. After 112 days post inoculation (DPI), three randomly selected mice from Group 3 (Group 3a) were administered a challenge dose of 5 x $10^5$ trypomastigotes of the Brazil strain. The remaining 3 mice (Group 3b) were administered a challenge dose of 5 x $10^5$ trypomastigotes of the FLOPO3 isolate. Four additional negative controls were similarly inoculated with an equivalent volume of culture medium.

All mice were observed daily for any physical or behavioral changes indicative of Chagas disease, such as lethargy, hind limb paralysis, weight loss, or ruffed coat. Three mice from each group were bled and plasma collected at 0, 1, 7, 14, and 21 DPI and/or days post challenge (DPC). At 28 DPI/DPC, all mice were humanely euthanized and ~0.75 mL of whole blood was

collected by cardiocentesis into ethylenediaminetetraacentic acid (EDTA) tubes. A necropsy was conducted and ~50 mg of heart and quadriceps tissue was collected. One half of each tissue sample was fixed in 10% buffered formalin for histological evaluation and the remaining half was frozen at -20C for future PCR analysis. Plasma was separated from blood and frozen until cytokine analysis. Groups of two age-matched control mice were sacrificed at the same time points and sampled as noted above.

*Histopathological Evaluation*

Formalin-fixed tissues were routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Slides were examined by light microscopy and scored blindly by a pathologist. Based on the severity of tissue damage and inflammation, each tissue was given a score of normal (0), mild (1), moderate (2), or severe (3). Presence of amastigote nests were also noted in tissues after scanning 40 fields at 400X magnification.

*Quantification of Murine Cytokines*

Levels of IL-2, IL-4, IL-6, IL-10, IL-17a, IFN-γ, and TNF cytokines in the sera of mice were quantified using the Mouse Th1/Th2/Th17 Cytometric Bead Array (CBA) kit from BD™ (BD Biosciences, San Jose, CA). The assay was performed according to the manufacturer's instructions, with modifications. Briefly, capture beads (25 μl) were added to a polystyrene round-bottom tube (Becton and Dickinson Inc, Franklin Lakes, NJ) combined with either 25 μl of cytokine standard (range of 0 to 5,000 pg/ml), or with 25 μl of plasma samples. Phycoerythrin (PE) -conjugated detection reagent (25 μl) was added to each tube and incubated for 2 h at room temperature in the dark. CBA wash buffer was added and tubes were centrifuged at 400 x g for 10 min. Supernatants were removed, and bead pellets were resuspended in 200 μl of CBA wash buffer. The fluorescence intensity of the CBA beads was measure on a BD™ LSRII flow

cytometer using the FACSDiva software (BD Biosciences, San Jose, CA) and analyzed using FCAP Array software (Soft Flow Inc., Burnsville, MN).

*Statistical Analyses*

The data was analyzed using Two Way Analysis of Variance to compare between more than two groups. For post-hoc analysis, Fisher's least significant difference method was used. P-values $<0.05$ were considered significant. Results in Figures 4.1 and 4.2 are expressed as means ± SE.

Results

None of the mice, regardless of inoculum, exhibited any signs of morbidity or mortality during the experiment. Weights of experimental mice were similar to weights of control mice (data not shown).

Differences in cytokine production were noticed in primary mice inoculated with the two different *T. cruzi* strains. Primary mice inoculated with the Brazil strain (Group 1) produced significantly higher IFN-γ on 7 and 14 DPI and higher IL-10 and IL-6 levels at 7 DPI than primary mice inoculated with FLOPO3 (Group 2) (Figures 4.1 A-B, D-E and 4.2 A and E). There was no significant difference in TNF, IL-2, IL-4, and IL-17a levels between primary mice inoculated with the Brazil strain (Group 1) or FLOPO3 (Group 2) (Figures 4.1 C and F and 4.2 B-D, F-H).

Primary and challenged mice displayed unique immune profiles with a general decrease in the levels of pro-inflammatory cytokines being induced in the challenged mice. In regards to the FLOPO3 groups, primary mice inoculated with FLOPO3 (Group 2) produced significantly higher levels of the pro-inflammatory cytokines IFN-γ and TNF compared with the challenged

group (Group 3b) on 14 and 21 DPI (Figure 4.1 A and B). In contrast, levels of IL-4, a Th2 signature cytokine, were generally higher in the challenged group across all time points (Figure 4.2 B). There was no difference in IL-2, IL-6, IL-10, and IL-17a levels between primary mice (Group 2) and challenged mice (Group 3b) inoculated with FLOPO3 (Figures 4.1 B. and 4.2 A, C and D).

Within groups 1 and 3a that were inoculated with the Brazil strain, challenged mice generally produced lower levels of all cytokines across all time points, except for the 1 DPC timepoint when cytokines of challenged mice were higher (Figures 4.1 D-F and 4.2. E-H). IFN-γ and IL-6 levels were significantly higher among primary mice at DPI 7 and 14 compared with challenged mice at DPC 7 and 14, respectively (Figure 4.1 D and E). Similarly, IL-10, IL-17a and TNF levels were higher for primary mice on DPI 7 (IL-17a, IL-10) and 14 (TNF) compared with challenged mice on DPC 7 and 14, respectively (Figures 4.1 E-F and 4.2 H).

No differences were noted in cytokine levels between mice challenged with Brazil (Group 3a) or FLOPO3 (Group 3b) isolates, except for IFN-γ. Mice challenged with the Brazil strain induced earlier and significantly higher IFN-γ levels at 1 DPC than mice challenged with FLOPO3 (Group 3b), but the latter group retained higher IFN-γ levels compared with Group 3a (Brazil) throughout the rest of the experiment (Figure 4. 1. A & D).

In primary mice inoculated with the Brazil strain, inflammation was generally more severe in quadriceps and heart tissue than primary mice inoculated with the FLOPO3 strain. Significantly milder lesion scores were determined for hearts and quadriceps from challenged mice (both FLOPO3 and Brazil groups combined) as compared with mice from the primary inoculations (data not shown). No difference was noted in the lesion scores between the Brazil

and FLOPO3 challenged mice heart or quadricep tissues. No pseudocysts were observed in any tissues.

Discussion

The ability of avirulent *T. cruzi* strains to induce host protection to virulent *T. cruzi* strains has been demonstrated by previous studies (Lauria-Pires and Teixeira, 1997; Soares et al., 2003). Several studies with strains of *T. cruzi* from the US have suggested that they are not virulent for laboratory mice, the standard host to study Chagas disease (Pietrzak and Pung, 1998; Yabsley and Noblet, 2002; Roellig and Yabsley, 2010). A recent study found outbred laboratory mice inoculated with a US *T. cruzi* isolate had no detectable parasitemias nor any morbidity in the acute phase, and upon re-inoculation with a virulent SA isolate (Brazil strain), the parasitemias were lower than mice not previously inoculated with the US strain (Hall et al., 2010). This host protection by avirulent strains against virulent strains may be an important factor in the evolution of virulence properties of this pathogen, as chronic nonfatal infections favor transmission of the parasite. The data from the current study demonstrates previous inoculation with a US isolate modulates the immune response of a challenge inoculation with a SA strain and decreases the severity of lesions during the acute phase in challenged mice.

The cytokine production profiles produced by the primary mice inoculated with either the FLOPO3 or Brazil strains were similar to other studies (Edwards, unpublished; Zhang and Tarleton, 1996; Cummings and Tarleton, 2004). As expected, levels of inflammatory cytokines (IFN-γ and IL-6) were significantly higher and increased levels of inflammation were noted in the quadriceps and heart tissues of mice inoculated with the Brazil strain compared with those from mice inoculated with FLOPO3. The difference in cytokine production and lesions scores

could also be a direct result of the high inflammatory profile of the Brazil strain and/or the increased parasite reproductive rate *in-vivo* compared with the FLOPO3 isolate. In similar studies, US isolates have displayed a reduced reproductive rate *in vivo* and *in vitro*, compared with SA isolates (Yabsley and Noblet, 2002; Hall et al., 2010; Roellig et al., unpublished data). This reduced parasite reproductive rate likely results in the lower levels of pro-inflammatory cytokines, and subsequent tissue inflammation, noted in the FLOPO3 mice.

For the challenged mice, there was a decrease in production of proinflammatory cytokines than compared with their primary counterparts. This same trend of increased IFN- γ was observed in a study in which BALB/c mice were inoculated with an avirulent clone of the CL strain (CL-14) and subsequently challenged with the virulent CL strain (Soares et al., 2003). Among the FLOPO3 challenge mice, IFN-γ and TNF levels were lower than that of primary infected mice, except at 1 and 7 DPI/DPC, respectively. This same pattern was also seen among the Brazil challenged mice, which exhibited decreased levels of IFN-γ, TNF, and IL-6 (excluding minor increases at 1 DPC). This earlier inflammatory response in the challenged mice suggests immune recognition of the parasite, and the decreased levels later in the infection show an apparent control of the parasite without the production of inflammatory compounds. In fact, parasite-infected cells of the challenged mice may actually have been targeted early in the infection by CD8+ specific cytotoxic T cells (CTLs), which do not require increased production of systemic inflammatory cytokines for parasite control (Tarleton, 1990; Paiva et al., 1999).

The cytokine profile of challenged mice also suggests a more regulatory response, in regards to IL-2, IL-4, IL-10, and IL-17a production. IL-2 is important in T-cell proliferation and is normally suppressed upon inoculation with *T. cruzi* strains (Soares et al., 2003; Ouaissi, 2007). Upon challenge with the Brazil strain, IL-2 levels increased within 24 hours, suggesting active T-

cell proliferation response while levels of IL-4, IL-10 and IL-17a levels also increased at that time, which suggests a more balanced Th1/Th2/Th17 response during the challenge infection. Similarly, mice previously infected with the avirulent C-14 strain and challenged with the virulent CL strain also had increased IL-2 production (Soares et al., 2003). Although some studies suggest that increased production of the Th2 signature cytokines IL-4 and IL-10 lead to increased host susceptibility (Barbosa de Oliveira et al., 1996; Hiyama et al., 2001); other studies fail to find an association of IL-4 and IL-10 levels with susceptibility (Zhang and Tarleton, 1996; D'Avila et al., 2009; Guedes et al., 2009). In the current study, we also failed to find a correlation between IL-4 and IL-10 with susceptibility. IL-17a is the signature cytokine of the Th17 response and has been shown to be necessary for host protection against *T. cruzi* (Miyazaki et al., 2010). The increased IL-17a production by the Brazil-challenged mice throughout the experiment, suggests a role in protection. After mice were challenged with the Brazil strain, their cytokine levels were not different from challenged mice inoculated with the FLOPO3 isolate, except for IFN-γ. Brazil induced significantly higher IFN-γ levels earlier in the infection after 1 DPC; however, FLOPO3-challenged mice had generally higher IFN-γ throughout the rest of the experiment, though levels were not significantly different from Brazil challenged mice. These similar cytokine levels are to be expected given the lack of inflammatory lesions in tissues of the challenged mice.

This study shows previous inoculation with the FLOPO3 isolate altered the immune response and decreased inflammatory lesions during the acute phase when challenged with the Brazil strain. These data suggest that infection with FLOPO3 may provide some protection against infection with a more virulent strain. Though the Brazil strain did not cause morbidity or mortality in our study, the inflammatory lesion scores and levels of cytokines produced by

primary mice inoculated with the Brazil strain were more pronounced than those of FLOPO3 primary mice. However, our study only examined mice during the acute phase following the challenge, additional studies are needed to determine if these alterations in cytokine levels and inflammation during the acute phase impacts the development of chronic disease. In addition, previous studies on the virulence of US isolates in laboratory mice have been short-term; therefore, studies are needed to determine if they result in chronic disease.

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Figure 4.1. Levels of IFN-γ, IL-10, and TNF in mice inoculated with the FLOPO3 strain (A-C) and Brazil strains (E-H) during the primary and challenge experiments.

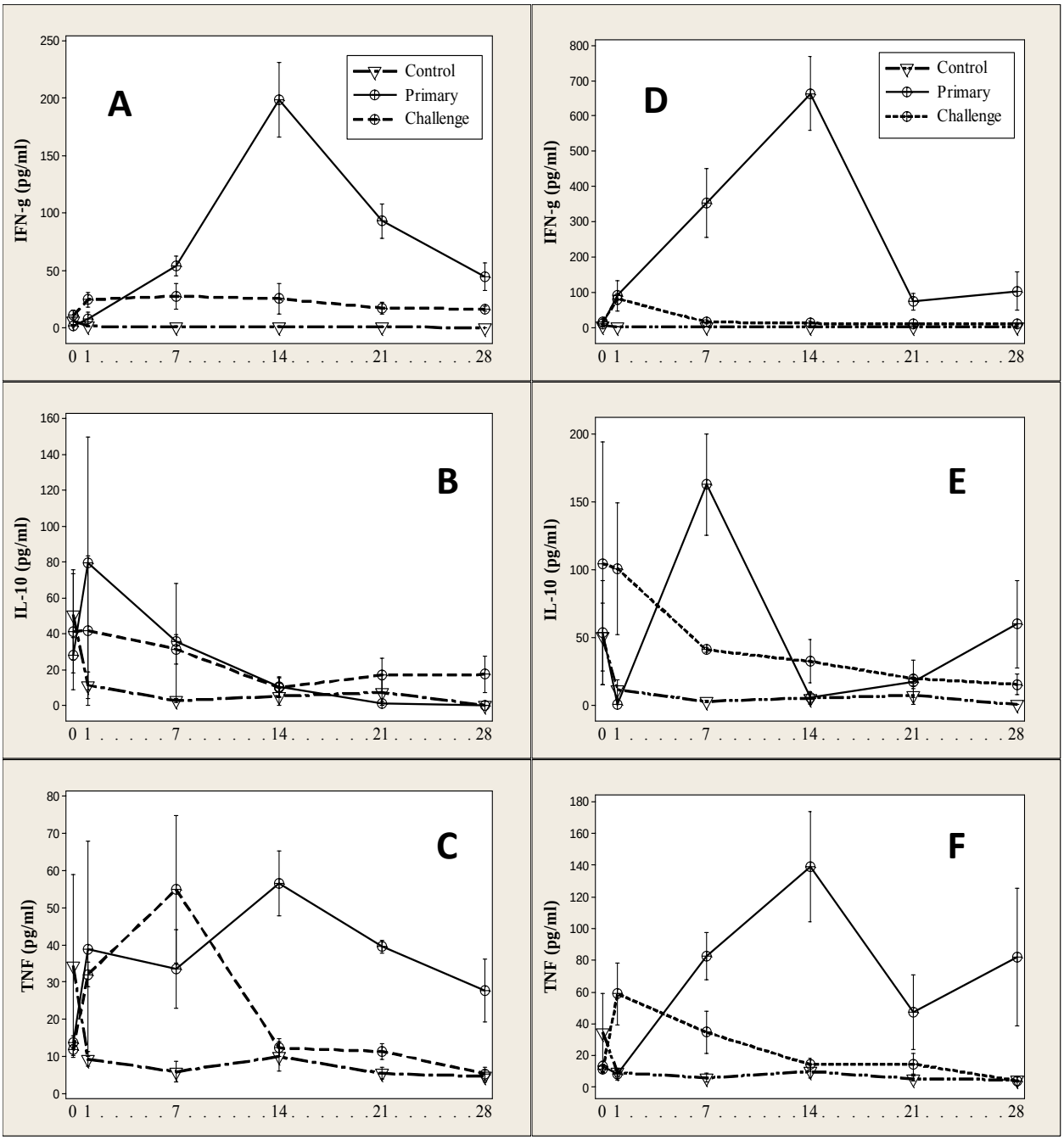
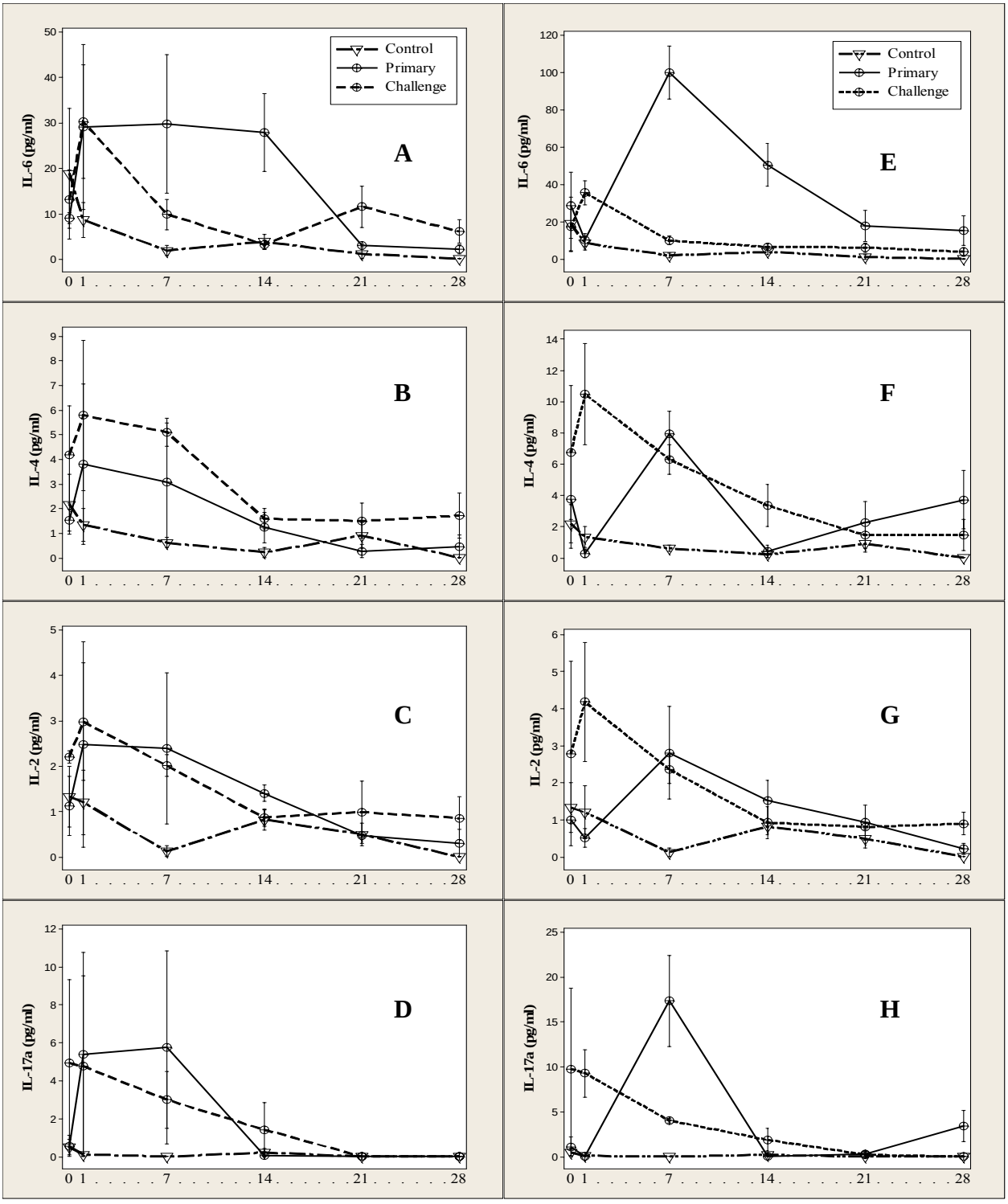


Figure 4.2. Levels of IL-6, IL-4, IL-2, and IL-17a for mice inoculated with the FLOPO3 strain (A-D) and Brazil strain (E-H) during primary and challenge experiments



# CHAPTER 5

## CONCLUSIONS

The overall goal of this thesis was to characterize the immunological response to infection with a diverse set of *Trypanosoma cruzi* isolates from the United States (US) in an experimental mouse model and to determine if a primary infection with a US isolate alters the immune response during a challenge infection. *T. cruzi* is very genetically and biologically diverse species and only limited studies have been conducted on US strains of *T. cruzi*. In the US, the diversity of *T. cruzi* genotypes (two genotypes) is lower than in South America (six genotypes) (Roellig et al., 2008). In South America, certain Tc types are associated with geographical regions and has been hypothesized that certain phenotypic pathologies of Chagas disease are associated with various genotypes (Ramírez et al., 2010; Díaz et al., 2011). Several studies have characterized the virulence of US strains in laboratory mice and have been classified as less virulent than many South American strains due to a lack of morbidity and mortality and low parasitemia levels (Yabsley and Noblet, 2002; Hall et al., 2010; Roellig and Yabsley, 2010). We hypothesized that the US strains in our study would have an overall lower inflammatory response as measured by cytokine production and that previous exposure to a US strain would provide the host with protection against challenge with a virulent South American *T. cruzi* strain.

Study 1 (Chapter 3)

In this study, our experimental data indicated that infection with different *T. cruzi* strains elicited diverging immune responses in mice, with South American strains eliciting a higher pro-inflammatory profile. The four South American strains, in general, induced greater levels of IFN-γ, TNF, IL-6, and IL-10 than the 13 US strains. One of the South American strains, Tulahuen, induced extremely high levels of TNF which was followed by morbidity. Although general trends in cytokine production were noted, each of the 17 strains included in this study exhibited a unique cytokine profile, most likely attributing to biological variability such as cell preference and replication rates, and/or parasite induced immunological modulation. Although little genetic variation has been noted among TcI and TcIV isolates from the US (Roellig et al., unpublished), we observed a high degree of variation in cytokine responses between and within *T. cruzi* I and IV from the US. Interestingly, the cytokine profiles of US TcIV strains were lower and less variable than US TcI strains. Similar to our cytokine findings, histologic lesion scores in the heart and quadriceps muscles were lower for US strains compared with South America strains. Collectively, these data described the infection strategies and immunological modulations of this unique subset of *T. cruzi* within the US.

Study 2 (Chapter 4)

In this study, our data indicated that mice previously inoculated with the FLOPO3 isolate and subsequently challenged with Brazil or FLOPO3, exhibited lower pro-inflammatory cytokine profiles and mild to normal tissue lesions, suggesting a protective response of the US isolate during the acute infection. Though the SA strain did not induce mortality during the acute phase, primary mice inoculated with the Brazil strain exhibited more severe inflammation

in the quadriceps and heart tissues compared with FLOPO3 primary mice. Collectively, our data indicate that an initial infection with an avirulent US strain results in an altered immunological response during subsequent infections.

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