FUNCTIONAL $CD8^+$ T CELLS MEDIATE PARASITE CONTROL THROUGHOUT EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION

by

ANGELA DELORES PACK

(Under the Direction of Rick L. Tarleton)

ABSTRACT

Trypanosoma cruzi establishes life-long persistent infection in most mammalian hosts and is the causative agent of Chagas disease in humans. The development of persistent intracellular infection is frequently attributed to the functional compromise of pathogen-specific $CD8^+$ T cells during chronic viral and protozoal infections. $CD8^+$ T cells are crucial for the survival of the acute phase of *T. cruzi* infection, but the ability of these cells to retain their functional capacity over time is often questioned. This work shows that neither $CD8^+$ T cell regulation nor failed responses, during chronic infection, can explain *T. cruzi* persistence. In fact, *T. cruzi*-specific $CD8^+$ T cells continue to function as potent effectors, demonstrated by the *in situ* production of key effector molecules. In addition, antibody-mediated depletion reveals that $CD8^+$ T cells are crucial for the suppression of parasite outgrowth during chronic infection. Because *T. cruzi* establishes persistent infection in muscle, despite the maintenance of functional parasite-specific $CD8^+$ T cells in that tissue, the ability of $CD8^+$ T cells to recognize *T. cruzi* infected cells was in question. The data herein suggest that overexpression of muscle MHC class I increases skeletal muscle surveillance by $CD8^+$ T cells. This initially has a positive impact on *T. cruzi* control, but ultimately results in the systemic compromise of *T. cruzi*-specific $CD8^+$ T

cells. These data suggest that normal muscle MHC I expression is at a level which permits *Trypanosoma cruzi* control and the preservation of anti-parasite $CD8^+$ T cell function; a balance which may come at the expense of infection resolution.

INDEX WORDS: *Trypanosoma cruzi*, Chagas disease, $CD8^+$ T cell, chronic infection, exhaustion, antigen presentation, MHC class I, muscle

FUNCTIONAL $CD8^+$ T CELLS MEDIATE PARASITE CONTROL THROUGHOUT EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION

by

ANGELA DELORES PACK

B.S., University of Tennessee, 2009

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2016

FUNCTIONAL $CD8^+$ T CELLS MEDIATE PARASITE CONTROL THROUGHOUT EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION

by

ANGELA DELORES PACK

Major Professor: Rick L. Tarleton
Committee: Daniel G. Colley
Boris Striepen
Eric V. Stabb

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2016

DEDICATION

This work is dedicated to my wonderful parents and their never-ending belief in my abilities. Their sacrifices and support have made this entire process possible.

ACKNOWLEDGEMENTS

I would like to thank my major professor Dr. Rick Tarleton for providing me with a place to explore my interests in immunology and the opportunity to grow as scientist in your laboratory. I'm certain that your commitment and training in the practice of exacting science will serve me well in my future positions. For my committee members, Drs. Daniel Colley, Boris Striepen, and Eric Stabb, you've been a great source of support and advice during my time here at UGA. You all are clear examples that a person can be both kind-hearted and academically successful. Many thanks go to Dr. Kim Klonowski for her support and perspectives on science and life. To my laboratory mentors, Juan Bustamante and Angel Padilla, thank you for training me and serving as 'sounding boards' during the good times and the bad. To the TRG lunch crew, Weibo Zhang, Duo Peng, and Phil Yao, your global perspectives and our experiences together have truly enriched my life. I would like to thank the rest of the Tarleton Research Group for helpful discussions, technical assistance, and continual optimism regarding my projects and future. I would also like to thank Julie Nelson for wonderful conversation and patience during my endless hours at the CTEGD Cytometry Shared Resource Laboratory. I am grateful for my classmates for their camaraderie and the reminder that life exists beyond the lab. I am thankful for my dear friend Ashley and her surprise packages and conversations that keep me going. I am truly indebted to my loving boyfriend Andre, for his willingness to listen to my endless talk about the lab and for always being there for me. I am most thankful for my parents and family who have encouraged me, even when I was at the point of giving up. I am eternally grateful that they chose me as their daughter and they have been a true blessing every day of my life.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 *Trypanosoma cruzi* and Chagas Disease

Since the introduction of the 'germ theory of disease' and the determination that microorganisms are the causative agents for many human and animal diseases, a great deal of effort has been applied to alleviate suffering and the debilitating economic effects of infection. These efforts to control and eradicate many communicable agents represent a commendable pursuit to improve the human condition and to promote the stability of human populations. Marginalized members of society, of lower economic position, frequently shoulder the greatest burden of infectious disease. This disparity is clearly illustrated upon evaluation of infection incidence by the protozoan *Trypanosoma cruzi,* a leading cause of parasite-associated disease in the Americas (1,2).

Herein, I will provide a brief background of the biology of *T. cruzi*, the associated disease manifestations and treatment options, and the immunity directed against this parasite. Finally, I will describe my experimental strategy to better understand the factors that enable *T. cruzi* persistence in mammalian hosts.

1.1.1 History and *T. cruzi* lineage

American trypanosomiasis is caused by infection with the single-cell protozoan *Trypanosoma cruzi*. A zoonotic infection that is endemic to countries of Central and South America, infection with *T. cruzi* has been a public health challenge for human populations for at least nine thousand years (3). *T. cruzi* is a member of the Euglenozoa phylum and the Kinetoplastida class, named for their unique mitochondria-like structure known as the kinetoplast. Kinetoplastids are responsible for a number of debilitating diseases in humans and

domestic livestock, and the group includes the causative agent of African trypanosomiasis or "Sleeping Sickness" (*Trypanosoma brucei*) and *Leishmania* species (4).

1.1.2 Elucidation of life cycle and transmission

Historically, human *T. cruzi* infection, referred to as Chagas disease, has been localized to rural areas of Latin America. This infection syndrome is named for the Brazilian clinician, Carlos Chagas, who is credited with the description, vector determination, and association of clinical disease manifestations with *T. cruzi* infection. Like many protozoan parasites, the life cycle of *T. cruzi* is complex. *T. cruzi* dissemination is dependent on the conversion of parasites between four distinct life stages in the digestive tract of hematophagous insect vectors of the *Triatominae* subfamily and within the tissues of a broad range of mammalian hosts, including primates and rodents (5-7). Each stage possesses a unique protein expression profile, reflecting the many adaptations required for survival in the different metabolic and immunologic environments encountered by *T. cruzi* based upon its broad host range (8-10).

In triatomine insects, epimastigotes replicate in the insect midgut, prior to migration to the hindgut and conversion to a flagellated form, known as metacyclic trypomastigotes. Insect feeding causes breaks in the protective skin barrier, permitting the coincidental transfer of metacyclics in infected feces, following vector defecation during blood meals. Two additional ways that *T. cruzi* metacyclics infect humans are through parasite contact with mucosal surfaces of the eye and by ingestion of contaminated food products (11,12). This highly motile parasite form can then invade nearly any nucleated cell through the generation and escape from a parasitophorous vacuole (13-15). Following an additional conversion event, *T. cruzi* amastigotes replicate in the cytoplasm of host cells. 4-5 days later, following several rounds of replication via binary fission and conversion to a new flagellated trypomastigote form, parasites escape from

ruptured host cells (16). The trypomastigotes can go on to infect nearby cells, and following bloodstream entry, can infect distal cells or new insect vectors during a blood meal. The trypomastigote and amastigote forms of *T. cruzi,* are the most relevant to consider for the purposes of this thesis, as mammalian immunity to *T. cruzi* infection is the primary focus.

1.1.3 Clinical manifestations and epidemiology of Chagas' Disease

Although parasites demonstrate great promiscuity in the selection of target cells for invasion in mammals, *T. cruzi* DNA and amastigotes pseudocysts (sites of replication) are most frequently detected in muscle, gastrointestinal, and nervous tissue during chronic infection (15,17). Localization within these tissues can be especially problematic, due to the compromises in tissue structure and function that can occur as a consequence of immune responses in these sites. Infection of cardiac tissue is often associated with cardiomyopathies capable of progressing to heart failure (18), while gastrointestinal infection can induce megalies of the colon and esophagus (19). In addition, infection of the nervous system significantly increases the risk of stroke in infected subjects with persistent *T. cruzi* infection (20-22).

Clinicians have defined three general phases of Chagas disease presentation: acute, indeterminate, and chronic (23,24). The acute phase is often undiagnosed due to the development of mild, indiscriminate (flu-like) symptoms. Acute diagnoses are made in a small subset of patients who develop severe disease following parasite ingestion or upon observation of a typically unremarkable inflammatory nodule, known as a chagoma, at the insect bite wound (25). During the acute phase (2-4 months), trypomastigote forms are easily detected in blood smears and infection is generally well controlled. The majority of patients remain unaware of their infection status and progress to an indeterminate phase, where *T. cruzi* detection via microscopy becomes challenging, as parasite levels in the blood are extremely low.

Approximately thirty percent of *T. cruzi*-infected subjects develop severe Chagas disease complications following decades of infection. The debate regarding the factors that contribute to this progression remains unresolved, although there is consensus that the genetic diversity of the infecting strain(s) and host genetic predisposition are likely contributors (26,27). Elucidation of Chagas disease etiology is further complicated by the extreme amount of time (decades) required for disease progression, and the fact that parasites typically persist below the limits of detection of classical methods like histology. Two conflicting hypotheses to explain cardiac disease progression arose in the field: 1) autoimmunity and 2) persistent parasites as key determinants for disease. The autoimmunity hypothesis is primarily supported by the detection of antibodies against human cardiac proteins, with *T. cruzi* proteins performing a type of molecular mimicry inducing host-targeted responses (28,29). The foundation of this theory is the technical challenge of regularly detecting parasites in chronically infected subjects via histology (30-32). With improvements in molecular diagnosis, through sensitive PCR technology, this theory has largely been displaced by a body of literature defining parasite persistence as the key determinant for disease (33,34). Support for this hypothesis is based on the detection of *T. cruzi* DNA in cardiac tissue of infected patients using highly sensitive PCR techniques, demonstrating continued infection (35-37). Enhanced disease, including intensified meningoencephalitis and myocarditis, and increased parasite levels upon immunosuppression for transplantation and during HIV infection (38,39), also support the conclusion that persistent parasites are present following decades of *T. cruzi* infection. A final observation supporting *T. cruzi* persistence as a determinant for chronic Chagas disease, is that infection can be transferred during transplantation of infected tissue; a finding that has convinced most in the field that disease progression is dependent on *T. cruzi* persistence (40,41).

Despite decades of investigation into *T. cruzi* and Chagas disease, progress to eliminate *T. cruzi* infection as a public health concern has been slow. Currently, there is no vaccine for use in humans or animals and the treatment options for Chagas disease are limited. There are only two approved anti-*T. cruzi* chemotherapeutics available, benznidazole and nifurtimox, and tolerance to these compounds is quite variable due to toxic side effects (42). Prescription of these compounds is typically restricted to patients diagnosed during the acute phase, as treatment efficacy during chronic infection is difficult to assess with few established metrics to gauge treatment success and cure.

As a consequence of these limitations in vaccine and treatment options, the social and economic impact of Chagas disease remains substantial. An estimated 14,000 deaths per year and 700,000 disability adjusted life years (DALY), due to lost productivity, are attributed to Chagas disease (43). Large-scale control efforts have focused on: 1) improvements in the poor housing conditions that serve as vector habitats, and 2) insecticide usage in domestic structures. These programs have been successful in significantly reducing the number of newly acquired infections in endemic areas, but have failed to eradicate infection transmission (44). In addition, *T. cruzi* infection in sylvatic populations, which live in close proximity to domestic animals and human domiciles, will likely prevent the success of *T. cruzi* eradication efforts (45). Historically, human *T. cruzi* infection has primarily been restricted to endemic areas of Latin America. Increased migration from Latin America has expanded the range of Chagas disease, with an estimated 100,000 infected individuals now residing in the United States and Europe (46). Other modes of transmission including congenital infection, transfer during blood and organ transplant, and autochthonous transmission in the southeastern United States (47,48) also contribute to increased infection incidence. Based upon the severity of disease that can be associated with *T.*

cruzi infection and increased infection incidence beyond endemic areas, surveillance efforts in the U.S. and elsewhere require expansion.

1.2 Immunity to *T. cruzi* infection

A detailed understanding of the immune responses generated against *T. cruzi* infection is imperative, if researchers want to improve disease prognosis and reduce infection rates through the development of vaccines and therapies. Complex parasite biology, including the presence of multiple life stages within a given host, complicate studies defining the relative contributions of different immune cells in *T. cruzi* infection. Despite these challenges, a great deal of work has been completed evaluating anti-*T. cruzi* immunity. It is clear that survival of acute *T. cruzi* infection requires the coordinated actions of innate, humoral, and cellular immunity. Here, I will provide a brief summary of primary themes in *T. cruzi* immunity in mammals.

1.2.1 Innate immunity

Initial pathogen recognition and priming of the adaptive immune response is coordinated by the innate immune system. The primary defenses against *T. cruzi* entry are protective barriers like the skin. Breaches of this critical defense, by insect bite or mucosal surface contact with infected feces (49,50), permit *T. cruzi* invasion of local host cells or engulfment by phagocytic cells. These phagocytes, namely macrophages, dendritic cells, or neutrophils (16,51), are involved in parasite killing through the production of the inflammatory cytokine IL-12, and production of reactive oxygen species or nitric oxide (52,53). They also participate in the priming of adaptive responses for the production of IFNγ, which supports phagocyte activation (54). While *T. cruzi* induces responses of the innate immune system, is it primarily restricted to the production of Type I IFNs and the magnitude of IFN stimulated gene induction is dependent on the virulence of the infecting *T. cruzi* strain(s) (55-58). These deficiencies in host stimulation

are largely attributed to low levels of endogenous pathogen associated molecular patterns (PAMPs) expression by *T. cruzi*. *T. cruzi* fails to rapidly induce TLR-receptor mediated parasite detection, and the modest amount of induction that does occur, is delayed compared to other intracellular pathogens (59). Transgenic expression by *T. cruzi* of classical PAMPs, like flagellin, can overcome this stimulation deficiency, leading to significant improvements in innate stimulation and parasite control (60).

1.2.2 Adaptive immunity

As a result of *T. cruzi*'s cyclic infection patterns, immune cells encounter parasites in intra and extracellular locations; necessitating the actions of a number of lymphocyte subsets for *T. cruzi* control. Rodent models have been used to determine the relative contributions of different lymphocyte populations, through the infection of knock-out (KO) mouse strains and antibody-based depletion to eliminate lymphocyte subsets or their effector functions.

Antibodies, produced by B cells, are directed at the surface of pathogens, targeting them for phagocyte uptake or destruction by the complement system. Antibody production is essential for survival of acute *T. cruzi* infection, as mice deficient in B cells (μMT mice) are capable of controlling parasites early on, but succumb by 3 months post infection (61). Antibody levels tend to remain quite stable throughout the course of *T. cruzi* infection. This allows anti-*T. cruzi* antibody titers to serve as biomarkers of trypanocidal treatment efficacy in Chagas patients; with decreased antibody levels reflecting reductions in parasite burden during longitudinally monitoring (62,63).

$CD4^+$ T cells are another lymphocyte population capable of recognizing peptide epitopes derived from extracellular proteins. Classically, $CD4^+$ T cells perform a number of functions to support pathogen control including the development of protective B cells and antibody class

switching. In addition, $CD4^+$ T cells serve as "helpers," and are crucial for priming and activation of effector $CD8^+$ T cell responses (64).

During *T. cruzi* infection, $CD4^+$ T cells are critical for survival of acute infection, but their exact role is not easily defined (65-67). The classical role of $CD4^+$ T cells is as helpers in the initiation of $CD8^+$ T cell responses, but mice deficient in $CD4^+$ T cells are still capable of generating robust immunodominant *T. cruzi*-specific $CD8^+$ T cell responses (66). Despite this surprising observation, it is clear that CD4 cells are involved in the development of anti-*T. cruzi* responses through the induction of responses driven by the inflammatory cytokine IFNγ. IFNγ production is essential for survival of acute *T. cruzi* infection (68-70) and the cytokine polarization of the anti-*T. cruzi* response is driven by the actions of T helper 1 (Th1) $CD4^+$ T cells. In addition, Th17 $CD4^+$ T cells, another pro-inflammatory CD4 lineage, are also important for CD8 activation via IL-21 signaling and the development of protective $CD8^+$ T cell responses during *T. cruzi* infection (71,72).

$CD8^+$ T cells are the final lymphocyte population that play an essential role in survival of acute *T. cruzi* infection (61,73). The actions of this cell population are the focus of this thesis and an in-depth discussion of the specificity and function of *T. cruzi*-specific $CD8^+$ T cells is described below.

1.3 *T. cruzi*-specific $CD8^+$ T cells

$CD8^+$ T cells are responsible for the surveillance of mammalian cells for signs of intracellular infection or cancerous transformation via surface bound peptide-major histocompatibility class I (MHC I) complexes. Upon T cell receptor (TCR) engagement with specific peptide complexes, $CD8^+$ T cells become activated and can produce a number of effector molecules such as IFNγ, TNFα, perforin and granzymes.

1.3.1 $CD8^+$ T cell function during *T. cruzi* infection

T. cruzi replicates in the cytoplasm in mammalian host cells; an intracellular niche where parasite proteins can be degraded and presented via the class I MHC presentation pathway to $CD8^+$ T cells (74,75). $CD8^+$ T cells directed against *T. cruzi* proteins are essential for host survival of early parasite infection, as antibody-mediated CD8 depletion and infection of β2-microglobulin deficient mice is lethal during the acute phase (65,76,77). It is unclear which effector functions are critical for $CD8^+$ T cell elimination of *T. cruzi* infected cells, although it is generally well accepted that IFNγ production by $CD8^+$ T cells is essential. There are conflicting reports regarding the roles of perforin and granzymes in *T. cruzi* control and cardiac pathology (78,79). These conflicts likely reflect redundancies in the pathways of $CD8^+$ T cell cytotoxicity (61). The common conclusion of these studies is that no single cytolytic pathway is essential for survival of *T. cruzi* infection, as infection of mice with single knockouts of these molecules is not lethal. Regardless, $CD8^+$ T cells, are very important for acute *T. cruzi* control and the presence of $CD8^+$ T cells in cardiac infiltrates has been positively associated with less severe disease in humans and mice (80,81).

1.3.2 Specificity and phenotype of *T. cruzi*-specific $CD8^+$ T cells

$CD8^+$ T cells express T cell receptors (TCRs) capable of interacting with specific MHC class I peptide complexes. Using epitope prediction software, researchers can identify candidate peptides from proteins of interest. The best candidates contain canonical proteasome cut sites, ideal MHC binding motifs, and favorable immunogenicity predictions (82). Using fluorescent reagents containing four complexed MHC-peptide molecules (tetramers), it is possible to track the responses of $CD8^+$ T cells that bind the tetramers and are specific for these peptide sequences.

In *T. cruzi* infection, a sizeable portion of the $CD8^+$ T cell population is specific for peptides derived from a highly abundant group of *T. cruzi* invasion-associated surface proteins called trans-sialidases (83-87). Identification of these parasite-specific cells has permitted investigation into the phenotype and stability of *T. cruzi*-specific $CD8^+$ T cell responses over time. Following a primary host exposure to *T. cruzi*, it takes nearly 9 days for detectable $CD8^+$ T cell activation; a significant delay compared to model infection systems (59). After this period, the kinetics of the systemic response (in the spleen) progress as expected. There is a substantial clonal T cell expansion, with nearly 30% of all activated $CD8^+$ T cells capable of recognizing a single trans-sialidase derived peptide, ANYFKTLV (TSKB20), in the spleen at the peak (20-30 days) of *T. cruzi* infection (88). As parasite levels decrease, there is a sharp contraction in TSKB20+ $CD8^+$ T cell frequency.

The surface expression of activation (Killer Cell Lectin Like Receptor G1 (KLRG1)) and memory associated (CD62L and CD127) markers, can be used to define the phenotype and functional status of T cells (89). T cell expression of these markers can also provide clues about the general abundance of a pathogen, with heightened activation marker expression coinciding with higher antigen levels (89). During acute *T. cruzi* infection, $CD8^+$ T cells possess an effector phenotype, with a significant percentage of cells expressing high levels of KLRG1 and low expression of the memory markers, CD62L and CD127. As mice progress to the chronic phase of infection (~100 days post infection (dpi)), a small proportion of parasite-specific (TSKB20+) cells are converted to central memory (Tcm) cells. These cells have high expression levels of the memory markers mentioned above, exhibit antigen-independent survival, and are detectable in the spleen and in peripheral blood mononuclear cells (PBMCs) (90). Concurrently, the bulk of splenic cells have a phenotype ($KLRG1^+CD127^{lo}$) consistent with resting effector memory

(Tem) cells (91), reflective of a low-level persistent infection. *T. cruzi* establishes persistent infection in the muscle—presumably making the inflammatory environment and frequency of antigen counter significantly different than that experienced by splenic CD8s. The exact phenotype of these cells is not well defined in the literature, but reports suggest that muscle-derived $CD8^+$ T cells possess an effector/effector memory phenotype during acute and chronic *T. cruzi* infection (92). This phenotype of this $CD8^+$ T cell population is explored extensively in chapter 2 of this thesis.

1.4 Factors that impact the quality of $CD8^+$ T cell immunity

T. cruzi persistence, despite the presence of a robust immune response, is a defining feature of *T. cruzi* infection. Chronic infections in long-lived hosts, like humans, can span decades. This prolonged antigen exposure may impact the quality of parasite-specific $CD8^+$ T cell responses, through highly regulated T cell function. $CD8^+$ T cell regulation and compromised immunity are common features of some viral, protozoal, and bacterial infections (93). In addition, the presence of parasites in non-lymphoid peripheral tissues, may impact the quality of *T. cruzi*-specific $CD8^+$ T cell responses. A number of important factors influence the generation and maintenance of potent $CD8^+$ T cell responses against intracellular pathogens. The two evaluated in this dissertation, are described below.

1.4.1 Regulation of $CD8^+$ T cells during persistent infection

Immune regulation is a strategy that likely evolved to minimize lethal immunopathology and contributes to the development of persistence in a number of infection models, through the restraint of T cell function. In the case of clone 13 lymphocytic choriomeningitis virus (LCMV) infection, likely the most heavily studied model of chronic infection, extremely high viral titers and continuous TCR stimulation drive $CD8^+$ T cells to a

state of functional exhaustion (94). Functional exhaustion is characterized by limited cytokine production in response to *ex vivo* polyclonal stimulation, with sequential loss in the capacity to produce IL-2, TNFα, and IFNγ (95). Exhaustion can also limit the development of quality memory populations, even following pathogen clearance (94-96).

This regulated, and typically reversible state, is usually driven by the actions of regulatory T cells (Tregs), anti-inflammatory cytokines, and inhibitory receptor expression. Tregs play a significant role in the resolution of intracellular infections like *Plasmodium* and *Mycobacterium tuberculosis*, and selective depletion of regulatory $CD4^+$ T cells can improve infection control (97-99). Regulatory cytokines like interleukin-10 (IL-10) and transforming growth factor β (TGF-β), often produced by Tregs, also restrain T cell responses during persistent infections. When the actions of IL-10 or TGF-β are blocked or when IL-10 deficient mice are infected, with the intracellular pathogens *Leishmania* (100) and *Mycobacterium tuberculosis* (101), and during persistent viral infection (100-103), pathogen control is significantly improved.

The most rapidly growing body of literature in chronic infection research is focused on the impact of inhibitory receptor expression on $CD8^+$ T cell function (104). Inhibitory receptor expression is upregulated in the presence inflammatory cytokines and as a consequence of repetitive TCR stimulation (105). CTLA-4 and programmed death 1 (PD-1) receptor upregulation and crosslinking with their B7-family ligands (PD-L1 and PD-L2), has a significant impact on infection resolution in some model systems (106-109). Preventing receptor-ligand crosslinking, through PD-1 blockade during infection with LCMV, *Plasmodium (110)*, *Leishmania (111)*, *Toxoplasma (112)*, and *Mycobacterium tuberculosis* (113) can improve CD8 recognition of target cells, restore T cell replicative potential, and ultimately, improve infection

control (114). While this receptor is a major regulator of T cell function during chronic infection, it has a minor impact in a number of infections (115), and it is actually expressed on the surface of T cells in some healthy patients (116).

The exact contribution of regulated $CD8^+$ T cell responses to development of *T. cruzi* persistence is unknown. The extended duration and the antigen environment encountered by muscle $CD8^+$ T cells during chronic *T. cruzi* infection, has brought the functional capacity of this population into question. While the phenotype of muscle $CD8^+$ T cells during chronic *T. cruzi* infection has not been thoroughly investigated, the capacity to respond to polyclonal *ex vivo* stimulation, a traditional indicator of T cell fitness, has been evaluated. Fewer muscle $CD8^+$ T cells produce IFNγ in response to *in vitro* stimulation compared to their splenic counterparts during chronic infection (92). Despite these functional differences, muscle $CD8^+$ T cells appear neither anergic nor apoptotic (92), which suggests that extreme T cell regulation is not occurring during chronic *T. cruzi* infection (117). Studies have sought to link *T. cruzi* persistence to this perceived dysfunction, but were unsuccessful at identifying a regulatory mechanism responsible for restraining $CD8^+$ T cell function (118,119). Limiting the actions of Tregs in *T. cruzi* infection results in severe immune related pathology but has a negligible impact on T cell *ex vivo* responses or parasite control (119). Blockade of the above mentioned regulatory cytokines also failed to prevent *T. cruzi* persistence (118). During acute *T. cruzi* infection, parasitemia is significantly reduced in mice treated with anti-IL-10 and the cytokine appears to temper T cell activation to limit immunopathology, but fails to alter parasite burden (120-122). Following decades of human *T. cruzi* infection, a subset of chronically infected patients exhibit deficiencies in *ex vivo* cytokine production (123,124) and increased inhibitory receptor (PD-1) expression (125-127). It is important to note that infection duration is a key factor impacting the appearance

of these restrained responses, as functional $CD8^+$ T cells are consistently observed in subjects with shorter infection exposure (children) (87,124).

The inability to alter $CD8^+$ T cell responses to improve *T. cruzi* control, through manipulation of key regulatory pathways, suggests that highly regulated CD8 function is unlikely to be the primary factor enabling *T. cruzi* persistence during chronic mouse infection.

1.4.2 Antigen availability is key for $CD8^+$ T cell function

Additional requirements for optimal $CD8^+$ T cell responses are antigen and cognate peptide availability. Successful antigen presentation via the MHC class I processing pathway requires: 1) the degradation of proteins in cytoplasmic proteasomes into 8-11 amino acid peptides prior to translocation via the transporter associated with antigen processing (TAP) 2) MHC I folding and loading in the ER 3) MHC I complex trafficking through the Golgi 4) and stable expression on the surface of nucleated cells.

Pathogens, particularly viruses of the cytomegalovirus and adenovirus families, have evolved strategies to actively disrupt nearly every step of this pathway to evade immune elimination (128). A few well studied examples include the proteasomal processing inhibitor, Epstein-Barr virus nuclear antigen 1 (129), the TAP inhibitors: human cytomegalovirus protein US6 (130) and herpes simplex virus protein ICP47 (131). E3-19K, an adenovirus protein, retains MHC I complexes in the ER (132), while the HIV protein Nef prevents surface MHC I complex expression by diverting stable molecules from the Golgi to a lysosome for degradation (133). While these strategies of MHC I manipulation have not been heavily investigated in the field of parasitology, a single report questioning the capacity of *T. cruzi* to restrict host MHC class I expression *in vitro* is available (134).

While all nucleated cell populations are capable of MHC class I expression, serious regulation of this molecule in muscle and cells of the nervous system, restricts surface MHC class I expression (135). This deficiency limits the surveillance potential of these tissue types, but can be overcome if MHC class I is upregulated by proinflammatory cytokines like IFNγ. Pathogens take advantage of this normal host regulation, with low MHC class I expression associated with the establishment of chronic infection by *Toxoplasma* and herpes simplex virus 1 in neural tissue (136,137).

In the case of *T. cruzi* infection, an additional factor may delay the recognition of infected targets and the capacity of specific $CD8^+$ T cells to eliminate parasites. Based upon parasite stage specific gene expression and protein availability in host cell cytoplasm following life cycle conversion events by *T. cruzi* (9,138), the timing of immunodominant epitope (TSKB20) display is likely suboptimal. When *T. cruzi* infected cells are co-incubated *in vitro* with T cells specific for TSKB20, there is an approximately 24-hour delay in IFNγ production, compared to <6 hours by $CD8^+$ T cells directed against epitopes (flagellar protein derived) available earlier in cytoplasm of host cells (138). These data, combined with the frequent association of *T. cruzi* with cells of highly regulated MHC expression during persistent *T. cruzi* infection, have challenged us to consider the impact of infected cell recognition on the development of persistent infection.

1.5 Summary and experimental approach

The factors that enable intracellular pathogen persistence remain challenging to define, as a single conserved strategy is unlikely. These studies are especially complicated during infection with protozoans, like *T. cruzi*, with complex life cycles and tropisms for tissues that are technically challenging to investigate in mice and that are rarely available from patients.

Although critical for survival of early *T. cruzi* infection, the contribution of $CD8^+$ T cell function to parasite control beyond the acute phase remains controversial. In chapter 2, I evaluated the hypothesis that $CD8^+$ T cells remain critical for control of *T. cruzi* throughout the course of infection, despite the presence of persistent parasites. T cell phenotype and function were evaluated directly *in situ* in lymphoid and tissues of persistence, and the role of $CD8^+$ T cells role was ultimately defined using specific CD8 cell depletion. In addition, regulatory pathways that inhibit T cell function were experimentally manipulated and the impact on *T. cruzi* persistence was determined. There were also questions regarding the ability of $CD8^+$ T cells to rapidly recognize and eliminate *T. cruzi* infected cells in non-lymphoid tissue (muscle). In chapter 3, I investigated the hypothesis that parasite persistence in skeletal muscle is related to the immunosurvelliance potential via MHC class I in this tissue. Through the use of a transgenic mouse model, the impact of MHC I expression on *T. cruzi* survival in skeletal muscle and the $CD8^+$ T cells that mediate control was evaluated.

Knowledge from these studies can inform decisions about the best strategies to improve *T. cruzi* control and elimination through vaccination or immune therapies. In addition, they build on the foundation in the fields of immunology and microbial pathogenesis, to reveal strategies that pathogens, particularly parasites, exploit for survival.

1.6 References

1. Pan American Health Organization, W. P. o. N. T., and Diseases. (2007) Estimación cuantitativa de la enfermedad de Chagas en les Américas. Pan American Health Organization, Washington

2. Schofield, C. J., Jannin, J., and Salvatella, R. (2006) The future of Chagas disease control. *Trends in parasitology* **22**, 583-588

3. Aufderheide, A. C., Salo, W., Madden, M., Streitz, J., Buikstra, J., Guhl, F., Arriaza, B., Renier, C., Wittmers, L. E., Jr., Fornaciari, G., and Allison, M. (2004) A 9,000-year record of Chagas' disease. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 2034-2039

4. Simpson, A. G., Stevens, J. R., and Lukes, J. (2006) The evolution and diversity of kinetoplastid flagellates. *Trends in parasitology* **22**, 168-174

5. De Souza, W. (2002) Basic cell biology of Trypanosoma cruzi. *Current pharmaceutical design* **8**, 269-285

6. Massad, E. (2008) The elimination of Chagas' disease from Brazil. *Epidemiol Infect* **136**, 1153-1164

7. Noireau, F., Diosque, P., and Jansen, A. M. (2009) Trypanosoma cruzi: adaptation to its vectors and its hosts. *Vet Res* **40**, 26

8. Queiroz, R. M., Charneau, S., Bastos, I. M., Santana, J. M., Sousa, M. V., Roepstorff, P., and Ricart, C. A. (2014) Cell surface proteome analysis of human-hosted Trypanosoma cruzi life stages. *Journal of proteome research* **13**, 3530-3541

9. Atwood, J. A., 3rd, Weatherly, D. B., Minning, T. A., Bundy, B., Cavola, C., Opperdoes, F. R., Orlando, R., and Tarleton, R. L. (2005) The Trypanosoma cruzi proteome. *Science* **309**, 473-476

10. Roberts, S. B., Robichaux, J. L., Chavali, A. K., Manque, P. A., Lee, V., Lara, A. M., Papin, J. A., and Buck, G. A. (2009) Proteomic and network analysis characterize stage-specific metabolism in Trypanosoma cruzi. *BMC Syst Biol* **3**, 52

11. Shikanai-Yasuda, M. A., and Carvalho, N. B. (2012) Oral transmission of Chagas disease. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **54**, 845-852

12. Lupi, O., Bartlett, B. L., Haugen, R. N., Dy, L. C., Sethi, A., Klaus, S. N., Machado Pinto, J., Bravo, F., and Tyring, S. K. (2009) Tropical dermatology: Tropical diseases caused by protozoa. *J Am Acad Dermatol* **60**, 897-925; quiz 926-898

13. Burleigh, B. A., and Woolsey, A. M. (2002) Cell signalling and Trypanosoma cruzi invasion. *Cellular microbiology* **4**, 701-711

14. Andrade, L. O., and Andrews, N. W. (2004) Lysosomal fusion is essential for the retention of Trypanosoma cruzi inside host cells. *The Journal of experimental medicine* **200**, 1135-1143

15. Melo, R. C., and Brener, Z. (1978) Tissue tropism of different Trypanosoma cruzi strains. *The Journal of parasitology* **64**, 475-482

16. Andrade, L. O., and Andrews, N. W. (2005) The Trypanosoma cruzi-host-cell interplay: location, invasion, retention. *Nature reviews. Microbiology* **3**, 819-823

17. Marcon, G. E., de Albuquerque, D. M., Batista, A. M., Andrade, P. D., Almeida, E. A., Guariento, M. E., Teixeira, M. A., and Costa, S. C. (2011) Trypanosoma cruzi: parasite persistence in tissues in chronic chagasic Brazilian patients. *Memorias do Instituto Oswaldo Cruz* **106**, 85-91

18. Marin-Neto, J. A., Cunha-Neto, E., Maciel, B. C., and Simoes, M. V. (2007) Pathogenesis of chronic Chagas heart disease. *Circulation* **115**, 1109-1123

19. de Oliveira, R. B., Troncon, L. E., Dantas, R. O., and Menghelli, U. G. (1998) Gastrointestinal manifestations of Chagas' disease. *Am J Gastroenterol* **93**, 884-889

20. Carod-Artal, F. J., and Gascon, J. (2010) Chagas disease and stroke. *Lancet Neurol* **9**, 533-542

21. Jesus, P. A., Neville, I., Cincura, C., Menezes, D. F., Vieira-de-Melo, R. M., Lacerda, A. M., Viana, L. C., Pereira, D. F., Ribeiro-dos-Santos, V., Jr., Reis, F. J., Macedo, C., and Oliveira-Filho, J. (2011) Stroke history and Chagas disease are independent predictors of silent cerebral microembolism in patients with congestive heart failure. *Cerebrovasc Dis* **31**, 19-23

22. Carod-Artal, F. J., Vargas, A. P., Melo, M., and Horan, T. A. (2003) American trypanosomiasis (Chagas' disease): an unrecognised cause of stroke. *J Neurol Neurosurg Psychiatry* **74**, 516-518

23. Barrett, M. P., Burchmore, R. J., Stich, A., Lazzari, J. O., Frasch, A. C., Cazzulo, J. J., and Krishna, S. (2003) The trypanosomiases. *Lancet* **362**, 1469-1480

24. Dutra, W. O., Rocha, M. O., and Teixeira, M. M. (2005) The clinical immunology of human Chagas disease. *Trends in parasitology* **21**, 581-587

25. Patel, S., and Sethi, A. (2009) Imported tropical diseases. *Dermatol Ther* **22**, 538-549

26. Williams-Blangero, S., VandeBerg, J. L., Blangero, J., and Correa-Oliveira, R. (2003) Genetic epidemiology of Trypanosoma cruzi infection and Chagas' disease. *Front Biosci* **8**, e337-345

27. Buscaglia, C. A., and Di Noia, J. M. (2003) Trypanosoma cruzi clonal diversity and the epidemiology of Chagas' disease. *Microbes and infection / Institut Pasteur* **5**, 419-427

28. Girones, N., Cuervo, H., and Fresno, M. (2005) Trypanosoma cruzi-induced molecular mimicry and Chagas' disease. *Curr Top Microbiol Immunol* **296**, 89-123

29. Bonney, K. M., and Engman, D. M. (2008) Chagas heart disease pathogenesis: one mechanism or many? *Curr Mol Med* **8**, 510-518

30. Hyland, K. V., and Engman, D. M. (2006) Further thoughts on where we stand on the autoimmunity hypothesis of Chagas disease. *Trends in parasitology* **22**, 101-102; author reply 103

31. Kierszenbaum, F. (2005) Where do we stand on the autoimmunity hypothesis of Chagas disease? *Trends in parasitology* **21**, 513-516

32. Engman, D. M., and Leon, J. S. (2002) Pathogenesis of Chagas heart disease: role of autoimmunity. *Acta tropica* **81**, 123-132

33. Tarleton, R. L. (2001) Parasite persistence in the aetiology of Chagas disease. *International journal for parasitology* **31**, 550-554

34. Tarleton, R. L., Grusby, M. J., and Zhang, L. (2000) Increased susceptibility of Stat4-deficient and enhanced resistance in Stat6-deficient mice to infection with Trypanosoma cruzi. *J Immunol* **165**, 1520-1525

35. Jones, E. M., Colley, D. G., Tostes, S., Lopes, E. R., Vnencak-Jones, C. L., and McCurley, T. L. (1993) Amplification of a Trypanosoma cruzi DNA sequence from inflammatory lesions in human chagasic cardiomyopathy. *The American journal of tropical medicine and hygiene* **48**, 348-357

36. Vago, A. R., Macedo, A. M., Oliveira, R. P., Andrade, L. O., Chiari, E., Galvao, L. M., Reis, D., Pereira, M. E., Simpson, A. J., Tostes, S., and Pena, S. D. (1996) Kinetoplast DNA signatures of Trypanosoma cruzi strains obtained directly from infected tissues. *The American journal of pathology* **149**, 2153-2159

37. Vago, A. R., Macedo, A. M., Adad, S. J., Reis, D. D., and Correa-Oliveira, R. (1996) PCR detection of Trypanosoma cruzi DNA in oesophageal tissues of patients with chronic digestive Chagas' disease. *Lancet* **348**, 891-892

38. Jardim, E., and Takayanagui, O. M. (1994) Chagasic meningoencephalitis with detection of Trypanosoma cruzi in the cerebrospinal fluid of an immunodepressed patient. *J Trop Med Hyg* **97**, 367-370

39. Prata, A. (2001) Clinical and epidemiological aspects of Chagas disease. *Lancet Infect Dis* **1**, 92-100

40. Tarleton, R. L., Zhang, L., and Downs, M. O. (1997) "Autoimmune rejection" of neonatal heart transplants in experimental Chagas disease is a parasite-specific response to infected host tissue. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3932-3937

41. Tarleton, R. L., and Zhang, L. (1999) Chagas disease etiology: autoimmunity or parasite persistence? *Parasitol Today* **15**, 94-99

42. Castro, J. A., and Diaz de Toranzo, E. G. (1988) Toxic effects of nifurtimox and benznidazole, two drugs used against American trypanosomiasis (Chagas' disease). *Biomed Environ Sci* **1**, 19-33

43. Stuart, K., Brun, R., Croft, S., Fairlamb, A., Gurtler, R. E., McKerrow, J., Reed, S., and Tarleton, R. (2008) Kinetoplastids: related protozoan pathogens, different diseases. *The Journal of clinical investigation* **118**, 1301-1310

44. Tarleton, R. L., Reithinger, R., Urbina, J. A., Kitron, U., and Gurtler, R. E. (2007) The challenges of Chagas Disease-- grim outlook or glimmer of hope. *PLoS medicine* **4**, e332

45. Costales, J. A., Jara-Palacios, M. A., Llewellyn, M. S., Messenger, L. A., Ocana-Mayorga, S., Villacis, A. G., Tibayrenc, M., and Grijalva, M. J. (2015) Trypanosoma cruzi population dynamics in the Central Ecuadorian Coast. *Acta tropica* **151**, 88-93

46. Bern, C., Montgomery, S. P., Herwaldt, B. L., Rassi, A., Jr., Marin-Neto, J. A., Dantas, R. O., Maguire, J. H., Acquatella, H., Morillo, C., Kirchhoff, L. V., Gilman, R. H., Reyes, P. A., Salvatella, R., and Moore, A. C. (2007) Evaluation and treatment of chagas disease in the United States: a systematic review. *JAMA : the journal of the American Medical Association* **298**, 2171-2181

47. Beard, C. B., Pye, G., Steurer, F. J., Rodriguez, R., Campman, R., Peterson, A. T., Ramsey, J., Wirtz, R. A., and Robinson, L. E. (2003) Chagas disease in a domestic transmission cycle, southern Texas, USA. *Emerging infectious diseases* **9**, 103-105

48. Dorn, P. L., Perniciaro, L., Yabsley, M. J., Roellig, D. M., Balsamo, G., Diaz, J., and Wesson, D. (2007) Autochthonous transmission of Trypanosoma cruzi, Louisiana. *Emerging infectious diseases* **13**, 605-607

49. Giddings, O. K., Eickhoff, C. S., Smith, T. J., Bryant, L. A., and Hoft, D. F. (2006) Anatomical route of invasion and protective mucosal immunity in Trypanosoma cruzi conjunctival infection. *Infection and immunity* **74**, 5549-5560

50. Hoft, D. F., Farrar, P. L., Kratz-Owens, K., and Shaffer, D. (1996) Gastric invasion by Trypanosoma cruzi and induction of protective mucosal immune responses. *Infection and immunity* **64**, 3800-3810

51. Burleigh, B. A., and Andrews, N. W. (1995) The mechanisms of Trypanosoma cruzi invasion of mammalian cells. *Annu Rev Microbiol* **49**, 175-200

52. Fichera, L. E., Albareda, M. C., Laucella, S. A., and Postan, M. (2004) Intracellular growth of Trypanosoma cruzi in cardiac myocytes is inhibited by cytokine-induced nitric oxide release. *Infection and immunity* **72**, 359-363

53. Cummings, K. L., and Tarleton, R. L. (2004) Inducible nitric oxide synthase is not essential for control of Trypanosoma cruzi infection in mice. *Infection and immunity* **72**, 4081-4089

54. Kayama, H., and Takeda, K. (2010) The innate immune response to Trypanosoma cruzi infection. *Microbes and infection / Institut Pasteur* **12**, 511-517

55. Bafica, A., Santiago, H. C., Goldszmid, R., Ropert, C., Gazzinelli, R. T., and Sher, A. (2006) Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection. *J Immunol* **177**, 3515-3519

56. Gazzinelli, R. T., and Denkers, E. Y. (2006) Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nature reviews. Immunology* **6**, 895-906

57. Oliveira, A. C., de Alencar, B. C., Tzelepis, F., Klezewsky, W., da Silva, R. N., Neves, F. S., Cavalcanti, G. S., Boscardin, S., Nunes, M. P., Santiago, M. F., Nobrega, A., Rodrigues, M. M., and Bellio, M. (2010) Impaired innate immunity in Tlr4(-/-) mice but preserved CD8+ T cell responses against Trypanosoma cruzi in Tlr4-, Tlr2-, Tlr9- or Myd88-deficient mice. *PLoS pathogens* **6**, e1000870

58. Chessler, A. D., Unnikrishnan, M., Bei, A. K., Daily, J. P., and Burleigh, B. A. (2009) Trypanosoma cruzi triggers an early type I IFN response in vivo at the site of intradermal infection. *J Immunol* **182**, 2288-2296

59. Padilla, A. M., Simpson, L. J., and Tarleton, R. L. (2009) Insufficient TLR activation contributes to the slow development of CD8+ T cell responses in Trypanosoma cruzi infection. *J Immunol* **183**, 1245-1252

60. Kurup, S. P., and Tarleton, R. L. (2013) Perpetual expression of PAMPs necessary for optimal immune control and clearance of a persistent pathogen. *Nat Commun* **4**, 2616

61. Kumar, S., and Tarleton, R. L. (1998) The relative contribution of antibody production and CD8+ T cell function to immune control of Trypanosoma cruzi. *Parasite immunology* **20**, 207-216

62. Cooley, G., Etheridge, R. D., Boehlke, C., Bundy, B., Weatherly, D. B., Minning, T., Haney, M., Postan, M., Laucella, S., and Tarleton, R. L. (2008) High throughput selection of effective serodiagnostics for Trypanosoma cruzi infection. *PLoS neglected tropical diseases* **2**, e316

63. Laucella, S. A., Mazliah, D. P., Bertocchi, G., Alvarez, M. G., Cooley, G., Viotti, R., Albareda, M. C., Lococo, B., Postan, M., Armenti, A., and Tarleton, R. L. (2009) Changes in Trypanosoma cruzi-specific immune responses after treatment: surrogate markers of treatment efficacy. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **49**, 1675-1684

64. Bold, T. D., and Ernst, J. D. (2012) CD4+ T cell-dependent IFN-gamma production by CD8+ effector T cells in Mycobacterium tuberculosis infection. *J Immunol* **189**, 2530-2536

65. Rottenberg, M. E., Bakhiet, M., Olsson, T., Kristensson, K., Mak, T., Wigzell, H., and Orn, A. (1993) Differential susceptibilities of mice genomically deleted of CD4 and CD8 to infections with Trypanosoma cruzi or Trypanosoma brucei. *Infection and immunity* **61**, 5129-5133

66. Padilla, A., Xu, D., Martin, D., and Tarleton, R. (2007) Limited role for CD4+ T-cell help in the initial priming of Trypanosoma cruzi-specific CD8+ T cells. *Infection and immunity* **75**, 231-235

67. Tarleton, R. L., Grusby, M. J., Postan, M., and Glimcher, L. H. (1996) Trypanosoma cruzi infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *International immunology* **8**, 13-22

68. Kumar, S., and Tarleton, R. L. (2001) Antigen-specific Th1 but not Th2 cells provide protection from lethal Trypanosoma cruzi infection in mice. *J Immunol* **166**, 4596-4603

69. Torrico, F., Heremans, H., Rivera, M. T., Van Marck, E., Billiau, A., and Carlier, Y. (1991) Endogenous IFN-gamma is required for resistance to acute Trypanosoma cruzi infection in mice. *J Immunol* **146**, 3626-3632

70. Michailowsky, V., Silva, N. M., Rocha, C. D., Vieira, L. Q., Lannes-Vieira, J., and Gazzinelli, R. T. (2001) Pivotal role of interleukin-12 and interferon-gamma axis in controlling tissue parasitism and inflammation in the heart and central nervous system during Trypanosoma cruzi infection. *The American journal of pathology* **159**, 1723-1733

71. Cai, C. W., Blase, J. R., Zhang, X., Eickhoff, C. S., and Hoft, D. F. (2016) Th17 Cells Are More Protective Than Th1 Cells Against the Intracellular Parasite Trypanosoma cruzi. *PLoS pathogens* **12**, e1005902

72. Cobb, D., and Smeltz, R. B. (2012) Regulation of proinflammatory Th17 responses during Trypanosoma cruzi infection by IL-12 family cytokines. *J Immunol* **188**, 3766-3773

73. Tarleton, R. L. (1990) Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with Trypanosoma cruzi. *J Immunol* **144**, 717-724

74. Frevert, U., Schenkman, S., and Nussenzweig, V. (1992) Stage-specific expression and intracellular shedding of the cell surface trans-sialidase of Trypanosoma cruzi. *Infection and immunity* **60**, 2349-2360

75. Garg, N., Nunes, M. P., and Tarleton, R. L. (1997) Delivery by Trypanosoma cruzi of proteins into the MHC class I antigen processing and presentation pathway. *J Immunol* **158**, 3293-3302

76. Tarleton, R. L., Koller, B. H., Latour, A., and Postan, M. (1992) Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection. *Nature* **356**, 338-340

77. Rottenberg, M. E., Riarte, A., Sporrong, L., Altcheh, J., Petray, P., Ruiz, A. M., Wigzell, H., and Orn, A. (1995) Outcome of infection with different strains of Trypanosoma cruzi in mice lacking CD4 and/or CD8. *Immunol Lett* **45**, 53-60

78. Muller, U., Sobek, V., Balkow, S., Holscher, C., Mullbacher, A., Museteanu, C., Mossmann, H., and Simon, M. M. (2003) Concerted action of perforin and granzymes is critical for the elimination of Trypanosoma cruzi from mouse tissues, but prevention of early host death is in addition dependent on the FasL/Fas pathway. *European journal of immunology* **33**, 70-78

79. Silverio, J. C., de-Oliveira-Pinto, L. M., da Silva, A. A., de Oliveira, G. M., and Lannes-Vieira, J. (2010) Perforin-expressing cytotoxic cells contribute to chronic cardiomyopathy in Trypanosoma cruzi infection. *Int J Exp Pathol* **91**, 72-86

80. Sun, J., and Tarleton, R. L. (1993) Predominance of CD8+ T lymphocytes in the inflammatory lesions of mice with acute Trypanosoma cruzi infection. *The American journal of tropical medicine and hygiene* **48**, 161-169

81. Zhang, L., and Tarleton, R. L. (1999) Parasite persistence correlates with disease severity and localization in chronic Chagas' disease. *The Journal of infectious diseases* **180**, 480-486

82. Vita, R., Overton, J. A., Greenbaum, J. A., Ponomarenko, J., Clark, J. D., Cantrell, J. R., Wheeler, D. K., Gabbard, J. L., Hix, D., Sette, A., and Peters, B. (2015) The immune epitope database (IEDB) 3.0. *Nucleic Acids Res* **43**, D405-412

83. Hall, B. F., Webster, P., Ma, A. K., Joiner, K. A., and Andrews, N. W. (1992) Desialylation of lysosomal membrane glycoproteins by Trypanosoma cruzi: a role for the

surface neuraminidase in facilitating parasite entry into the host cell cytoplasm. *The Journal of experimental medicine* **176**, 313-325

84. Schenkman, S., Jiang, M. S., Hart, G. W., and Nussenzweig, V. (1991) A novel cell surface trans-sialidase of Trypanosoma cruzi generates a stage-specific epitope required for invasion of mammalian cells. *Cell* **65**, 1117-1125

85. Wizel, B., Nunes, M., and Tarleton, R. L. (1997) Identification of Trypanosoma cruzi trans-sialidase family members as targets of protective CD8+ TC1 responses. *J Immunol* **159**, 6120-6130

86. Peterson, D. S., Wrightsman, R. A., and Manning, J. E. (1986) Cloning of a major surface-antigen gene of Trypanosoma cruzi and identification of a nonapeptide repeat. *Nature* **322**, 566-568

87. Alvarez, M. G., Postan, M., Weatherly, D. B., Albareda, M. C., Sidney, J., Sette, A., Olivera, C., Armenti, A. H., Tarleton, R. L., and Laucella, S. A. (2008) HLA Class I-T cell epitopes from trans-sialidase proteins reveal functionally distinct subsets of CD8+ T cells in chronic Chagas disease. *PLoS neglected tropical diseases* **2**, e288

88. Martin, D. L., Weatherly, D. B., Laucella, S. A., Cabinian, M. A., Crim, M. T., Sullivan, S., Heiges, M., Craven, S. H., Rosenberg, C. S., Collins, M. H., Sette, A., Postan, M., and Tarleton, R. L. (2006) CD8+ T-Cell responses to Trypanosoma cruzi are highly focused on strain-variant trans-sialidase epitopes. *PLoS pathogens* **2**, e77

89. Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708-712

90. Bixby, L. M., and Tarleton, R. L. (2008) Stable CD8+ T cell memory during persistent Trypanosoma cruzi infection. *J Immunol* **181**, 2644-2650

91. Martin, D. L., and Tarleton, R. L. (2005) Antigen-specific T cells maintain an effector memory phenotype during persistent Trypanosoma cruzi infection. *J Immunol* **174**, 1594-1601

92. Leavey, J. K., and Tarleton, R. L. (2003) Cutting edge: dysfunctional CD8+ T cells reside in nonlymphoid tissues during chronic Trypanosoma cruzi infection. *J Immunol* **170**, 2264-2268

93. Davenport, M. P., Belz, G. T., and Ribeiro, R. M. (2009) The race between infection and immunity: how do pathogens set the pace? *Trends in immunology* **30**, 61-66

94. Mueller, S. N., and Ahmed, R. (2009) High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 8623-8628

95. Wherry, E. J., Blattman, J. N., Murali-Krishna, K., van der Most, R., and Ahmed, R. (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *Journal of virology* **77**, 4911-4927

96. Wherry, E. J., Barber, D. L., Kaech, S. M., Blattman, J. N., and Ahmed, R. (2004) Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16004-16009

97. Hisaeda, H., Maekawa, Y., Iwakawa, D., Okada, H., Himeno, K., Kishihara, K., Tsukumo, S., and Yasutomo, K. (2004) Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. *Nature medicine* **10**, 29-30

98. Shafiani, S., Tucker-Heard, G., Kariyone, A., Takatsu, K., and Urdahl, K. B. (2010) Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *The Journal of experimental medicine* **207**, 1409-1420

99. Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P., and Yamaguchi, T. (2009) Regulatory T cells: how do they suppress immune responses? *International immunology* **21**, 1105-1111

100. Belkaid, Y., Hoffmann, K. F., Mendez, S., Kamhawi, S., Udey, M. C., Wynn, T. A., and Sacks, D. L. (2001) The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. *The Journal of experimental medicine* **194**, 1497-1506

101. Beamer, G. L., Flaherty, D. K., Assogba, B. D., Stromberg, P., Gonzalez-Juarrero, M., de Waal Malefyt, R., Vesosky, B., and Turner, J. (2008) Interleukin-10 promotes Mycobacterium tuberculosis disease progression in CBA/J mice. *J Immunol* **181**, 5545-5550

102. Couper, K. N., Blount, D. G., and Riley, E. M. (2008) IL-10: the master regulator of immunity to infection. *J Immunol* **180**, 5771-5777

103. Ejrnaes, M., Filippi, C. M., Martinic, M. M., Ling, E. M., Togher, L. M., Crotty, S., and von Herrath, M. G. (2006) Resolution of a chronic viral infection after interleukin-10 receptor blockade. *The Journal of experimental medicine* **203**, 2461-2472

104. Leibson, P. J. (2004) The regulation of lymphocyte activation by inhibitory receptors. *Current opinion in immunology* **16**, 328-336

105. Riella, L. V., Paterson, A. M., Sharpe, A. H., and Chandraker, A. (2012) Role of the PD-1 pathway in the immune response. *Am J Transplant* **12**, 2575-2587

106. Freeman, G. J., Wherry, E. J., Ahmed, R., and Sharpe, A. H. (2006) Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *The Journal of experimental medicine* **203**, 2223-2227

107. Iwai, Y., Terawaki, S., Ikegawa, M., Okazaki, T., and Honjo, T. (2003) PD-1 inhibits antiviral immunity at the effector phase in the liver. *The Journal of experimental medicine* **198**, 39-50

108. Collins, M., Ling, V., and Carreno, B. M. (2005) The B7 family of immune-regulatory ligands. *Genome biology* **6**, 223

109. Sansom, D. M. (2000) CD28, CTLA-4 and their ligands: who does what and to whom? *Immunology* **101**, 169-177

110. Horne-Debets, J. M., Faleiro, R., Karunarathne, D. S., Liu, X. Q., Lineburg, K. E., Poh, C. M., Grotenbreg, G. M., Hill, G. R., MacDonald, K. P., Good, M. F., Renia, L., Ahmed, R., Sharpe, A. H., and Wykes, M. N. (2013) PD-1 dependent exhaustion of CD8+ T cells drives chronic malaria. *Cell reports* **5**, 1204-1213

111. Liang, S. C., Greenwald, R. J., Latchman, Y. E., Rosas, L., Satoskar, A., Freeman, G. J., and Sharpe, A. H. (2006) PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous leishmaniasis. *European journal of immunology* **36**, 58-64

112. Wilson, E. H., Harris, T. H., Mrass, P., John, B., Tait, E. D., Wu, G. F., Pepper, M., Wherry, E. J., Dzierzinski, F., Roos, D., Haydon, P. G., Laufer, T. M., Weninger, W., and Hunter, C. A. (2009) Behavior of parasite-specific effector CD8+ T cells in the brain and visualization of a kinesis-associated system of reticular fibers. *Immunity* **30**, 300-311

113. Jeong, Y. H., Jeon, B. Y., Gu, S. H., Cho, S. N., Shin, S. J., Chang, J., and Ha, S. J. (2014) Differentiation of antigen-specific T cells with limited functional capacity during Mycobacterium tuberculosis infection. *Infection and immunity* **82**, 132-139

114. Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., Freeman, G. J., and Ahmed, R. (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* **439**, 682-687

115. Snyder, C. M., Cho, K. S., Bonnett, E. L., van Dommelen, S., Shellam, G. R., and Hill, A. B. (2008) Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. *Immunity* **29**, 650-659

116. Keir, M. E., Butte, M. J., Freeman, G. J., and Sharpe, A. H. (2008) PD-1 and its ligands in tolerance and immunity. *Annual review of immunology* **26**, 677-704

117. Schwartz, R. H. (2003) T cell anergy. *Annual review of immunology* **21**, 305-334

118. Martin, D. L., Postan, M., Lucas, P., Gress, R., and Tarleton, R. L. (2007) TGF-beta regulates pathology but not tissue CD8+ T cell dysfunction during experimental Trypanosoma cruzi infection. *European journal of immunology* **37**, 2764-2771

119. Kotner, J., and Tarleton, R. (2007) Endogenous CD4(+) CD25(+) regulatory T cells have a limited role in the control of Trypanosoma cruzi infection in mice. *Infection and immunity* **75**, 861-869

120. Reed, S. G., Brownell, C. E., Russo, D. M., Silva, J. S., Grabstein, K. H., and Morrissey, P. J. (1994) IL-10 mediates susceptibility to Trypanosoma cruzi infection. *J Immunol* **153**, 3135-3140

121. Hunter, C. A., Ellis-Neyes, L. A., Slifer, T., Kanaly, S., Grunig, G., Fort, M., Rennick, D., and Araujo, F. G. (1997) IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. *J Immunol* **158**, 3311-3316

122. Holscher, C., Mohrs, M., Dai, W. J., Kohler, G., Ryffel, B., Schaub, G. A., Mossmann, H., and Brombacher, F. (2000) Tumor necrosis factor alpha-mediated toxic shock in Trypanosoma cruzi-infected interleukin 10-deficient mice. *Infection and immunity* **68**, 4075-4083

123. Albareda, M. C., Laucella, S. A., Alvarez, M. G., Armenti, A. H., Bertochi, G., Tarleton, R. L., and Postan, M. (2006) Trypanosoma cruzi modulates the profile of memory CD8+ T cells in chronic Chagas' disease patients. *International immunology* **18**, 465-471

124. Laucella, S. A., Postan, M., Martin, D., Hubby Fralish, B., Albareda, M. C., Alvarez, M. G., Lococo, B., Barbieri, G., Viotti, R. J., and Tarleton, R. L. (2004) Frequency of interferon- gamma -producing T cells specific for Trypanosoma cruzi inversely correlates with disease severity in chronic human Chagas disease. *The Journal of infectious diseases* **189**, 909-918

125. Lasso, P., Mateus, J., Pavia, P., Rosas, F., Roa, N., Thomas, M. C., Lopez, M. C., Gonzalez, J. M., Puerta, C. J., and Cuellar, A. (2015) Inhibitory Receptor Expression on CD8+ T Cells Is Linked to Functional Responses against Trypanosoma cruzi Antigens in Chronic Chagasic Patients. *J Immunol* **195**, 3748-3758

126. Arguello, R. J., Albareda, M. C., Alvarez, M. G., Bertocchi, G., Armenti, A. H., Vigliano, C., Meckert, P. C., Tarleton, R. L., and Laucella, S. A. (2012) Inhibitory receptors are expressed by Trypanosoma cruzi-specific effector T cells and in hearts of subjects with chronic Chagas disease. *PloS one* **7**, e35966

127. Arguello, R. J., Vigliano, C., Cabeza-Meckert, P., Viotti, R., Garelli, F., Favaloro, L. E., Favaloro, R. R., Laguens, R., and Laucella, S. A. (2014) Presence of antigen-experienced T cells with low grade of differentiation and proliferative potential in chronic Chagas disease myocarditis. *PLoS neglected tropical diseases* **8**, e2989

128. Hansen, T. H., and Bouvier, M. (2009) MHC class I antigen presentation: learning from viral evasion strategies. *Nature reviews. Immunology* **9**, 503-513

129. Levitskaya, J., Sharipo, A., Leonchiks, A., Ciechanover, A., and Masucci, M. G. (1997) Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 12616-12621

130. Ahn, K., Gruhler, A., Galocha, B., Jones, T. R., Wiertz, E. J., Ploegh, H. L., Peterson, P. A., Yang, Y., and Fruh, K. (1997) The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity* **6**, 613-621

131. York, I. A., Roop, C., Andrews, D. W., Riddell, S. R., Graham, F. L., and Johnson, D. C. (1994) A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell* **77**, 525-535

132. Burgert, H. G., Maryanski, J. L., and Kvist, S. (1987) "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 1356-1360

133. Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D., and Baltimore, D. (1998) HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391**, 397-401

134. Camargo, R., Faria, L. O., Kloss, A., Favali, C. B., Kuckelkorn, U., Kloetzel, P. M., de Sa, C. M., and Lima, B. D. (2014) Trypanosoma cruzi infection down-modulates the immunoproteasome biosynthesis and the MHC class I cell surface expression in HeLa cells. *PloS one* **9**, e95977

135. Daar, A. S., Fuggle, S. V., Fabre, J. W., Ting, A., and Morris, P. J. (1984) The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation* **38**, 287-292

136. Cabral, C. M., Tuladhar, S., Dietrich, H. K., Nguyen, E., MacDonald, W. R., Trivedi, T., Devineni, A., and Koshy, A. A. (2016) Neurons are the Primary Target Cell for the Brain-Tropic Intracellular Parasite Toxoplasma gondii. *PLoS pathogens* **12**, e1005447

137. Joly, E., Mucke, L., and Oldstone, M. B. (1991) Viral persistence in neurons explained by lack of major histocompatibility class I expression. *Science* **253**, 1283-1285

138. Kurup, S. P., and Tarleton, R. L. (2014) The Trypanosoma cruzi flagellum is discarded via asymmetric cell division following invasion and provides early targets for protective CD8(+) T cells. *Cell host & microbe* **16**, 439-449

CHAPTER 2

$CD8^+$ T CELLS CONTINUE TO CONTROL PATHOGEN LOAD THROGHOUT CHRONIC *TRYPANOSOMA CRUZI* INFECTION[1]

[1] Pack, A.D. and Tarleton, R.L., *et al*. 2016. Manuscript in preparation for submission to *The Journal of Immunology*.

2.1 Abstract

Trypanosoma cruzi infection is characterized by chronic parasitism of non-lymphoid tissues and is rarely eliminated despite strong adaptive immune responses. This failure to cure could be caused by loss or impairment of anti-*T. cruzi* T cell responses over time, similar to T cell dysfunction that is described for other persistent infections. In this study, we have evaluated the role of $CD8^+$ T cells during chronic *T. cruzi* infection (>100 days post infection), with a focus on sites of pathogen persistence. Consistent with repetitive antigen exposure during chronic infection, parasite specific $CD8^+$ T cells from multiple tissues expressed high levels of KLRG1, but exhibit a preferential accumulation of $CD69^+$ cells in skeletal muscle, indicating recent antigen encounter in a niche for *T. cruzi* persistence. A significant proportion of $CD8^+$ T cells in the muscle also produced IFNγ, TNFα and granzyme B *in situ*, an indication of their detection of and functional response to *T. cruzi in vivo*. $CD8^+$ T cell function was crucial for the control of parasite burden during chronic infection, as exacerbation of parasite load was observed upon antibody-mediated depletion of this population. Attempts to improve T cell function by blocking PD-1 or IL-10, potential negative regulators of T cells, failed to increase IFNγ and TNFα production or to enhance *T. cruzi* clearance. These results highlight the capacity of $CD8^+$ T cells to retain essential *in vivo* function despite chronic antigen stimulation and support a model in which $CD8^+$ T cell dysfunction plays a negligible role in the ability of *Trypanosoma cruzi* to persist in mice.

2.2 Introduction

Chagas disease, caused by the protozoal parasite *Trypanosoma cruzi*, is the leading global cause of infectious myocarditis and infects at least 10 million individuals (1,2). Over decades of infection, many infected individuals develop clinical disease including mega colon and dilated cardiomyopathy for which treatment options are limited (3). The pathophysiology of Chagas disease is best described as chronic inflammatory damage driven by persistent parasitism of the affected tissues. A better understanding of the immune mechanisms regulating the host-parasite interaction and disease outcomes is needed to improve development of therapeutic and preventive strategies for this neglected tropical disease and is the focus of this study.

Immune control of intracellular pathogens like *T. cruzi* is dependent on MHC class I presentation of cytoplasmic antigens (Ag) and the subsequent destruction of infected cells as a result of inflammatory cytokine production or cytolysis by $CD8^+$ T cells (4,5). In many infections, effective immunity results in acute phase pathogen clearance, with recognition and elimination of infected host cells early in the infection cycle, thus preventing pathogen spread and contributing to rapid infection resolution. During infections where complete pathogen clearance does not occur, or is significantly delayed, persistent antigen can drive the emergence of 'exhausted' T cells with diminished capacity to produce key cytokines, reduced replicative potential, anergy, and in extreme cases, T cell deletion by apoptosis (6-8). In some cases, this exhausted state is reversible, by interrupting one or more of a number of regulatory mechanisms responsible for restraining $CD8^+$ T cell activity, e.g. regulatory T cells (Tregs), inhibitory cytokines, or inhibitory receptors such as programmed cell death-1 (PD-1) (9). While these regulatory programs minimize immunopathology, they may also compromise infection resolution (10-13).

$CD8^+$ T cells are essential for host survival of acute *T. cruzi* infection (14,15), but the significance of this population in control of chronic infection is not well defined. The high parasite load characteristic of acute *T. cruzi* infection, is ultimately reduced to nearly undetectable levels in most hosts, a feat that involves, and in fact depends upon the extensive expansion and effector function of anti-*T. cruzi* $CD8^+$ T cells (16). Based upon the evidence for persistence of *T. cruzi* in infected hosts and the demonstration that persistent Ag stimulation is associated with reduced T cell function in other systems, including several intracellular protozoans (17-19), investigation of the contribution of T cell exhaustion in *T. cruzi* persistence is warranted.

The lack of direct evidence for an *in vivo* contribution of $CD8^+$ T cells to control of chronic *T. cruzi* infection and the emergence of reports questioning the validity of *ex vivo* assays to predict such function (20-22), prompted us to attempt to conclusively determine whether reduced $CD8^+$ T cell function is the primary factor enabling the persistence of *T. cruzi*. For this, we examined the stability and *in vivo* functional capacity of *T. cruzi* specific cells in spleen and skeletal muscle during chronic *T. cruzi* infection and evaluated the relative contribution of immunoregulation in the forms of PD-1 and IL-10 to T cell activity and parasite persistence. Our findings indicate that despite chronic antigen stimulation, $CD8^+$ T cells continue to exert critical effector functions to limit parasite expansion throughout the course of *T. cruzi* infection.

2.3 Materials and Methods

2.3.1 Mice and parasites

For *T. cruzi* infections, mice of 8-12 weeks old were infected via intraperitoneal (i.p.) injection of 10^3 or 10^4 trypomastigotes of the Brazil or TCC strain. The plasmid, pTRIX2-RE9h, a kind gift from Dr. John Kelly, London School of Hygiene and Tropical Medicine, London, UK

(23), was used to generate Colombiana strain parasites that express red-shifted firefly luciferase for the indirect determination of parasite levels by quantification of luminescent signal. Parasites were maintained in culture using serial passage through Vero cells.

C57BL/6 mice (CD45.2+), B6.SJL (CD45.1+), B6.129S4-Ifng$^{tm3.1Lky}$/J (IFNγ eYFP "GREAT"), and B6.129P2-*Il*10^{tm1Cgn}/J (IL-10 KO) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), the National Cancer Institute (Frederick, MD) or were bred and maintained under specific pathogen-free conditions at the Coverdell Vivarium (University of Georgia, Athens, GA). PD-L1 KO mice were a kind gift from Dr. Arlene Sharp, Brigham and Women's Hospital, Boston, MA. All mice were euthanized by CO_2. All animal use was performed in accordance with protocols approved by the University of Georgia Institutional Animal Care and Use Committee.

2.3.2 Lymphocyte isolation from peripheral tissues

In a small number of experiments, lymphocytes were isolated from peripheral tissues as previously described (24). Most frequently, the following protocol was utilized with a few modifications (25). Prior to tissue removal, mice were perfused by injection of 20 mL of 0.8% sodium citrate solution in PBS. Tissues were then minced and stirred in Hank's balanced salt solution (Corning) with 1.25mM EDTA for 30 min at 37^{o}C. This treatment was followed by incubation with 150U/mL collagenase (Gibco) in RPMI for at least 1h. The digested tissue was then filtered through a 70μM nylon cell strainer (BD Biosciences) and pelleted via centrifugation. The pellet was resuspended in 44% Percoll (GE Healthcare) then underlain 67% Percoll in PBS. Following centrifugation at 600x g, cells were collected from the gradient interface and washed in RPMI.

2.3.3 T cell phenotyping

$CD8^+$ T cell phenotypes were determined by staining with the MHC class I tetramer TSKB20 (ANYKFTLV/K^b) synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA) and the following: CD8, CD44, KLRG1 (eBioscience), CD69, PD-1 (BD Pharmingen), and CD8 (Accurate Chemical). RBCs in single-cell suspensions of spleen cells were lysed in a hypotonic ammonium chloride solution and washed in staining buffer [2% BSA and 0.02% azide in PBS (PAB)]. Cells were stained at 4°C, washed in PAB, and fixed in 2% formaldehyde. At least 100,000 lymphocytes were acquired using a HyperCyAn or CyAn flow cytometer (DakoCytomation) and analyzed with FlowJo software (Tree Star).

2.3.4 Standard T cell stimulation and intracellular staining

Lymphocytes isolated from spleen or peripheral tissue were stimulated with 1.5 μg of plate-bound anti-mouse CD3ε (eBioscience) or 1 μM *T. cruzi* peptide TSKB20 (ANYKFTLV) (GenScript) for 5h at 37°C in the presence of Golgi Plug (BD Pharmingen). Cells were surface stained, washed, fixed and permeabilized, and intracellular cytokine staining was performed to detect IFNγ (eBioscience) and TNFα (BD Pharmingen) using a Cytofix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer's instructions. For CD107 labeling, CD107a antibody (BD Pharmingen) was added during stimulation at 37°C.

2.3.5 Direct intracellular cytokine staining and in vivo activation

For direct intracellular staining (dICS) (20), mice were injected with 0.25 mg of brefeldin A (Sigma) in PBS and 6-8h later, tissues were collected and processed as described above with the addition of brefeldin (10 μg/mL) to isolation solutions. For intracellular granzyme B (BD Pharmingen) staining, cells were stained directly *ex vivo* without stimulation using the Cytofix/Cytoperm kit (BD Pharmingen) following surface staining. For *in vivo* stimulation with

synthetic peptides (26), mice with injected i.p. with 100 μg of TSKB20 or control SIINFEKL peptide simultaneously with brefeldin A administration. Cells were then processed using the typical dICS protocol.

2.3.6 In vivo BrdU incorporation

A bromodeoxyuridine (BrdU) (Sigma) solution (0.8mg/mL) was prepared fresh every 2d and administered *ad libitum* in drinking water to naïve and chronically infected mice for 21d. Lymphocytes were isolated from skeletal muscle and spleen as described above. Staining was then performed with anti-BrdU antibody in accordance with manufacturer instructions in the BrdU Flow Kit (BD Pharmingen).

2.3.7 Treatments

Blocking or depleting monoclonal antibodies (mAb) were administered by i.p. injection every third day for 30 days with the exception of anti-CD8a which was administered every other day for 21 days. The clone, specificity, and amount administered with each treatment were as follows: anti-IL-10R (1B1.3A) 250 μg; anti-CD8a (YTS 169.4) 200 μg; anti-VLA-4 (PS/2) 150 μg; anti-PD-L1 (MIH5) 200 μg. The anti-CD8a Ab used for detection by flow cytometry is a distinct clone from the depleting Ab used.

For some experiments, mice were briefly treated with the trypanocidal drug benznidazole (Roche) to reduce the parasite load in chronically infected mice. The compound was administered orally (100 mg/kg body weight) for 21 days.

2.3.8 Adoptive transfer of $CD8^+$ T cells

For transfers, spleens from chronically *T. cruzi*-infected mice were homogenized with frosted glass slides (Fisher Scientific) in a hypotonic ammonium chloride RBC lysis buffer and

washed in RPMI with 10% FBS. $CD8^+$ T cells were negatively selected through magnetic sorting with a $CD8a^+$ T cell isolation kit (Miltenyi). $CD8^+$ cells were transferred i.v. into infection-matched congenic mice, and recipients were terminated at various days post transfer to analyze donor cell populations in recipient tissues.

2.3.9 Quantitation of parasite burden

Parasite equivalents in tissue were determined as previously described (27). Briefly, tissues were collected from mice and finely minced. Samples were incubated at 55 °C in SDS-proteinase K lysis buffer. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 100% ethanol, and resuspended in nuclease free water. PCR reactions contained iQ SYBR Green Supermix (Biorad) and primers specific for *T. cruzi* or mouse genomic DNA. Samples were analyzed on an iCycler (Biorad) and *T. cruzi* equivalents were calculated as the ratio of *T. cruzi* satellite DNA divided by the quantity of mouse TNFα DNA in each sample.

Parasite burden in select tissues and organs was assessed via *ex vivo* imaging following infection with luciferase expressing parasites. Mice were perfused with 25 mL of D-luciferin (Gold Bio) at 0.3 mg/mL in PBS via the heart. Excised tissues were transferred to a culture dish, soaked in 0.3 mg/ml of D-luciferin in PBS, and then imaged using an IVIS Lumina II system (Xenogen). Exposure time was 5 minutes.

2.3.10 Histology

Skeletal muscle was obtained from *T. cruzi*-infected mice and controls, fixed in 10% buffered formalin and embedded in paraffin. Five-micron thick sections were obtained and stained with hematoxylin-eosin. Inflammation was evaluated qualitatively according to the presence or absence of myocyte necrosis and infiltrates according to distribution (focal,

confluent or diffuse) and extent of inflammatory cells present. Images of tissue sections were taken with an OLYMPUS DP70 digital camera on an OLYMPUS BX60 microscope.

2.3.11 Statistical Analysis

Statistical significance was calculated using a two-tailed Student's t-test or Mann-Whitney test. * indicates values (mean+/- SEM) that are significantly different between specified groups (* $P \leq 0.05$, ** $P \leq 0.01$,***$P \leq .001$).

2.4 Results

2.4.1 Pathogen-specific tissue-resident T cells exhibit markers of recent antigen exposure

The $CD8^+$ T cell response to *T. cruzi* infection is dominated by T cells recognizing strongly immunodominant epitopes from proteins of the trans-sialidase family (28). In B6 mice, TSKB20 (ANYKFTLV)–specific T cells expand to high levels in the spleen during acute *T. cruzi* infection before contracting and remaining at relatively low levels as the infection is controlled (Fig. 2.1A). In the skeletal muscle, a site of *T. cruzi* persistence, TSKB20 T cells maintain a higher stable fraction of the total Ag-experienced $CD8^+$ population throughout acute and chronic infection. Both splenic and skeletal muscle *T. cruzi*-specific $CD8^+$ T cells exhibited substantial KLRG1 expression, indicative of prior Ag exposure (Fig. 2.1B). However, a significantly higher frequency of $CD8^+$ T cells in the skeletal muscle exhibited PD-1 expression, consistent with continued T cell activation in a tissue of parasite persistence (Fig. 2.1C). Furthermore, only in skeletal muscle T cells (Fig. 2.1D) and other tissues of parasite-persistence such as heart and fat (Sup. Fig. 2.1A) was the marker of recent activation, CD69, expressed in a substantial proportion of *T. cruzi*–specific $CD8^+$ T cells.

The activation status and accumulation of *T. cruzi* specific T cells was related to parasite

antigen abundance in the tissues, as shown by the fact that CD69 expression was lower in the case of infection with the low level but persistent TCC infection (Sup. Fig. 2.1B,C) and in mice treated with the trypanocidal compound benznidazole (BZ) (Fig. 2.1E,F). As previously reported, BZ treatment also resulted in overall fewer inflammatory infiltrates in association with a reduction in parasite numbers in the muscle (Fig. 2.1G) (29). Collectively, these data provide strong evidence that *T. cruzi*-specific T cells regularly encounter and are responsive to parasites at sites of pathogen persistence in chronically infected hosts.

2.4.2 *T. cruzi*-specific $CD8^+$ T cells exhibit potent effector functions during chronic infection

We next assessed the effector function of parasite-specific T cells in peripheral tissues of mice with chronic *T.* cruzi infection. IFNγ production measured directly ex vivo without restimulation using both a YFP-reporter strain (Fig. 2.2A) and intracellular cytokine staining (Fig. 2.2B, Sup. Fig. 2.2A) demonstrated that parasite-specific $CD8^+$ T cells were significantly more activated for production of this critical cytokine in peripheral sites of parasite persistence (skeletal muscle) as compared to the spleen (20,30). Additionally, a higher frequency of $CD8^+$ T cells in the skeletal muscle showed granzyme B expression (Fig. 2.2C), a measure of $CD8^+$ T cell capacity to eliminate infected target cells through the delivery of cytotoxic granules, relative to splenic counterparts.

To further evaluate the capacity of parasite-specific T cells in peripheral tissues to respond to parasite Ag *in vivo* during chronic *T. cruzi* infection, mice were injected with control (SIINFEKL) or parasite (TSKB20) peptides (Fig. 2.2D) and assessed for cytokine production by ICS (26). $CD8^+$ T cells responded to specific peptide stimulation with significant fold increases in the frequency (Fig. 2.2E) and magnitude (Fig. 2.2F) of inflammatory cytokine production (IFNγ and TNFα) in both in the spleen and skeletal muscle; although there was no further

increase in the already high levels of TNFα production upon peptide stimulation.

2.4.3 $CD8^+$ T cell function is required for optimal control of chronic *T. cruzi* infection

The data presented to this point indicate that *T. cruzi*–specific T cells at sites of parasite persistence are capable of sensing and responding to *T. cruzi*. To evaluate whether these $CD8^+$ T cells provide a host-protective function in the chronic phase of infection, anti-CD8 antibody was administered to mice beginning at >100 dpi. Treatment significantly reduced circulating $CD8^+$ T cells (Fig. 2.3A) and when compared to IgG-treated controls, the parasite load in skeletal muscle and fat was significantly higher in the CD8-depleted group (Fig. 2.3B). Monitoring of luciferase-expressing parasites in intact organs also revealed a substantial increase in parasite levels, particularly in the gastrointestinal tract, in mice depleted of $CD8^+$ T cells (Fig. 2.3C). Notably, depletion of $CD8^+$ T cells also resulted in increased severity of inflammation and disruption of tissue integrity in skeletal muscle (Fig. 2.3D). Thus, tissue-homing *T. cruzi*–specific $CD8^+$ T cells retained important effector functions and are vital to infection control in chronic *T. cruzi* infection.

2.4.4 Maintenance of $CD8^+$ T cell response to *T. cruzi* in peripheral tissue

We next explored the question of whether T cells in the peripheral tissue, sites of parasite persistence, are being continuously recruited from the systemic population. $CD8^+$ T cells from the spleens of chronically *T. cruzi*-infected donor mice (CD45.1) were transferred to infection-matched congenic recipient (CD45.2) mice. Donor cells were promptly incorporated into the $CD8^+$ T cell population at sites of parasite persistence as early as 2 days post transfer and are detectable in the tissue up to 50 days after transfer (Fig. 2.4A). These data show that even in the setting of low-level chronic inflammation, $CD8^+$ T cell trafficking is a dynamic process, and $CD8^+$ T cells responding to *T. cruzi* move readily from the circulation into tissues harboring

parasite-infected cells. To determine if frequent recruitment of effector cells to infected tissue was a key mechanism for controlling *T. cruzi* during chronic infection, trafficking of T cells was disrupted by administering a blocking antibody to VLA-4, a molecule involved in leukocyte extravasation (31). Mice receiving anti-VLA-4 Ab exhibited an increased parasite load in skeletal muscle, indicating that infected tissues depend on continuous leukocyte trafficking to control parasite growth (Fig. 2.4B).

In addition to continued recruitment of Ag-experienced $CD8^+$ T cells into responses to persistent pathogens, recruitment of recently activated T cells during an ongoing immune response may be necessary to sustain effector cell levels. To determine whether new parasite-specific effectors are generated during chronic *T. cruzi* infection, the level of recently replicated (BrdU+) cells, was assessed following *ad libitum* BrdU administration for 21 days. A substantial fraction of $CD8^+$ and $TSKB20^+$ cells in muscle and spleen were BrdU+, with an increased accumulation in skeletal muscle (Fig. 2.4C). Many of these recently replicated cells become exposed to *T. cruzi* antigen and possessed an effector activation phenotype, with substantial frequencies of CD8 cells expressing CD69 in the muscle of infected animals (Fig. 2.4D). These data support a model of a very active immune response requiring T cell migration into parasite-infected tissues, where T cell replication continues to occur well into chronic infection.

2.4.5 Immunoregulation via PD-1 or IL-10 has a negligible impact on parasite persistence

The retention of effector function and participation in infection control of $CD8^+$ T cells in mice with chronic *T. cruzi* infection is in contrast to the often-reported loss of the quality of effector responses in multiple other chronic infectious diseases (32-34). In a number of these other models, checkpoint molecules such as PD-1 have been implicated to have a negative impact on pathogen clearance (19,35,36). In Figure 1, we noted that PD-1 expression is evident

on $CD8^+$ T cells in mice with chronic *T. cruzi* infection. To further explore the potential role of PD-1 in constraining T cell responses and supporting parasite persistence during chronic *T. cruzi* infection, we more carefully examined PD-1 expression. PD-1 expressers were always significantly higher in the muscle relative to spleen, although an overall decline in PD-1 expression on $CD8^+$ T cells was observed later in infection, coincident with reduced Ag levels as infection progressed to the chronic phase (Fig. 2.5A). Utilizing IFNγ YFP reporter mice and *in vivo* cytokine detection as described above, we found that PD-1 expression on a given cell did not correlate with IFNγ production potential (Fig. 2.5B) or the capacity of $TSKB20^+$ cells to respond to parasite peptide stimulation *in vivo* (Fig. 2.5C).

To directly address the impact of PD-L1 on control of parasite burden, parasite load and tissue inflammation were evaluated in PD-L1 KO mice. Inhibiting the effects of PD-1 failed to improve parasite control (Fig. 2.5D) or alter tissue pathology (Fig. 2.5E) during chronic infection. In addition, PD-L1 blockade failed to alter the capacity of $CD8^+$ T cells, in the muscle or spleen, to produce inflammatory cytokines in response to polyclonal *ex vivo* stimulation (Sup. Fig. 2.3A). Taken together, these data suggest a negligible role for PD-1 in modulating the T cell-dependent control of chronic *T. cruzi* infection in this model.

Interleukin-10 (IL-10) has also been shown to be a major regulator of T cell responses in chronic infections (12,37,38), so we examined whether this cytokine contributes to the control of T cell responses at sites of parasite persistence in *T. cruzi* infection. Our initial approach was to infect IL-10 KO mice and assess *T. cruzi*-specific T cell frequency and phenotype as mice progressed into the chronic phase. However this was not possible as IL-10 deficient mice succumbed to infection by 45 dpi, in agreement with previous studies (12). We did not observe enhanced parasite clearance (Fig. 2.6A), but inflammation in skeletal muscle was notably

increased (Fig. 2.6B) in acutely infected IL-10 KO mice compared to WT. Administration of an IL-10 receptor (IL-10R)-blocking antibody to mice with established *T. cruzi* infection allowed us to assess the role of IL-10 in parasite control in the chronic infection. The average parasite load detected in anti-IL-10R treated and rat IgG treated controls was not statistically different (Fig. 2.6C). These data suggest that neither PD-1 nor IL-10 are major factors inhibiting the clearance of *T. cruzi* during chronic infection.

2.5 Discussion

Persistent infections present special challenges for the immune system and the cause of persistence is often not clear; is pathogen persistence the result of failed or regulated immune responses or is it an inherent property of a host:pathogen interaction? In the latter case, persistence may or may not ultimately result in regulated immune responses due to persistent antigen stimulation.

The primary goal of this study was to determine the functional status of $CD8^+$ T cells during chronic *T. cruzi* infection and to assess the contribution of these cells to long-term control of persistent parasites. *T. cruzi* infection is thought to persist for the life of hosts; there are only a few anecdotal reports of apparent spontaneous cure in humans (39-41) and in mice (a natural host for this parasite) (42). Often the lack of parasite clearance in *T. cruzi* infection has been attributed to sub-par or highly regulated immune responses (24,43-45). We were particularly interested in evaluating anti-parasite $CD8^+$ T cell responses at the host-pathogen interface (in parasite infected tissues), as this cell population is most likely to be inhibited by immune regulatory mechanisms elicited by repetitive Ag exposure and/or tissue inflammation. We hypothesized that $CD8^+$ T cells retain critical effector function, and that suppression of *T. cruzi* to very low levels is actually a "successful" immunologic outcome that simultaneously limits

sequelae of uncontrolled infection and immunopathology in vital host tissue. This hypothesis is divergent from the paradigm that impaired pathogen clearance is associated with alterations in and regulation of host immunity (17,46-48).

Here, we have identified the effector $CD8^+$ T cell population as highly functional and essential for parasite control during chronic *T. cruzi* infection, despite persistent antigen activation. Persistent antigen activation is demonstrated by the expression of surface markers indicative of recent antigen exposure on as well as by the direct ex vivo detection of cytokine production by T cells from tissues known to harbor persistent parasites. Functionality is demonstrated by these same immunological traits, by the ability of a subset of the T cells to respond *in vivo* to parasite peptide, and by the essentiality of these cells in containing parasite load in these same tissues.

This is a significant observation, as retained $CD8^+$ T cell function is rarely reported in chronic infection systems, especially after hundreds of days of infection in mouse models. Our data suggest that highly regulated T cell responses and compromised immunity are not the sole outcome of persistent infection. In this mouse system of *T. cruzi* infection, well-described modes of immune regulation such as PD-1 and IL-10 appear to have negligible impacts on parasite persistence (19,49). Likewise, previous studies from our group demonstrate that deleting or blocking Treg action or TGF-β production also fail to alter parasite control in this infection (50,51). When combined with the additional evidence of the retained capacity for replication and continued effector function, characteristics often lost in systems with highly regulated T cells (52-54), we conclude that no one of these classically described modulators of $CD8^+$ T cell function is responsible for *T. cruzi* persistence.

Why is it that persistent *T. cruzi* infection in this natural host system fails to exhaust the immune system as in so many other persistent infections? One possibility is that the high efficiency of immune control in this infection dramatically limits parasite load, and thus prevents, or limits the degree of chronic and continuous T cell stimulation. A number of systems have been used to directly confirm the role of chronic antigen stimulation in T cell exhaustion (61,62). In one model, serial injection of live, but non-productive, influenza A virus was used to simulate chronic antigen stimulation in the absence of infection (55). This study revealed that chronic TCR stimulation alone, in the absence of infection-related inflammation, is sufficient to induce T cell exhaustion (55). Similar observations have been made during persistent viral infections (56). During HIV infection, administration of HAART during the first months following HIV exposure significantly reduces viral levels, and protects $CD8^+$ T cells from the extreme antigen stimulation that drives HIV-specific T cell exhaustion (57,58). Similarly, during chronic HCV infection, a model typically characterized by relatively low antigen levels like *T. cruzi*, $CD8^+$ T cell exhaustion is primarily observed in patients with heightened viral titers. In these subjects, high antigen levels induce aggressive immune regulation and compromise HCV control (59,60). In addition, patients with superior HCV control retain a pool of CD127+ antigen-specific memory $CD8^+$ T cells during chronic infection, which serve as an indicator for improved control and limited T cell regulation (59,60). A similar CD127+ Tcm population, capable of antigen-independent survival is evident during very chronic *T. cruzi* infections and after complete parasite clearance following benznidazole treatment (29,61). The maintenance of this memory population suggests that low antigen levels, characteristic of *T. cruzi* infection, likely preserve $CD8^+$ T cell function during chronic infection.

Then, nevertheless, one might still expect exhaustion to occur over very long periods of infection, even when antigen load is low. And that is what is seen in chronic human *T. cruzi* infections lasting decades (44). The level of retained T cell function varies significantly in *T. cruzi* infected patients; with the largest *ex vivo* responses observed in subjects with the shortest probable infection exposure (children) (62). In contrast, hallmarks of terminal differentiation, including inhibitory receptor expression, have been observed in some adult subjects with decades-long *T. cruzi* infections (63-65). It should be noted that, inhibitory receptor upregulation on $CD8^+$ T cells is not observed in all patients chronically infected with *T. cruzi*. These inconsistencies in T cell regulation likely reflect differences in the magnitude of antigen exposure by T cells, based upon infection duration and *T. cruzi* intensity in tissues (45,62). This variation in inhibitory receptor expression will undoubtedly prevent the use of checkpoint therapies, such as PD-1, as a standard treatment for Chagas patients, as they would prove unnecessary and inefficacious in patients with functional T cells. In addition, irreparable immunopathology despite improved parasite control, was observed following PD-1 blockade in one mouse model of *T. cruzi* infection (66). These data highlight the serious safety concerns associated with unrestrained T cell function in essential organs like the heart.

It is still unclear how *T. cruzi* persists in the presence of functional $CD8^+$ T cells, but a recent study has helped to expand our understanding of this phenomenon. A factor that may compromise *T. cruzi* elimination, in the presence of functional $CD8^+$ effectors, are complications in infected cell detection. $CD8^+$ T cell engagement with infected muscle cells is dependent on the presence of specific MHC class I peptide complexes on target cell surfaces. During *T. cruzi* infection, this process may be impacted by 1) delays in the availability of immunodominant *T. cruzi* antigens for infected cell presentation, and 2) low basal MHC I expression in muscle tissue

(67,68), a site frequently associated with persistent parasites (69). *In vitro* co-culture experiments suggest that there are likely delays in the responses made by immunodominant (TSKB20) $CD8^+$ T cells towards *T. cruzi* infected cells (70). When TSKB20+ $CD8^+$ T cells were co-incubated with *T. cruzi* infected cells, there was a delay of at least 24 hours before the production of IFNγ, compared to <6 hours for CD8 cells directed against subdominant epitopes available earlier within the parasite invasion cycle (flagellar proteins) (70). These data suggest that stage-specific protein expression and temporal restrictions in the availability of parasite proteins, based upon *T. cruzi*'s intracellular and extracellular form conversion events (71,72), may limit the speed of responses by $CD8^+$ T cells. As mentioned above, *T. cruzi* is frequently detected in skeletal muscle, which is unique from many of the other cell types that *T. cruzi* infects as it has minimal basal MHC I expression (67,68). Although MHC I expression is upregulated during *T. cruzi* infection (73), it's possible that this lower starting point limits myocyte surveillance by $CD8^+$ T cells, providing a slight survival advantage to muscle-dwelling parasites. Taken together, these factors may impact the recognition of *T. cruzi* infected cells in muscle. This might enable the survival of a nominal number of parasites, despite the presence of parasite-specific $CD8^+$ T cells, to support *T. cruzi* persistence and maintenance of chronic infection.

In this study, we have determined that immunity to *T. cruzi* infection is long lasting and that $CD8^+$ T cell exhaustion is an improbable explanation for parasite persistence. We propose that *T. cruzi* infection represents a system where anti-parasite immunity facilitates long-term host survival despite pathogen persistence; although, decades of infection maintenance may lead to deleterious effects in long-lived hosts such as humans. This study also highlights the value of examining immune response at the host-pathogen interface in the actual tissues of persistence. This approach permitted the detection of a previously unappreciated population of activated,

cytokine-producing, $CD8^+$ T cells that suppress *T. cruzi* expansion, which could not be identified by examining systemic T cell populations (spleen). As a number of pathogens of significant public health concern infect peripheral tissues and establish persistence, efforts to understand how these responses are maintained and how memory develops (during and post infection clearance), are likely to result in the development of therapies and vaccinations of improved efficacy.

Figure 2.1. Parasite-specific $CD8^+$ T cells encounter antigen and express markers of activation during chronic *T. cruzi* infection

A) The frequency of $CD8^+$ T cells specific for the immunodominant epitope, TSKB20, in the pool of all $CD44^+$ $CD8^+$ T cells was measured longitudinally in spleen (open square) and skeletal muscle (open triangle). B) $CD44^{hi}$ KLRG1+ cells were detected in the total (white bar) and parasite-specific (black bar) $CD8^+$ T populations in the spleen and muscle at 150 dpi. C) Expression of PD-1 on $CD44^+$ $CD8^+$ T cells decreases from acute (<40 dpi) to chronic infection (>100 dpi). D) Recently activated $CD8^+$ T cells are enriched in skeletal muscle. Representative flow plots show surface expression of CD69 for cells isolated from indicated tissue during chronic (150 dpi) *T. cruzi* infection. Plots gated on $CD44^+$ $CD8^+$ T cells. Numbers in upper right indicate the percentage of CD69+ TSKB20+ cells; numbers in lower right indicate the percentage of TSKB20- cells expressing CD69. Percentages of CD69+ cells in total (white) and TSKB20+ (black) bars are displayed graphically on the right. E) Reduction in parasite burden following brief treatment with the trypanocidal compound benznidazole (BZ) results in decreased CD69 expression and F) contraction of parasite specific cells in the muscle during chronic infection (>250 dpi). G) Antigen dependent inflammation observed during chronic infection is reduced following short-term benznidazole treatment. Skeletal muscle was fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. All data are representative of at least three independent experiments with n=3-5 with the exception of panels F and G which are representative of two independent experiments. Data are mean + SEM. * indicates percentage levels that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$,***$P \leq .001$) between specified groups.

Figure. 2.1

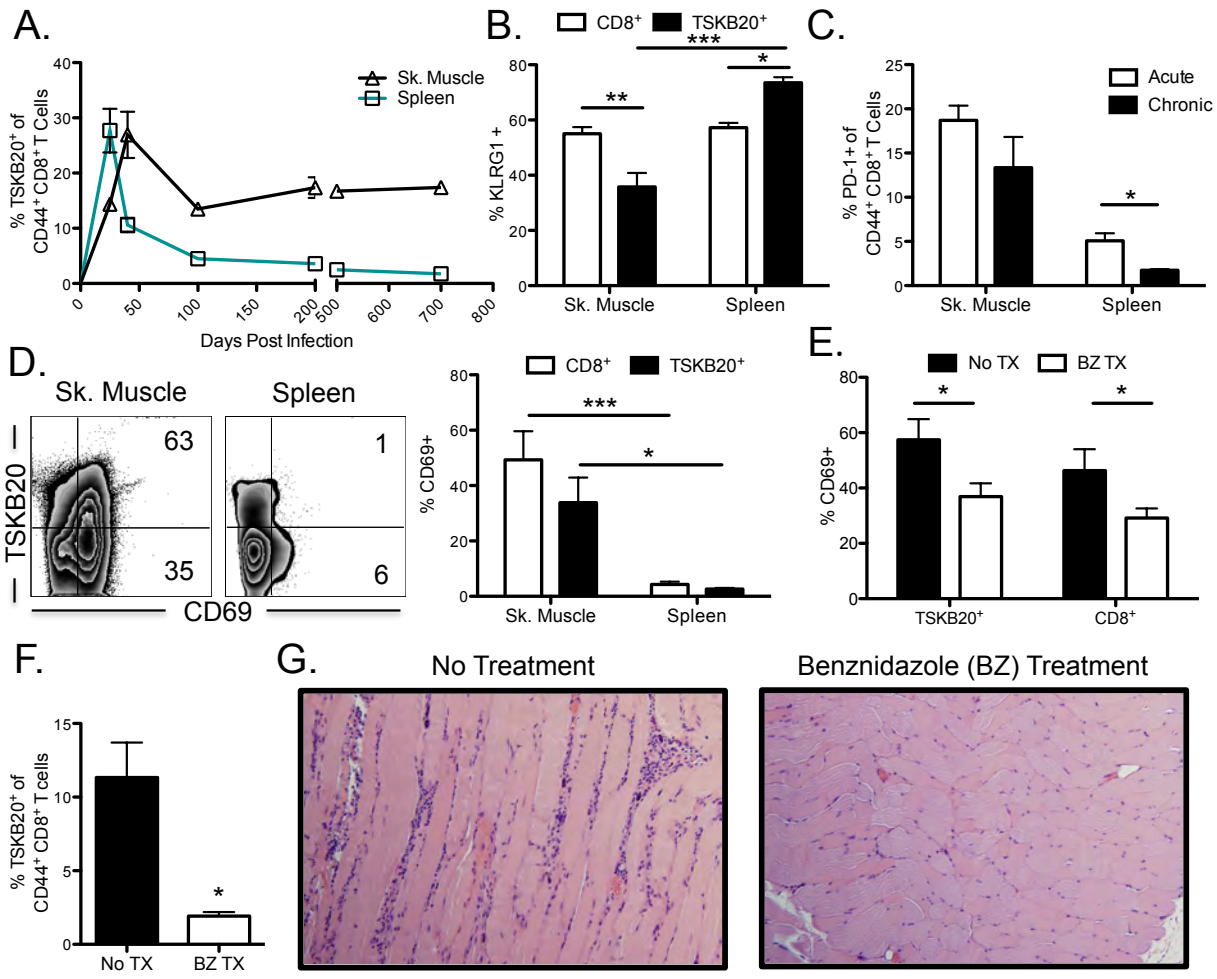

A.
B.
C.
D.
E.
F.
G.
Sk. Muscle
Spleen
Days Post Infection
% TSKB20+ of CD44+ CD8+ T Cells
CD8+
TSKB20+
% KLRG1 +
Acute
Chronic
% PD-1+ of CD44+ CD8+ T Cells
TSKB20
CD69
63
35
1
6
% CD69+
No TX
BZ TX
TSKB20+
CD8+
No Treatment
Benznidazole (BZ) Treatment

Figure 2.2. Parasite-specific $CD8^+$ T cells continue to produce effector molecules *in vivo* during chronic infection

A) *T. cruzi*-specific (TSKB20) $CD8^+$ T cells produce IFNγ during chronic infection. IFNγ production was assessed using an eYFP IFNγ transcription reporter model in the spleen and muscle at 90 dpi. B) Mice were injected with brefeldin A and approximately 6 hours later, tissues were harvested and stained to assess *in situ* cytokine production. Bar graphs represent IFNγ production by TSKB20+ $CD8^+$ T cells at 200 dpi in the absence of exogenous stimulation. C) Granzyme B production was measured directly *ex vivo* on parasite specific $CD8^+$ T cells during chronic infection. D) Schematic summarizes the protocol used to assess the capacity of parasite specific cell to respond to specific antigen stimulation *in vivo*. Mice were injected simultaneously with brefeldin A and TSKB20 or SIINFEKL control peptide prior to tissue collection 6-8 hours later. Cytokine production was evaluated without *ex vivo* stimulation. E) Fold change in the percentage of IFNγ and TNFα positive *T. cruzi*-specific cells following *in vivo* peptide administration (as described in D) was calculated using the equation: Fold change= (% of cytokine (+) cells in animals with specific peptide injection)/(% of cytokine (+) cells in animals with nonspecific peptide injection). F) The relative level of IFNγ and TNFα, as indicated by MFI, produced in animals receiving specific antigen stimulation (black bars) *in vivo* is higher than observed in animals with control peptide (white bars) injection in muscle (left) and spleen (right). Data are mean + SEM and are representative of 3-5 independent experiments with n=3. * indicates values that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$) between experimental and control peptide recipients.

Figure 2.2

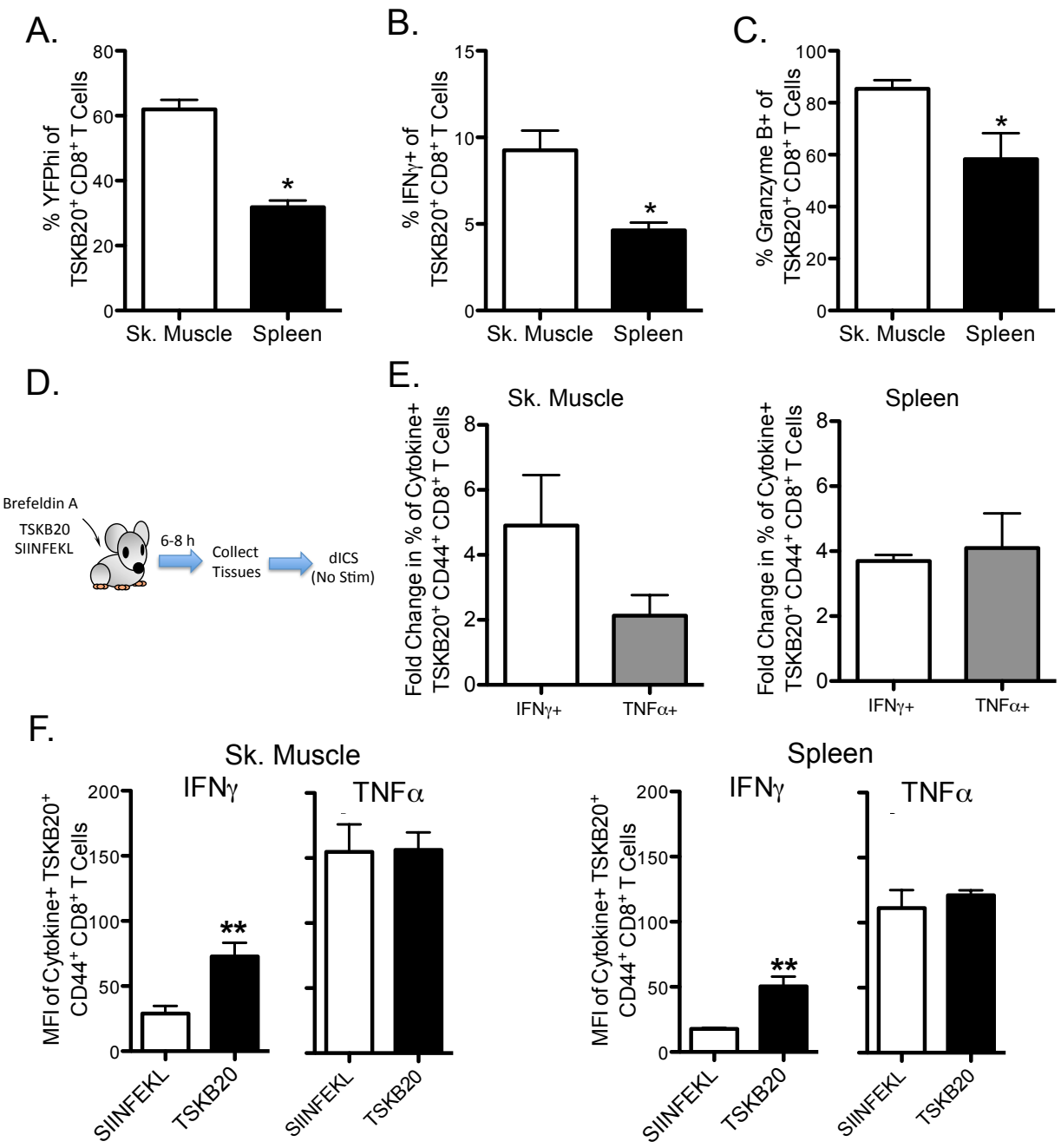

A.
% YFPhi of TSKB20+ CD8+ T Cells
Sk. Muscle
Spleen
B.
% IFNγ+ of TSKB20+ CD8+ T Cells
C.
% Granzyme B+ of TSKB20+ CD8+ T Cells
D.
Brefeldin A
TSKB20
SIINFEKL
6-8 h
Collect Tissues
dICS (No Stim)
E.
Sk. Muscle
Spleen
Fold Change in % of Cytokine+ TSKB20+ CD44+ CD8+ T Cells
IFNγ+
TNFα+
F.
Sk. Muscle
Spleen
IFNγ
TNFα
MFI of Cytokine+ TSKB20+ CD44+ CD8+ T Cells
SIINFEKL
TSKB20

Figure 2.3. $CD8^+$ T cell function is required for optimal control of chronic *T. cruzi* infection

Chronically infected mice (>100 dpi) were treated for 21d with αCD8 or IgG control antibody. A) A representative flow plot of the percentage of $CD8^+$ T cells remaining in the splenic lymphocyte population following CD8 depletion (left) and graphical representation of depletion efficacy (right). B) Depletion of $CD8^+$ T cells during chronic infection results in increased parasite burden in tissues of persistence: muscle (left) and fat (right). C) CD8 depletion results in parasite outgrowth in tissues of persistence, stomach (8), large intestine (10), and skeletal muscle (5) in mice chronically infected with luciferase-expressing parasites. The tissues included in the *ex vivo* luciferase assay are as follows: brain (1), heart (2), spleen (3), kidney (4), skeletal muscle (5), liver (6), mesenteric fat (7), stomach (8), small intestine (9), and large intestine (10). D) H&E staining of skeletal muscle reveals that CD8 depletion exacerbates tissue inflammation and deterioration of tissue integrity. All data are representative of two independent experiments with n=5 with the exception of panel C. Data are mean + SEM. * indicates values that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) between compared groups.

Figure 2.3

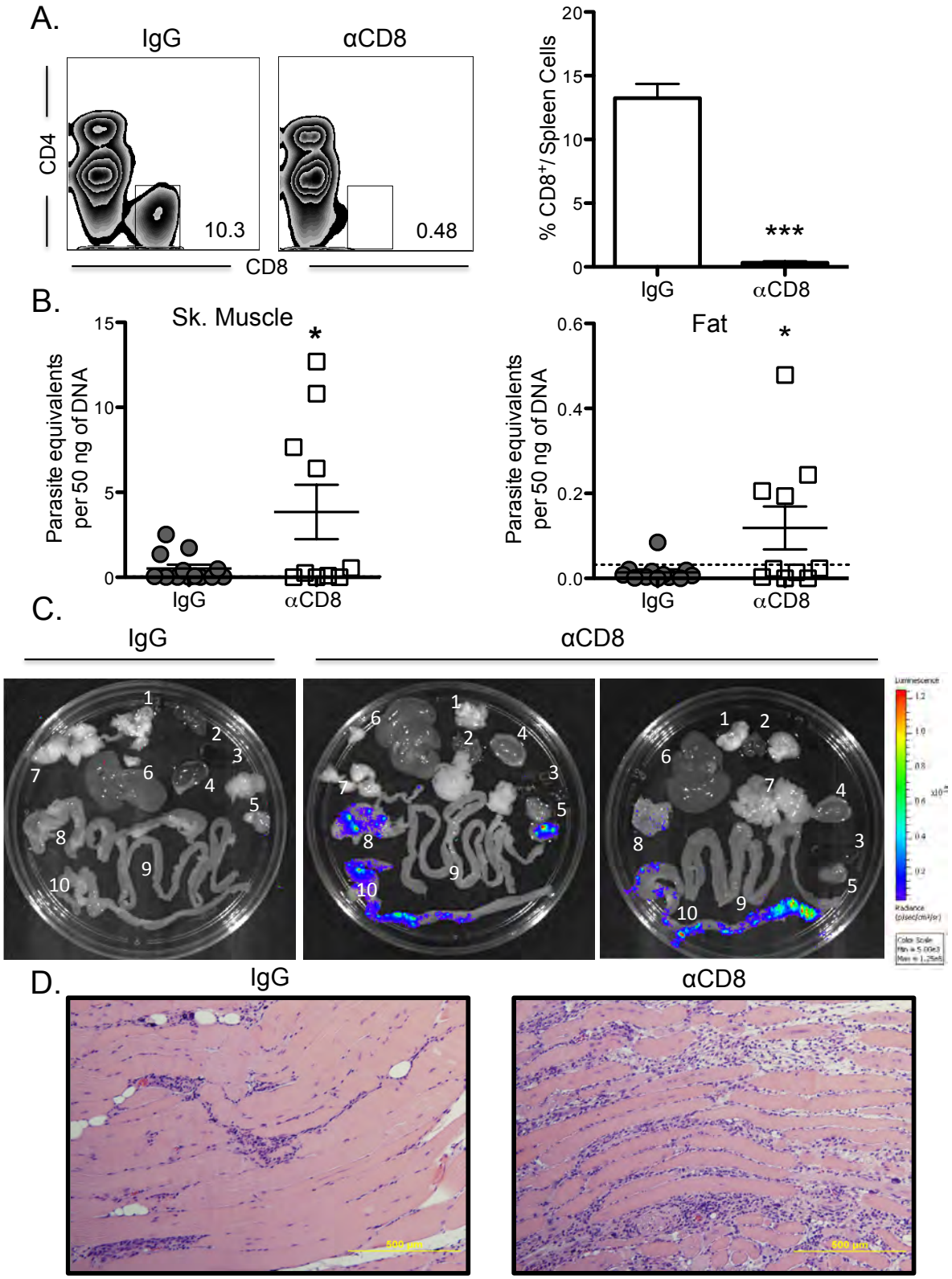

A.
IgG
αCD8
CD4
CD8
10.3
0.48
% CD8+/ Spleen Cells
***
B.
Sk. Muscle
Fat
Parasite equivalents per 50 ng of DNA
*
C.
IgG
αCD8
D.
IgG
αCD8

Figure 2.4. Trafficking and maintenance of $CD8^+$ T cell response to *T. cruzi* in peripheral tissue

A) $CD8^+$ T cells are recruited from a central pool to non-lymphoid tissues in chronic *T. cruzi* infection. $CD8^+$ T cells were magnetically purified from infection-matched CD45.1+ donor mice (>100 dpi) and transferred into CD45.2+ recipient mice. Cells were isolated from spleen (square) and skeletal muscle (triangle) at various time points after transfer. Mean ± SEM of two samples analyzed at each time point is shown and represents data obtained in three independent experiments. B) Interruption of leukocyte trafficking is detrimental to parasite control. Mice (>100 dpi) were treated with a blocking anti-VLA-4 Ab for 30 days, and parasite load was determined in skeletal muscle. * indicates $p<0.05$ compared to control by Mann-Whitney test. A similar trend ($p<0.05$) was observed in 2 of 3 additional experiments. C) Dividing $CD8^+$ T cells preferentially traffic to tissues of parasite persistence. Mice chronically infected with *T. cruzi* were given BrdU in their drinking water for 21 days. $CD8^+$ T cells were then isolated from lymphoid and non-lymphoid tissue. Numbers indicate percentage of $CD8^+$ cells that have incorporated BrdU. D) Dividing $CD8^+$ T cells develop into activated effectors at sites of parasite persistence. Expression of CD69 was examined among proliferating (BrdU+) $CD8^+$ T cells in muscle and spleen in naïve animals and mice with chronic *T. cruzi* infection. Data in panels C and D are mean + SEM and are representative two independent experiments with n=4-5. * indicates values that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$) between compared groups.

Figure 2.4

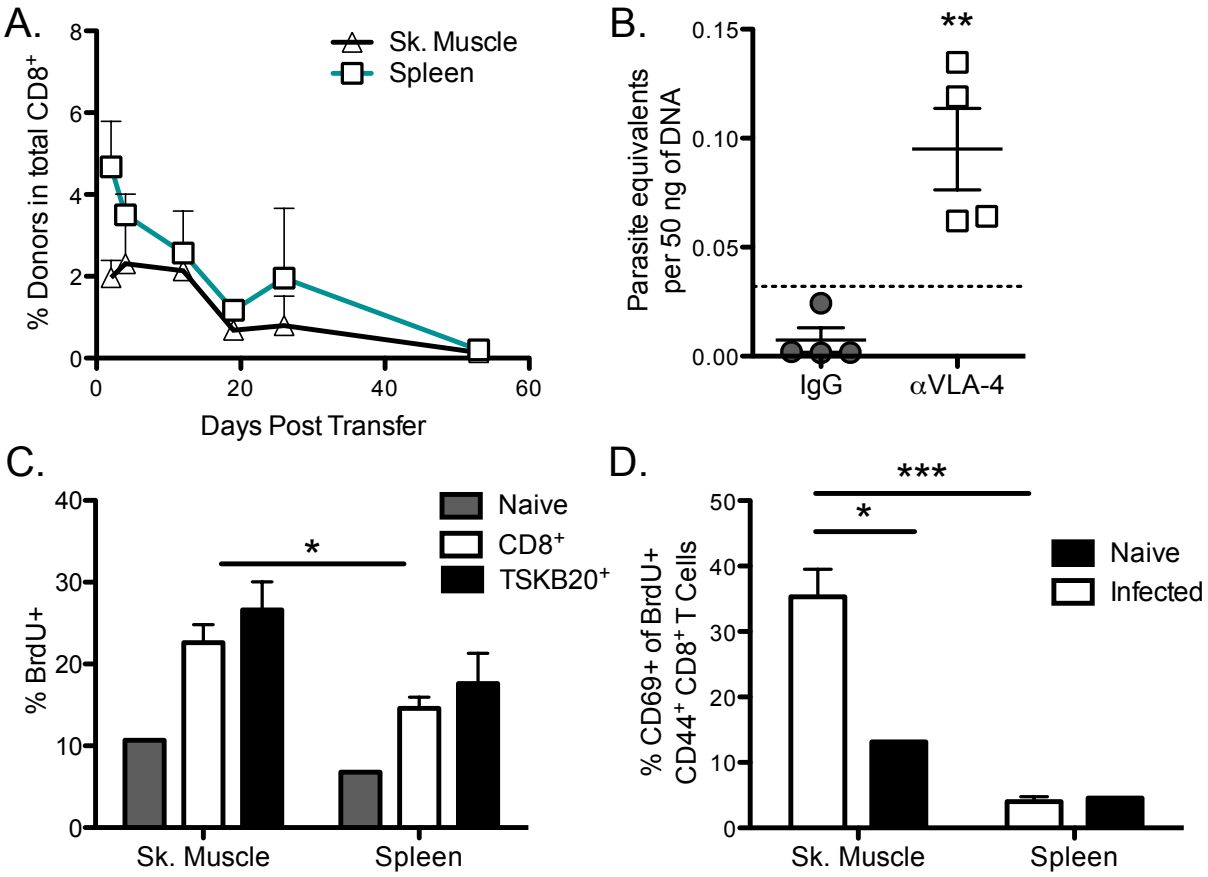

A.
% Donors in total CD8+
Sk. Muscle
Spleen
Days Post Transfer
B.
Parasite equivalents per 50 ng of DNA
**
IgG
αVLA-4
C.
% BrdU+
Naive
CD8+
TSKB20+
*
Sk. Muscle
Spleen
D.
% CD69+ of BrdU+ CD44+ CD8+ T Cells
***
*
Naive
Infected
Sk. Muscle
Spleen

Figure 2.5. CD8$^+$ T cells are not exhausted by a PD-1-dependent mechanism in chronic *T. cruzi* infection

A) The proportion of PD-1+ parasite-specific CD8$^+$ spleen and muscle derived cells decreases between acute (<40d) and chronic infection (>100d) in muscle and spleen.

B) Comparable portions of PD-1 positive and negative cells produce IFNγ at 90 days post infection, as indicated by YFP expression, in muscle (left) and spleen (right). C) PD-1 expression does not undermine the capacity of CD8$^+$ T cells to respond to antigen stimulation *in vivo* in skeletal muscle (left) or spleen (right) during chronic infection.

D) Mice lacking PD-L1 do not exhibit enhanced clearance of *T. cruzi* infection. Parasite load in skeletal muscle of PD-L1 KO and WT mice during and chronic (167 dpi) *T. cruzi* infection was assessed by qPCR. E) PD-L1 KO mice experience levels of inflammation similar to WT mice during chronic *T. cruzi* infection. H&E sections of skeletal muscle from infected PD-L1 KO and WT mice. Graphs represent mean + SEM. * indicates values that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$) between compared groups. All data are representative of at least two independent experiments with minimum n= 3.

Figure 2.5

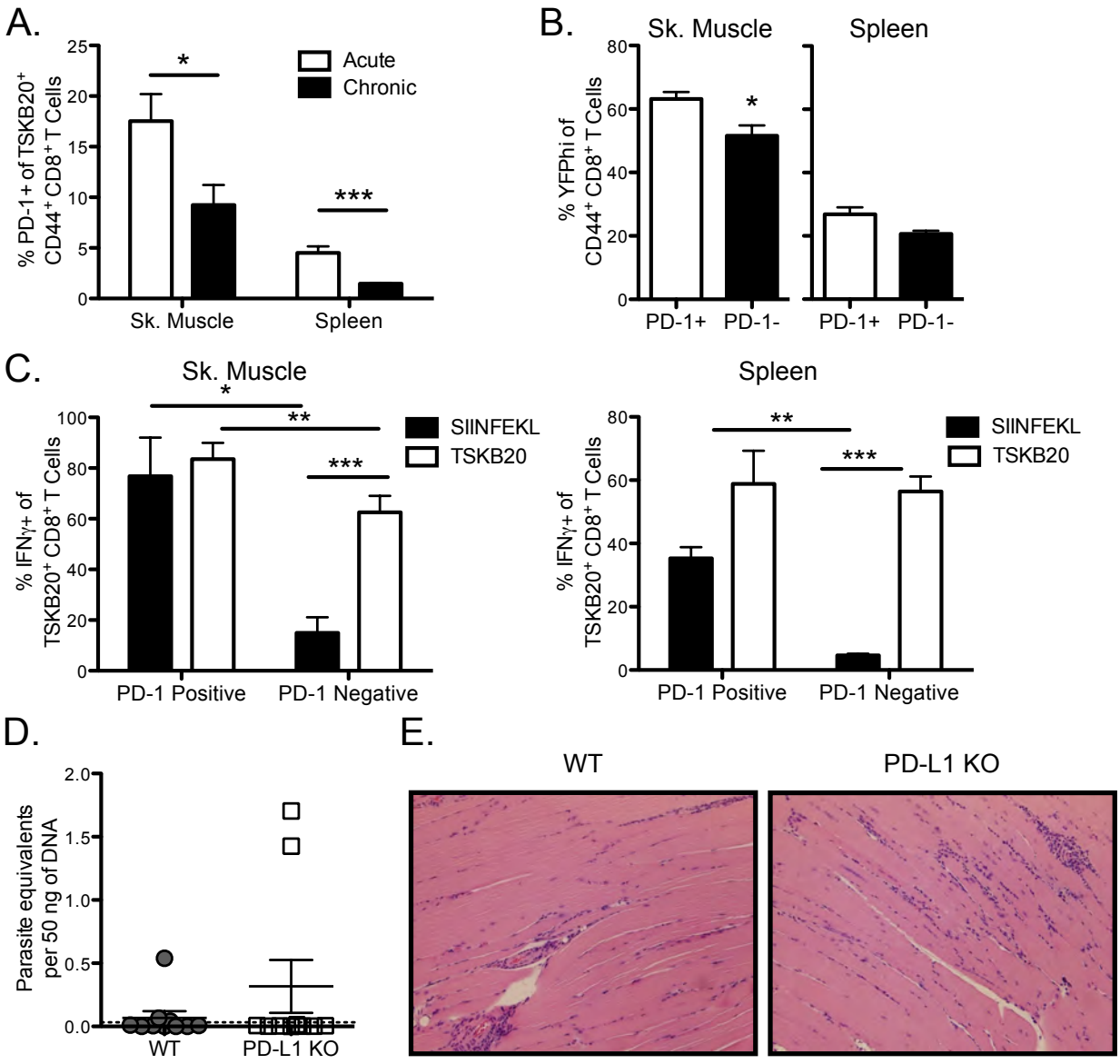
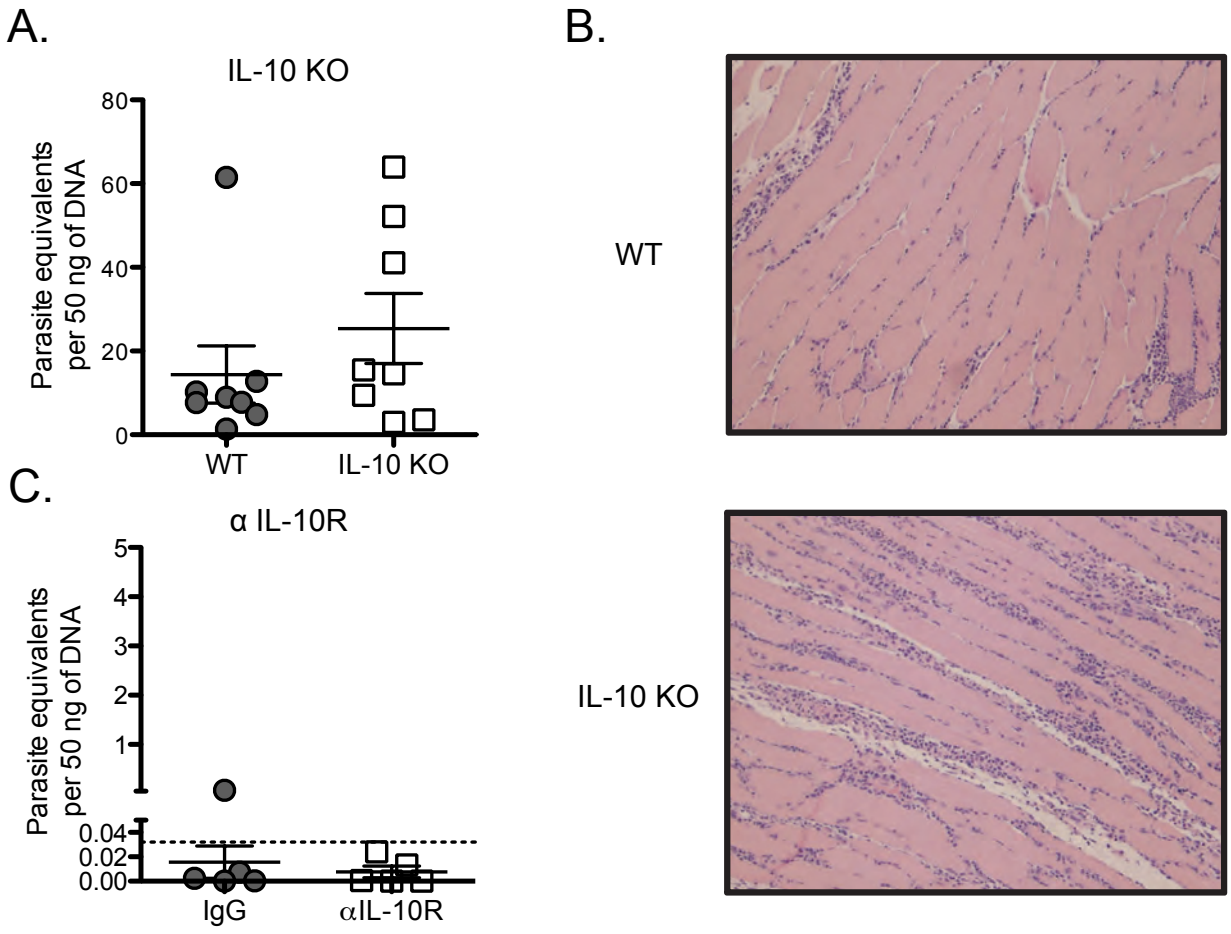

A.
% PD-1+ of TSKB20+ CD44+ CD8+ T Cells
Acute
Chronic
*
***
Sk. Muscle
Spleen
B.
Sk. Muscle
Spleen
% YFPhi of CD44+ CD8+ T Cells
*
PD-1+
PD-1-
PD-1+
PD-1-
C.
Sk. Muscle
Spleen
% IFNγ+ of TSKB20+ CD8+ T Cells
SIINFEKL
TSKB20
*
**
***
PD-1 Positive
PD-1 Negative
D.
Parasite equivalents per 50 ng of DNA
WT
PD-L1 KO
E.
WT
PD-L1 KO

Figure 2.6. IL-10 is not a major factor controlling $CD8^+$ T cells in *T. cruzi* infection

A) IL-10 KO and WT mice exhibit similar parasite burden. Parasite load in skeletal muscle of IL-10 KO and WT mice during acute (30 dpi) *T. cruzi* infection was assessed by real-time PCR. B) IL-10 KO mice cannot control the inflammatory response to *T. cruzi*. H&E sections of skeletal muscle from acutely-infected IL-KO and WT mice. C) Interrupting IL-10 signaling does not allow clearance of *T. cruzi*. Parasite loads in skeletal muscle of chronically *T. cruzi*-infected mice receiving anti-IL-10R Ab or rat IgG are plotted. Bars show mean, which were not statistically different by Mann-Whitney test. Similar results were obtained in a repeat of each experiment.

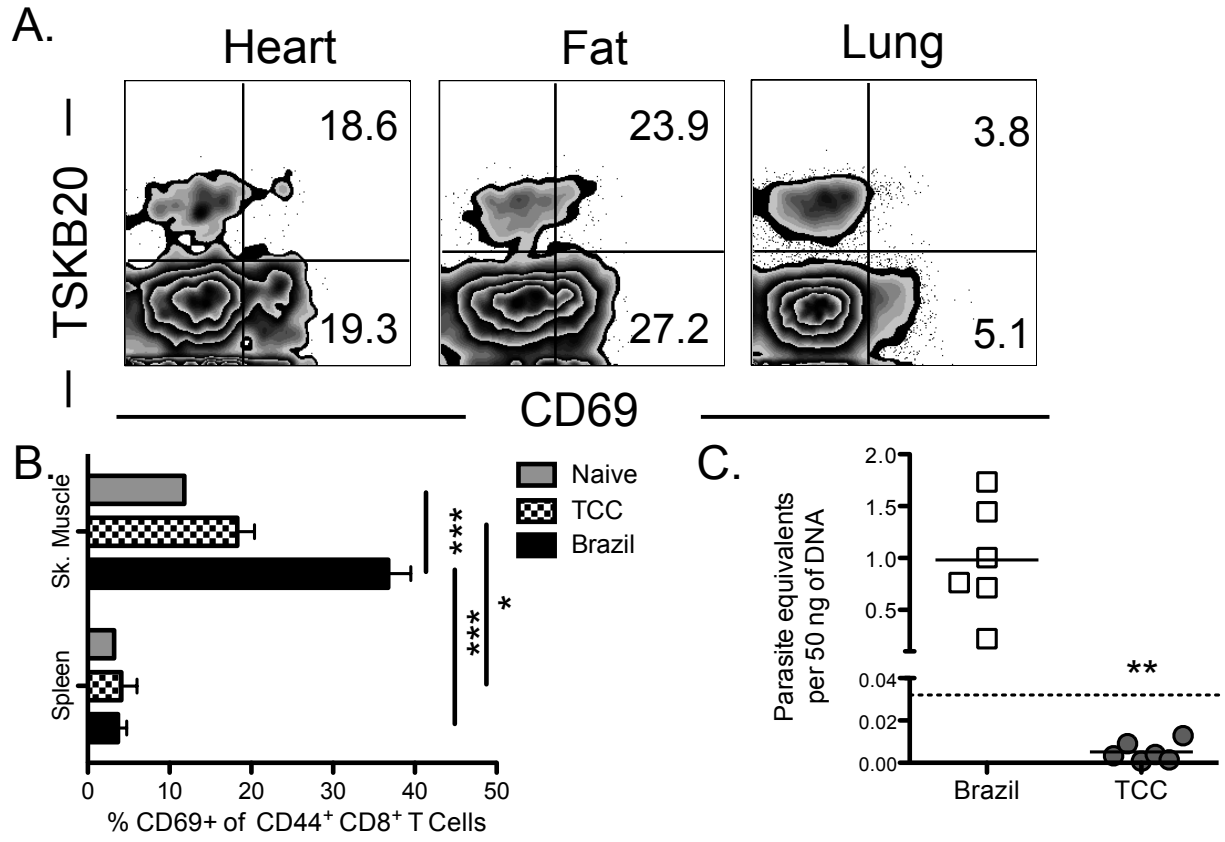
Figure 2.6


A.
IL-10 KO
Parasite equivalents per 50 ng of DNA
80
60
40
20
0
WT
IL-10 KO
B.
WT
IL-10 KO
C.
α IL-10R
Parasite equivalents per 50 ng of DNA
5
4
3
2
1
0.04
0.02
0.00
IgG
αIL-10R

Supplemental Figure 2.1. CD69 expression by $CD8^+$ T cells is related to parasite antigen level during chronic infection

A) Recently-activated $CD8^+$ T cells are preferentially found at sites of parasite persistence such as heart and fat. Representative flow plots show surface expression of CD69 for cells isolated from indicated tissue during chronic (~230 dpi) *T. cruzi* infection. Plots gated on $CD8^+$ T cells. Numbers in upper right indicate the percentage of CD69+TSKB20+ cells; numbers in lower right indicate the percentage of TSKB20- cells expressing CD69. B) The frequency of $CD8^+$ T cells that express CD69 in the muscle of mice chronically infected mice is greatest in those infected with muscle tropic (Brazil) verses the low level but persistent strain (TCC) infection. C) Quantification of chronic parasite burden in skeletal muscle via qPCR during chronic (>250 dpi) Brazil and TCC strain *T. cruzi* infection. Persistence of the TCC strain has been confirmed via qPCR and hemoculture following immunosuppression (data not shown). All data are representative of at 2-3 independent experiments with n=4-6 and depict mean+ SEM. * indicates percentage levels that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$) between specified groups.

Supplemental Figure. 2.1

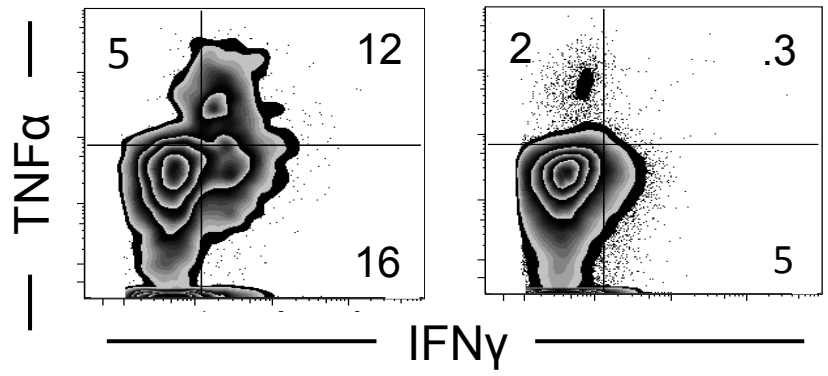


Supplemental Figure 2.2. More $CD8^+$ T cells in muscle produce IFNγ and TNFα *in vivo* than their splenic counterparts

A) Mice were injected with brefeldin A and approximately 6 hours later, tissues were harvested and stained to assess *in situ* cytokine production. Bar graphs represent IFNγ production by CD44+ $CD8^+$ T cells at 200 dpi in the absence of exogenous stimulation in the muscle (left) and spleen (right).

Supplemental Figure 2.2

A.

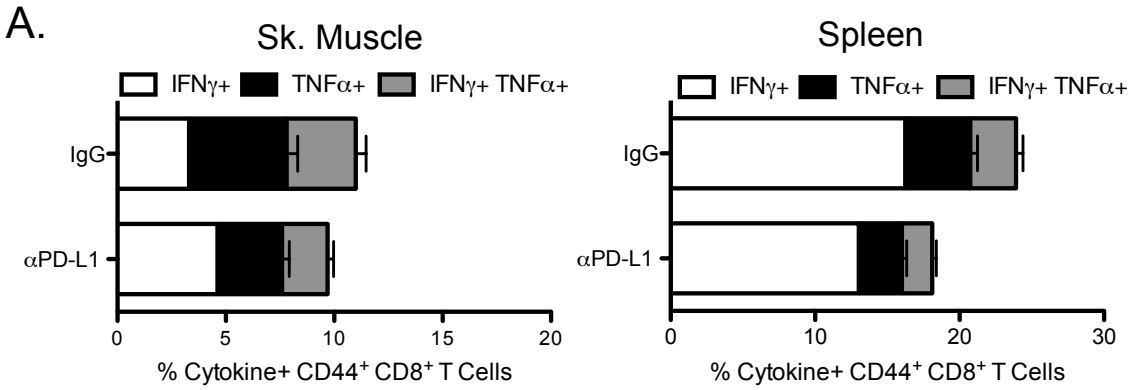


Supplemental Figure 2.3. PD-L1 blockade fails to improve cytokine production by $CD8^+$ T cells

$CD8^+$ T cells from chronically infected mice treated for 30 days with PD-L1 blocking antibody were stimulated for 5 hours with anti-mouse CD3ε.

A) The frequency of IFNγ+ (white), TNFα+ (black), and polyfunctional (grey) $CD8^+$ T cells in the muscle (left) and spleen (right) is not increased by PD-L1 blockade.

Supplemental Figure 2.3



2.6 References

1. Feldman, A. M., and McNamara, D. (2000) Myocarditis. *The New England journal of medicine* **343**, 1388-1398

2. Schofield, C. J., Jannin, J., and Salvatella, R. (2006) The future of Chagas disease control. *Trends in parasitology* **22**, 583-588

3. Bern, C., Montgomery, S. P., Herwaldt, B. L., Rassi, A., Jr., Marin-Neto, J. A., Dantas, R. O., Maguire, J. H., Acquatella, H., Morillo, C., Kirchhoff, L. V., Gilman, R. H., Reyes, P. A., Salvatella, R., and Moore, A. C. (2007) Evaluation and treatment of chagas disease in the United States: a systematic review. *Jama* **298**, 2171-2181

4. Harty, J. T., and Bevan, M. J. (1999) Responses of CD8(+) T cells to intracellular bacteria. *Current opinion in immunology* **11**, 89-93

5. Wong, P., and Pamer, E. G. (2003) CD8 T cell responses to infectious pathogens. *Annual review of immunology* **21**, 29-70

6. Wherry, E. J., Blattman, J. N., Murali-Krishna, K., van der Most, R., and Ahmed, R. (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *Journal of virology* **77**, 4911-4927

7. Fuller, M. J., Khanolkar, A., Tebo, A. E., and Zajac, A. J. (2004) Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. *J Immunol* **172**, 4204-4214

8. Mueller, S. N., and Ahmed, R. (2009) High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 8623-8628

9. Wherry, E. J., and Kurachi, M. (2015) Molecular and cellular insights into T cell exhaustion. *Nature Reviews Immunology* **15**, 486-499

10. Penaloza-MacMaster, P., Kamphorst, A. O., Wieland, A., Araki, K., Iyer, S. S., West, E. E., O'Mara, L., Yang, S., Konieczny, B. T., Sharpe, A. H., Freeman, G. J., Rudensky, A. Y., and Ahmed, R. (2014) Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. *The Journal of experimental medicine* **211**, 1905-1918

11. Brooks, D. G., Trifilo, M. J., Edelmann, K. H., Teyton, L., McGavern, D. B., and Oldstone, M. B. (2006) Interleukin-10 determines viral clearance or persistence in vivo. *Nature medicine* **12**, 1301-1309

12. Ejrnaes, M., Filippi, C. M., Martinic, M. M., Ling, E. M., Togher, L. M., Crotty, S., and von Herrath, M. G. (2006) Resolution of a chronic viral infection after interleukin-10 receptor blockade. *The Journal of experimental medicine* **203**, 2461-2472

13. Tinoco, R., Alcalde, V., Yang, Y., Sauer, K., and Zuniga, E. I. (2009) Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo. *Immunity* **31**, 145-157

14. Tarleton, R. L. (1990) Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with Trypanosoma cruzi. *J Immunol* **144**, 717-724

15. Tarleton, R. L., Koller, B. H., Latour, A., and Postan, M. (1992) Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection. *Nature* **356**, 338-340

16. Tarleton, R. L., Sun, J., Zhang, L., and Postan, M. (1994) Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. *Infection and immunity* **62**, 1820-1829

17. Joshi, T., Rodriguez, S., Perovic, V., Cockburn, I. A., and Stager, S. (2009) B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against Leishmania donovani infections. *PLoS pathogens* **5**, e1000431

18. Bhadra, R., Gigley, J. P., and Khan, I. A. (2012) PD-1-mediated attrition of polyfunctional memory CD8+ T cells in chronic toxoplasma infection. *The Journal of infectious diseases* **206**, 125-134

19. Horne-Debets, J. M., Faleiro, R., Karunarathne, D. S., Liu, X. Q., Lineburg, K. E., Poh, C. M., Grotenbreg, G. M., Hill, G. R., MacDonald, K. P., Good, M. F., Renia, L., Ahmed, R., Sharpe, A. H., and Wykes, M. N. (2013) PD-1 dependent exhaustion of CD8+ T cells drives chronic malaria. *Cell reports* **5**, 1204-1213

20. Liu, F., and Whitton, J. L. (2005) Cutting edge: re-evaluating the in vivo cytokine responses of CD8+ T cells during primary and secondary viral infections. *J Immunol* **174**, 5936-5940

21. Corbin, G. A., and Harty, J. T. (2005) T cells undergo rapid ON/OFF but not ON/OFF/ON cycling of cytokine production in response to antigen. *J Immunol* **174**, 718-726

22. Engelhardt, K. R., Richter, K., Baur, K., Staeheli, P., and Hausmann, J. (2005) The functional avidity of virus-specific CD8+ T cells is downmodulated in Borna disease virus-induced immunopathology of the central nervous system. *European journal of immunology* **35**, 487-497

23. Lewis, M. D., Fortes Francisco, A., Taylor, M. C., Burrell-Saward, H., McLatchie, A. P., Miles, M. A., and Kelly, J. M. (2014) Bioluminescence imaging of chronic Trypanosoma cruzi infections reveals tissue-specific parasite dynamics and heart disease in the absence of locally persistent infection. *Cellular microbiology* **16**, 1285-1300

24. Leavey, J. K., and Tarleton, R. L. (2003) Cutting edge: dysfunctional CD8+ T cells reside in nonlymphoid tissues during chronic Trypanosoma cruzi infection. *J Immunol* **170**, 2264-2268

25. Masopust, D., Vezys, V., Marzo, A. L., and Lefrancois, L. (2001) Preferential localization of effector memory cells in nonlymphoid tissue. *Science* **291**, 2413-2417

26. Bold, T. D., Banaei, N., Wolf, A. J., and Ernst, J. D. (2011) Suboptimal activation of antigen-specific CD4+ effector cells enables persistence of M. tuberculosis in vivo. *PLoS pathogens* **7**, e1002063

27. Cummings, K. L., and Tarleton, R. L. (2003) Rapid quantitation of Trypanosoma cruzi in host tissue by real-time PCR. *Molecular and biochemical parasitology* **129**, 53-59

28. Martin, D. L., Weatherly, D. B., Laucella, S. A., Cabinian, M. A., Crim, M. T., Sullivan, S., Heiges, M., Craven, S. H., Rosenberg, C. S., Collins, M. H., Sette, A., Postan, M., and Tarleton, R. L. (2006) CD8+ T-Cell responses to Trypanosoma cruzi are highly focused on strain-variant trans-sialidase epitopes. *PLoS pathogens* **2**, e77

29. Bustamante, J. M., Bixby, L. M., and Tarleton, R. L. (2008) Drug-induced cure drives conversion to a stable and protective CD8+ T central memory response in chronic Chagas disease. *Nat Med* **14**, 542-550

30. Reinhardt, R. L., Liang, H. E., and Locksley, R. M. (2009) Cytokine-secreting follicular T cells shape the antibody repertoire. *Nature immunology* **10**, 385-393

31. Siegelman, M. H., Stanescu, D., and Estess, P. (2000) The CD44-initiated pathway of T-cell extravasation uses VLA-4 but not LFA-1 for firm adhesion. *Journal of Clinical Investigation* **105**, 683-691

32. Day, C. L., Abrahams, D. A., Lerumo, L., Janse van Rensburg, E., Stone, L., O'Rie, T., Pienaar, B., de Kock, M., Kaplan, G., Mahomed, H., Dheda, K., and Hanekom, W. A. (2011) Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load. *J Immunol* **187**, 2222-2232

33. Jeong, Y. H., Jeon, B. Y., Gu, S. H., Cho, S. N., Shin, S. J., Chang, J., and Ha, S. J. (2014) Differentiation of antigen-specific T cells with limited functional capacity during Mycobacterium tuberculosis infection. *Infection and immunity* **82**, 132-139

34. Buggert, M., Tauriainen, J., Yamamoto, T., Frederiksen, J., Ivarsson, M. A., Michaelsson, J., Lund, O., Hejdeman, B., Jansson, M., Sonnerborg, A., Koup, R. A., Betts, M. R., and Karlsson, A. C. (2014) T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection. *PLoS pathogens* **10**, e1004251

35. Banga, R., Procopio, F. A., Noto, A., Pollakis, G., Cavassini, M., Ohmiti, K., Corpataux, J. M., de Leval, L., Pantaleo, G., and Perreau, M. (2016) PD-1 and follicular helper T cells are responsible for persistent HIV-1 transcription in treated aviremic individuals. *Nature medicine*

36. Tzeng, H. T., Tsai, H. F., Liao, H. J., Lin, Y. J., Chen, L., Chen, P. J., and Hsu, P. N. (2012) PD-1 blockage reverses immune dysfunction and hepatitis B viral persistence in a mouse animal model. *PloS one* **7**, e39179

37. Brooks, D. G., Lee, A. M., Elsaesser, H., McGavern, D. B., and Oldstone, M. B. (2008) IL-10 blockade facilitates DNA vaccine-induced T cell responses and enhances clearance of persistent virus infection. *The Journal of experimental medicine* **205**, 533-541

38. Beamer, G. L., Flaherty, D. K., Assogba, B. D., Stromberg, P., Gonzalez-Juarrero, M., de Waal Malefyt, R., Vesosky, B., and Turner, J. (2008) Interleukin-10 promotes Mycobacterium tuberculosis disease progression in CBA/J mice. *J Immunol* **181**, 5545-5550

39. Bertocchi, G. L., Vigliano, C. A., Lococo, B. G., Petti, M. A., and Viotti, R. J. (2013) Clinical characteristics and outcome of 107 adult patients with chronic Chagas disease and parasitological cure criteria. *Trans R Soc Trop Med Hyg* **107**, 372-376

40. Francolino, S. S., Antunes, A. F., Talice, R., Rosa, R., Selanikio, J., de Rezende, J. M., Romanha, A. J., and Dias, J. C. (2003) New evidence of spontaneous cure in human Chagas' disease. *Rev Soc Bras Med Trop* **36**, 103-107

41. Dias JC, D. E., Martins-Filho OA, Vitelli-Avelar D, Correia D, Lages E, Prata A. (2008) Further evidence of spontaneous cure in human Chagas disease. . *Rev Soc Bras Med Trop* **41**, 505-506

42. RL, T. (2013) The role of immunology in combatingTrypanosoma cruzi infection and Chagas disease. *Rev Esp Salud Publica* **86**, 33-39

43. Albareda, M. C., Olivera, G. C., Laucella, S. A., Alvarez, M. G., Fernandez, E. R., Lococo, B., Viotti, R., Tarleton, R. L., and Postan, M. (2009) Chronic human infection with Trypanosoma cruzi drives CD4+ T cells to immune senescence. *Journal of immunology* **183**, 4103-4108

44. Laucella, S. A., Postan, M., Martin, D., Hubby Fralish, B., Albareda, M. C., Alvarez, M. G., Lococo, B., Barbieri, G., Viotti, R. J., and Tarleton, R. L. (2004) Frequency of interferon- gamma -producing T cells specific for Trypanosoma cruzi inversely correlates with disease severity in chronic human Chagas disease. *The Journal of infectious diseases* **189**, 909-918

45. Arguello, R. J., Albareda, M. C., Alvarez, M. G., Bertocchi, G., Armenti, A. H., Vigliano, C., Meckert, P. C., Tarleton, R. L., and Laucella, S. A. (2012) Inhibitory receptors are

expressed by Trypanosoma cruzi-specific effector T cells and in hearts of subjects with chronic Chagas disease. *PloS one* **7**, e35966

46. Butler, N. S., Moebius, J., Pewe, L. L., Traore, B., Doumbo, O. K., Tygrett, L. T., Waldschmidt, T. J., Crompton, P. D., and Harty, J. T. (2012) Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. *Nature immunology* **13**, 188-195

47. Zajac, A. J., Blattman, J. N., Murali-Krishna, K., Sourdive, D. J. D., Suresh, M., Altman, J. D., and Ahmed, R. (1998) Viral immune evasion due to persistence of activated T cells without effector function. *Journal of Experimental Medicine* **188**, 2205-2213

48. Bhadra, R., Gigley, J. P., Weiss, L. M., and Khan, I. A. (2011) Control of Toxoplasma reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1-PDL-1 blockade. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 9196-9201

49. Gigley, J. P., Bhadra, R., Moretto, M. M., and Khan, I. A. (2012) T cell exhaustion in protozoan disease. *Trends in parasitology* **28**, 377-384

50. Kotner, J., and Tarleton, R. (2007) Endogenous CD4(+) CD25(+) regulatory T cells have a limited role in the control of Trypanosoma cruzi infection in mice. *Infection and immunity* **75**, 861-869

51. Martin, D. L., Postan, M., Lucas, P., Gress, R., and Tarleton, R. L. (2007) TGF-beta regulates pathology but not tissue CD8+ T cell dysfunction during experimental Trypanosoma cruzi infection. *European journal of immunology* **37**, 2764-2771

52. Kahan, S. M., Wherry, E. J., and Zajac, A. J. (2015) T cell exhaustion during persistent viral infections. *Virology* **479-480**, 180-193

53. Wherry, E. J., and Kurachi, M. (2015) Molecular and cellular insights into T cell exhaustion. *Nature reviews. Immunology* **15**, 486-499

54. Gupta, P. K., Godec, J., Wolski, D., Adland, E., Yates, K., Pauken, K. E., Cosgrove, C., Ledderose, C., Junger, W. G., Robson, S. C., Wherry, E. J., Alter, G., Goulder, P. J., Klenerman, P., Sharpe, A. H., Lauer, G. M., and Haining, W. N. (2015) CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. *PLoS pathogens* **11**, e1005177

55. Bucks, C. M., Norton, J. A., Boesteanu, A. C., Mueller, Y. M., and Katsikis, P. D. (2009) Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion. *J Immunol* **182**, 6697-6708

56. Richter, K., Brocker, T., and Oxenius, A. (2012) Antigen amount dictates CD8+ T-cell exhaustion during chronic viral infection irrespective of the type of antigen presenting cell. *European journal of immunology* **42**, 2290-2304

57. Streeck, H., Brumme, Z. L., Anastario, M., Cohen, K. W., Jolin, J. S., Meier, A., Brumme, C. J., Rosenberg, E. S., Alter, G., Allen, T. M., Walker, B. D., and Altfeld, M. (2008) Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells. *PLoS medicine* **5**, e100

58. Petrovas, C., Casazza, J. P., Brenchley, J. M., Price, D. A., Gostick, E., Adams, W. C., Precopio, M. L., Schacker, T., Roederer, M., Douek, D. C., and Koup, R. A. (2006) PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *The Journal of experimental medicine* **203**, 2281-2292

59. Wherry, E. J., Barber, D. L., Kaech, S. M., Blattman, J. N., and Ahmed, R. (2004) Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16004-16009

60. Bengsch, B., Seigel, B., Ruhl, M., Timm, J., Kuntz, M., Blum, H. E., Pircher, H., and Thimme, R. (2010) Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS pathogens* **6**, e1000947

61. Bixby, L. M., and Tarleton, R. L. (2008) Stable CD8(+) T cell memory during persistent Trypanosoma cruzi infection. *Journal of Immunology* **181**, 2644-2650

62. Albareda, M. C., De Rissio, A. M., Tomas, G., Serjan, A., Alvarez, M. G., Viotti, R., Fichera, L. E., Esteva, M. I., Potente, D., Armenti, A., Tarleton, R. L., and Laucella, S. A. (2013) Polyfunctional T cell responses in children in early stages of chronic Trypanosoma cruzi infection contrast with monofunctional responses of long-term infected adults. *PLoS neglected tropical diseases* **7**, e2575

63. Lasso, P., Mateus, J., Pavia, P., Rosas, F., Roa, N., Thomas, M. C., Lopez, M. C., Gonzalez, J. M., Puerta, C. J., and Cuellar, A. (2015) Inhibitory Receptor Expression on CD8+ T Cells Is Linked to Functional Responses against Trypanosoma cruzi Antigens in Chronic Chagasic Patients. *J Immunol* **195**, 3748-3758

64. Mateus, J., Lasso, P., Pavia, P., Rosas, F., Roa, N., Valencia-Hernandez, C. A., Gonzalez, J. M., Puerta, C. J., and Cuellar, A. (2015) Low frequency of circulating CD8+ T stem cell memory cells in chronic chagasic patients with severe forms of the disease. *PLoS neglected tropical diseases* **9**, e3432

65. Albareda, M. C., Laucella, S. A., Alvarez, M. G., Armenti, A. H., Bertochi, G., Tarleton, R. L., and Postan, M. (2006) Trypanosoma cruzi modulates the profile of memory CD8+ T cells in chronic Chagas' disease patients. *International immunology* **18**, 465-471

66. Gutierrez, F. R. S., Mariano, F. S., Oliveira, C. J. F., Pavanelli, W. R., Guedes, P. M. M., Silva, G. K., Campanelli, A. P., Milanezi, C. M., Azuma, M., Honjo, T., Teixeira, M. M.,

Aliberti, J. C. S., and Silva, J. S. (2011) Regulation of Trypanosoma cruzi-Induced Myocarditis by Programmed Death Cell Receptor. *Infection and immunity* **79**, 1873-1881

67. Frontera, W. R., and Ochala, J. (2015) Skeletal muscle: a brief review of structure and function. *Calcif Tissue Int* **96**, 183-195

68. Daar, A. S., Fuggle, S. V., Fabre, J. W., Ting, A., and Morris, P. J. (1984) The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation* **38**, 287-292

69. Melo, R. C., and Brener, Z. (1978) Tissue tropism of different Trypanosoma cruzi strains. *The Journal of parasitology* **64**, 475-482

70. Kurup, S. P., and Tarleton, R. L. (2014) The Trypanosoma cruzi flagellum is discarded via asymmetric cell division following invasion and provides early targets for protective CD8(+) T cells. *Cell host & microbe* **16**, 439-449

71. Queiroz, R. M., Charneau, S., Bastos, I. M., Santana, J. M., Sousa, M. V., Roepstorff, P., and Ricart, C. A. (2014) Cell surface proteome analysis of human-hosted Trypanosoma cruzi life stages. *Journal of proteome research* **13**, 3530-3541

72. Atwood, J. A., 3rd, Weatherly, D. B., Minning, T. A., Bundy, B., Cavola, C., Opperdoes, F. R., Orlando, R., and Tarleton, R. L. (2005) The Trypanosoma cruzi proteome. *Science* **309**, 473-476

73. Zhang, L., and Tarleton, R. L. (1996) Persistent production of inflammatory and anti-inflammatory cytokines and associated MHC and adhesion molecule expression at the site of infection and disease in experimental Trypanosoma cruzi infections. *Experimental parasitology* **84**, 203-213

CHAPTER 3

MHC CLASS I EXPRESSION IMPACTS TISSUE-SELECTIVE PATHOGEN PERSISTENCE AND T CELL EXHAUSTION IN MUSCLE[1]

[1]Pack, A.D. and Tarleton, R.L. 2016. Manuscript in preparation for submission to *PLOS Pathogens*.

3.1 Abstract

Muscle myocytes exhibit low basal levels of MHC I, and we hypothesized that this low MHC expression might impact the ability of pathogens to persist by avoiding surveillance by $CD8^+$ T cells. *Trypanosoma cruzi* has the capacity to infect most nucleated host cells but preferentially persists in a select set of non-lymphoid tissues, including muscle, suggesting a selective advantage in these particular tissues. To assess the contribution of skeletal muscle MHC expression on pathogen persistence in this tissue, we generated a mouse model wherein myocyte MHC I expression in skeletal muscle can be specifically upregulated by doxycycline administration and evaluated the impact of enhanced MHC expression on parasite control and immunity. MHC I overexpression during the early acute infection (days 1-20 of infection), resulted in enhanced T cell function in the skeletal muscle and a resulting reduction in parasite load. As expected, this reduction in parasites was dependent on $CD8^+$ T cells, as CD8 depletion superseded the impact of increased MHC expression. Continued overexpression of MHC I beyond 30 days, however, ultimately led to greatly increased tissue parasite load in association with reduced parasite-specific T cell function and evidence of immune exhaustion, that was in part reversible by blocking PD-1:PD-L1 interactions. These studies demonstrate a surprisingly strong and systemically dominant effect of skeletal muscle MHC expression on maintaining T cell function and pathogen control and argue that the normally low MHC I expression in skeletal muscle is host-protective by allowing for pathogen control while preventing immune exhaustion.

3.2 Introduction

The protozoan parasite, *Trypanosoma cruzi*, infects at least 10 million individuals globally and is a leading cause of parasite-associated disease in the Americas (1,2). After decades of infection, approximately 30% of individuals develop clinical manifestations of Chagas Disease, a syndrome involving megalies of the heart, colon, and esophagus, for which treatment options are limited (3). Although, *T. cruzi* demonstrates great promiscuity in cell range for invasion and can replicate within nearly all nucleated cells, parasites in chronically infected hosts are most frequently detected in muscle tissue (4-6). Understanding how *T. cruzi* avoids elimination from muscle and the subsequent impact on parasite persistence, generally for the lifetime of most infected hosts, is critical for the development of therapeutic strategies for this infection and is the focus of this study.

Muscle is a broad term used to describe filamentous tissue consisting primarily of actin and myosin, and is classically divided into three types that vary in capacity to function in a voluntary manner, fiber composition, and metabolic requirements (7). While a number of factors may impact the capacity of *T. cruzi* to survive in this tissue type, one characteristic that distinguishes muscle from epithelial or endothelial cells, cell types also commonly infected by *T. cruzi*, and may have an impact on the immunological response to parasites, is highly regulated expression of major histocompatibility complex class I (MHC I). Under basal conditions, muscle MHC I expression is nearly undetectable (8,9) and a number of debilitating muscle disorders are characterized by heightened MHC class I expression, resulting in deficiencies in muscle differentiation, regeneration, and general tissue function (10-13).

MHC class I expression on the surface of mammalian cells permits surveillance and elimination of targets with aberrant function (ex. tumors) and pathogen infection, and is essential

for efficient immune control of many intracellular infections (14-16). A number of pathogens have evolved strategies to interfere with MHC I expression including bacteria and viruses such *Chlamydia trachomatis* and *Mycobacterium tuberculosis*, human cytomegalovirus (HCMV) and human immunodeficiency virus (HIV) (17,18). These pathogens have been documented to down-regulate MHC class I, shift its expression towards alternative class types, and even compromise immune detection by mimicry of host peptides (19-24). Other infectious agents such as *Toxoplasma gondii* and herpes simplex virus 1, employ a more 'passive' evasion strategy, having evolved to survive in tissues such as neurons which also have with limited MHC class I expression (25,26). As a parasite that replicates within the cytoplasm of host cells, *T. cruzi* proteins are available for processing and presentation on host surface MHC I-peptide complexes (27-29). A robust $CD8^+$ T cell response is generated against *T. cruzi*-derived peptides and this response is essential for host survival of *T. cruzi* infection (30-32). Despite these responses, *T. cruzi* manages to evade elimination by CD8 cells.

We hypothesize that despite the capacity to infect many cell types, *T. cruzi* persistence is maintained through infection of tissues, namely skeletal muscle, with reduced capacity for immune surveillance. While *T. cruzi*-specific T cells are present in this tissue, we speculate that the limited MHC class I expression therein may lower efficient detection and clearance of pathogen-infected cells, thus allowing for parasite persistence. Here we describe a model for modulating class I MHC expression specifically in skeletal muscle and describe the initially productive but ultimately highly damaging impact of this modulation in the course of *T. cruzi* infection.

3.3 Materials and Methods

3.3.1 Mice and parasites

Mice capable of inducible major histocompatibility complex class I (MHC I) overexpression in skeletal muscle myocytes were generated by the crossing of two transgenic mouse models. Tg(ACTA1-rtTA, tet-O-cre)102Monk (also known as HSA-rtTA/TRE-Cre) were obtained from The Jackson Laboratory and possess a doxycycline inducible cre system that is specific for myocytes of skeletal muscle, with the reverse tet-controlled transactivator (rtTA) under the control of the skeletal muscle promoter actin alpha 1(ACTA1) (33). These mice were crossed with mice carrying the tetracycline-response element (TRE-$H2K^b$) transgene, a kind gift from Dr. Kanneboyina Nagaraju (Children's National Medicine Center, Washington, D.C.) (10). Mice positive for both transgene constructs ($H2K^b$-rtTA) were bred and maintained under specific pathogen-free conditions at the Coverdell Vivarium (University of Georgia, Athens, GA).

For *T. cruzi* infections, mice of 8-12 weeks old were infected via intraperitoneal (i.p.) injection with 10^3 trypomastigotes of the Brazil strain. Parasites were maintained in culture using serial passage through Vero cells. For *Listeria monocytogenes* infections, mice were infected by oral gavage with 10^4 bacteria expressing a plasmid for the production of the albumin derived protein ova (*Listeria*-ova). All mice were euthanized by CO_2. All animal use was performed in accordance with protocols approved by the University of Georgia Institutional Animal Care and Use Committee.

3.3.2 Lymphocyte isolation from skeletal muscle

Lymphocytes were isolated from muscle as described with a few modifications (34). Tissues were collected, minced, then stirred in Hank's balanced salt solution (Corning) with

1.25mM EDTA for 30 min at 37°C. This treatment was followed by incubation in a 150U/mL Collagenase (Gibco) RPMI solution for at least 1h. The digested tissue was then filtered through a 70μM nylon cell strainer (BD Biosciences) and pelleted via centrifugation. The pellet was resuspended in 44% Percoll (GE Healthcare) underlain with a 67% Percoll-PBS solution. Following centrifugation at 600xg, cells were collected from the gradient interface and washed in RPMI.

3.3.3 T cell phenotyping

$CD8^+$ T cell phenotypes were determined by staining with MHC I tetramer TSKB20 (ANYKFTLV/K^b) synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA) and the following: CD8 (Accurate Chemical), CD44 (eBioscience), CD69, and PD-1 (BD Pharmingen). Red blood cells in single-cell suspensions of spleen cells were lysed in a hypotonic ammonium chloride solution and washed in staining buffer (2% BSA and 0.02% azide in PBS (PAB)). Cells were stained at 4°C, washed in PAB, and fixed in 2% formaldehyde. A minimum of 200,000 lymphocytes were evaluated using a Hyper CyAn flow cytometer (DakoCytomation) and analyzed with FlowJo software (Tree Star).

3.3.4 Standard T cell stimulation and intracellular staining

Lymphocytes isolated from spleen or skeletal muscle were stimulated with 1 μM *T. cruzi* peptide TSKB20 (ANYKFTLV) (GenScript) for 5h at 37°C in the presence of 1 μg/ml Golgi Plug (BD Pharmingen). Cells were surface stained, washed, fixed and permeabilized, and intracellular cytokine staining was performed to detect IFNγ (eBioscience) and TNFα (BD Pharmingen) using a Cytofix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer's instructions.

3.3.5 Direct intracellular cytokine staining

To evaluate *in situ* cytokine production, a direct intracellular staining (dICS) protocol was applied (35). Briefly, mice were injected with 0.25 mg of brefeldin A (Sigma) in PBS. 6-8h later, tissues were collected and processed as described above with the addition of brefeldin (10 μg/mL) to isolation solutions.

3.3.6 Antibody Treatment

200 μg of anti-CD8a (Clone: YTS 169.4) was administered via i.p. injection every third day for 10 days and PD-L1 (Clone: MIH5 and 10F.9G2) was given for 20 days. The anti-CD8a antibody used for detection by flow cytometry is a distinct clone from the depleting antibody.

3.3.7 Quantitation of parasite burden

Parasite equivalents in tissue were determined as previously described (36). Briefly, tissue was collected from mice and finely minced. Samples were incubated at 55 °C in SDS-proteinase K lysis buffer. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 100% ethanol, and resuspended in nuclease free water. PCR reactions contained iQ SYBR Green Supermix (Biorad) and primers specific for *T. cruzi* or mouse genomic DNA. Samples were analyzed on an iCycler (Biorad) and *T. cruzi* equivalents were calculated as the ratio of *T. cruzi* satellite DNA divided by the quantity of mouse TNFα DNA in each sample.

3.3.8 Determination of MHC class I expression

Skeletal muscle from mice with wild-type or doxycycline induced MHC I overexpression (200 μg/mL doxycycline (Sigma)+ 5% sucrose in drinking water) was collected, minced and incubated for 30 min at 37^{o}C in Hank's balanced salt solution (Corning) with 1.25mM EDTA.

Samples were then incubated in a RPMI solution supplemented with 150U/mL collagenase (Gibco) for at least 1h. Tissue homogenate was then filtered through a 100 μM nylon cell strainer (BD Biosciences) and pelleted via centrifugation prior to surface staining with $H2K^b$ antibody (BD Biosciences).

For western blot analysis, muscle was dissociated using an electric tissue homogenizer (Omni TH International) in a solution of 50 mM Tris/10 mM EDTA and then mixed with a 0.125M Tris/4% SDS/20% glycerol/10% MCE treatment buffer. The homogenate was then centrifuged at 16,000xg for 20 mins. The supernatant was collected and the protein concentration was determined using a Thermo Scientific Pierce BCA Protein Assay Kit. Specific antibody staining for alpha-tubulin (Sigma-Aldrich) and $H2K^b$ (BD Biosciences) was measured using a GE Typhoon 7000 and band intensity calculated using Image J software.

3.3.9 Histology

Skeletal muscle was fixed in 10% buffered formalin and embedded in paraffin. Five-micron thick sections were obtained and stained with hematoxylin-eosin. The number of infected cells per 50 or 100 microscope fields and infection intensity (number of amastigotes/infected cell) were determined following blinded analysis of tissue sections. Images of tissue sections were taken with an OLYMPUS DP70 digital camera on an OLYMPUS BX60 microscope.

3.3.10 Statistical Analysis

We calculated statistical significance with a Student's two-tailed t-test or by One-way ANOVA with Tukey post-test analysis. * indicates values (mean+ SEM) that are significantly different between specified groups (* $P \leq 0.05$, ** $P \leq 0.01$,***$P \leq .001$).

3.4 Results

3.4.1 Inducible upregulation of MHC class I on skeletal muscle

In order to evaluate the hypothesis that muscle MHC expression impacts parasite success and persistence, we required a tool for tissue-specific manipulation of MHC class I expression. Towards that goal, we generated a transgenic mouse model where MHC I expression on skeletal muscle myocytes is transiently increased by the administration of the tetracycline-analog doxycycline. This "tet-on" model was generated through the cross of a strain carrying the reverse tetracycline-controlled transactivator under the control of the skeletal muscle promoter actin alpha 1 (HSA-rtTA) and mice carrying a tetracycline-response element for control of the MHC class I $H2K^b$ (TRE-$H2K^b$) promoter (Fig. 3.1A) (10,33). In animals with both transgenes ($H2K^b$-rtTA), the administration of doxycycline for 20 days results in a nearly two-fold increase in $H2K^b$ expression in whole tissue lysates (Fig. 3.1B) in the non-lymphocyte (CD45 negative) population (Fig. 3.1C) of the muscle relative to non-induced mice with wild-type levels of MHC I expression.

3.4.2 Increased antigen presentation results in improved infection control

To determine if increased MHC expression on skeletal muscle impacted control of acute *T. cruzi* infection, $H2K^b$-rtTA mice were induced with doxycycline beginning on the day of infection with *T. cruzi.* By day 20 of infection/doxycycline treatment, there was a significant reduction in the muscle parasite load as assessed by quantitative PCR (qPCR) and by evaluation of histology sections of the infection rate (number of infected cells/100 microscope fields) (Fig. 3.2A,B). In individual infected host cells, infection intensity (number of amastigotes per infected cell) was also altered upon MHC overexpression, with significant decreases in the proportion of cells containing ≥50 amastigotes (Fig. 3.2C,D).

To evaluate the contribution of $CD8^+$ cells in the enhanced parasite clearance during MHC overexpression, $CD8^+$ T cells were depleted from doxycycline-treated mice beginning at day 12 of infection/induction (Fig 3.3A). CD8 depletion reversed the impact of MHC overexpression on parasite control as assessed by qPCR (Fig. 3.3B) and histologically (Fig. 3C). Taken together, these data indicate that during early acute infection the elevated expression of MHC class I in skeletal muscle increased the immunosurvelliance of this tissue by $CD8^+$ T cells, resulting in enhanced control of *T. cruzi* in this tissue.

3.4.3 Increased frequency of highly activated effectors upon MHC class I upregulation

To further explore the impact of muscle MHC class I overexpression on *T. cruzi*–specific $CD8^+$ T cells, we examined parasite-specific T cell numbers, phenotype, and *in vivo* effector activity in muscle. An increase in the total number of T cells responsive to the immunodominant *T. cruzi* TSKB20 epitope (ANYFKLV) was observed in the tissues of *T. cruzi*–infected, doxycycline-treated $H2K^b$-rtTA mice (Fig. 3.4A), and a higher percentage of muscle *T. cruzi*–specific T cells showed signs of recent activation (CD69 expression; Fig 3.4B) and effector activity (cytokine production; Fig 3.4C), as compared to uninduced mice.

These effects on T cells in muscle tissue in doxycycline-treated mice were parasite antigen-dependent, as shown by the fact that a similar influx or enhanced activation was not observed in OVA-specific T cells induced by a prior infection with *Listeria*-ova (Fig. 3.4A,D). In addition, the contribution of $CD4^+$ T cells to the inflammatory response in *T. cruzi* infected muscle was not altered by MHC class I overexpression in the $H2K^b$-rtTA mice (Fig. 4E) Taken together, these data indicate that increased MHC expression facilitates improved CD8 surveillance of and response to *T. cruzi*- infected cells leading to enhanced parasite control.

3.4.4 Prolonged MHC overexpression results in compromised parasite control

The reduction in muscle parasite load in the $H2K^b$-rtTA mice induced for overexpression of class I MHC for the first ~20 days of infection was encouraging, so we extended the treatment further to determine if the infection might be completely resolved. However, instead of a further reduction of parasite numbers, $H2K^b$-rtTA mice induced for MHC overexpression for >30 days showed the opposite effect, with significantly elevated parasite load (Fig 3.5A,B) and a dramatic increase in inflammation in muscle tissue and severe deterioration of tissue integrity (Fig. 3.5C). Induction of MHC overexpression beyond 40 days resulted in further deterioration in the health of mice including significant weight loss ultimately requiring euthanasia.

The loss in parasite control was not restricted to the site of transgene induction (skeletal muscle), as higher parasite levels were also observed in the heart (Fig. 3.5D), another common tissue of *T. cruzi* persistence. This lost control was also associated with a decrease in the frequency of IFNγ producing CD8+ and TSKB20-specific T cells in muscle tissue (Fig. 3.5E) and reduced potential of T cells systemically to respond to parasite peptide *ex vivo* (Fig. 3.5F).

We next asked if the parasite-controlling impact of increased MHC expression might be productive if initiated later during the controlling phase of the acute infection or during a chronic, persisting infection. Doxycycline was thus administered to $H2K^b$-rtTA mice from days 15 to 35 post infection (Fig. 3.7A) or beginning at >120 days post-infection (Fig 3.7B). In both cases, MHC resulted in increased, rather than reduced parasite load.

3.4.5 Immune regulation via PD-1 contributes to compromised parasite control

The rapid change from enhanced control of *T. cruzi* infection after a 20 day period of MHC overexpression to a nearly complete collapse of parasite control associated with reduced T cell function after just 10 additional days of overexpression, suggested that increased antigen

presentation in skeletal muscle for >30 days might be exhausting *T. cruzi*–specific T cells. To examine this possibility we looked first at the expression of PD-1, a key regulator of T cell function, particularly in systems characterized by high antigen load (42-44). MHC overexpression for >30 days of *T. cruzi* infection resulted in a significant increase in PD-1-expressing CD8 T cells and *T. cruzi*-specific CD8 T cells in muscle tissue (Fig. 3.6A). To determine if this PD-1 expression was actually linked to T cell dysfunction and the associated loss of infection control, we blocked PD-1:PD-L1 interactions using anti-PD-L1 during days 15-35 of MHC overexpression (Fig. 3.6B). This treatment resulted in a restoration of *T. cruzi*-specific CD8 T cells numbers (Fig. 3.6C) and activation response (Fig. 3.6D) to the levels observed in non-induced, MHC-normal $H2K^b$-rtTA mice. More remarkably, PD-L1 blockade reestablished control of tissue parasite numbers and substantially reduced tissue inflammation in the muscle of mice with >30 day MHC overexpression (Fig. 3.6E,F).

3.5 Discussion

Through centuries of interactions with mammalian hosts, parasites have evolved to prevent elimination from their hosts, with the most successful pathogens, like *T. cruzi,* persisting for decades in long-lived hosts. Does *T. cruzi* exploit naturally occurring host regulation to support infection persistence? Under what pressures have these evolutionary adaptations developed? Can manipulation of host immune surveillance improve anti-parasite immunity and facilitate *T. cruzi* clearance?

We were particularly interested in evaluating the hypothesis that persistence of the kinetoplastid, *T. cruzi*, is associated with muscle class I MHC expression. We initiated our investigation into MHC I expression as a determinant for parasite persistence, based upon the following observations: 1) the knowledge that *T. cruzi* persistence is frequently associated with

tissues (muscle, nervous) of highly regulated MHC expression (4,6,37), 2) the anecdotal evidence of infected cells with limited local inflammation in MHC normal muscle and 3) the recent demonstration that $CD8^+$ T cells obtained from muscle are highly functional and contribute to the regulation of parasite numbers in muscle tissue throughout the course of *T. cruzi* infection (Chapter 2). Together, these factors suggest that persistence in restricted tissues could be associated with a relative inefficiency in detection of *T. cruzi* infected cells in muscle, perhaps due to the known low MHC expression of that tissue.

Here, we have determined that increasing muscle MHC expression initially results in improved *T. cruzi* control; which suggests that muscle class I levels during normal *T. cruzi* infection may be insufficient to completely eliminate parasites. Improved *T. cruzi* control is demonstrated by an overall reduction in parasite numbers and a significant decrease in infection intensity (parasites/infected cell) on the individual cell level in $H2K^b$-rtTA mice with <30d of MHC overexpression. Muscle $CD8^+$ T cells are likely responsible for this enhanced control, as CD8 depletion in induced $H2K^b$-rtTA mice reverses these improvements in *T. cruzi* control. The increased accumulation of activated (CD69+) *T. cruzi*-specific effectors in the skeletal muscle of induced $H2K^b$-rtTA mice, also serves as additional evidence to support this interpretation. The effector nature of these cells is demonstrated by their production of the key inflammatory cytokines, IFNγ and TNFα, in the skeletal muscle of infected mice. This was an exciting result, as it suggested that increasing the frequency of anti-*T. cruzi* effectors might serve as an effective strategy for improving *T. cruzi* control and potentially even infection clearance.

However, these effects could not be extended to resolve *T. cruzi* infection, and in fact the opposite occurred. Continuing muscle MHC I overexpression for >30 days compromised *T. cruzi* control in this tissue. Even more surprising, was the systemic loss of parasite control in distal

tissues (heart). Lost parasite control in tissues with "normal" MHC levels, unaffected by doxycycline administration, was an unexpected observation and suggested that there might be a systemic compromise in anti-*T. cruzi* immunity. When cytokine production by *T. cruzi*-specific $CD8^+$ T cells was evaluated, lower *in situ* cytokine production was observed in mice with prolonged MHC overexpression (>30d). This functional compromise extended to splenic $CD8^+$ T cells, as the capacity to respond to *ex vivo* stimulation with specific peptides, a common measure of T cell functionality, was significantly reduced in mice with continued (>30 days) MHC overexpression. In addition, this dysfunction appears to be associated with increased expression of the inhibitory receptor, PD-1, by $CD8^+$ T cells in overexpression mice. Additional support that PD-1 expression is involved in CD8 dysfunction in the overexpression system, is the success of PD-1 blockade at restoring specific cell frequency and activation potential, reversing tissue pathology, and ultimately, restoring *T. cruzi* control.

The impact that increasing skeletal muscle MHC I levels had on T cell function systemically was intriguing. Local effects on $CD8^+$ T cells in the muscle are easily explained, as studies of polymyositis, an inflammatory disease of the muscle, suggest that heightened muscle-MHC expression increases the frequency and potentiates the cytolytic function of infiltrating $CD8^+$ T cells specific for muscle proteins (38-40). The impact that altered MHC levels have on $CD8^+$ T cell function has also been evaluated in models of viral infection and cancer (20,41,42). In systems of downregulated or low MHC expression, treatments to restore MHC levels, lead to improved tumor and viral clearance through the actions of effector $CD8^+$ T cells (43-45). These results of increased surveillance promoting $CD8^+$ T cell action are comparable to our observations in induced $H2K^b$-rtTA mice with <30 days of MHC I overexpression. Conversely, attempts to extend augmented MHC class I expression in model systems, and presumably $CD8^+$

T cell stimulation, results in compromised infection control due to dysfunctional T cells (46,47); as seen in animals with >30 days of muscle class I overexpression. The idea that sustained high antigen levels can compromise the quality of $CD8^+$ T cell responses has been repeatedly confirmed through infection with different viral strains or interrogation of epitopes that vary in abundance; with high antigen levels consistently driving T cell dysfunction (46,48,49). The exact mechanisms by which *T. cruzi*-specific cells *systemically* are compromised by MHC overexpression in the muscle remains unclear; although studies of inflammatory muscle disorders influence our current understanding. In these systems, increased muscle MHC expression impacts the systemic distribution of autoreactive $CD8^+$ T cells in subjects with severe inflammatory muscle disorders (50). In these patients, $CD8^+$ T cells specific for muscle proteins are detectable in the periphery and these peripheral cells are believed to contribute to the persistence of inflammatory muscle disease throughout the body (50). Additional experimentation would be required to determine if a similar phenomenon is occurring in $H2K^b$-rtTA mice with broadly compromised $CD8^+$ T cells.

In this work, we have identified a unique system capable of inducible $CD8^+$ T cell exhaustion that does not require pathogen manipulation and is not reliant on the monitoring of T cells with differing specificities to assess the impact of MHC expression on T cell function. In addition, the induction of T cell regulation is rapid (~30 days), reversible through the administration of a classical immune checkpoint molecule (PD-1), and reaches beyond the tissue with increased class I expression. This innovative system also allowed us to evaluate the development of regulated $CD8^+$ T cell responses using mouse *T. cruzi* infection. While often implicated in *T. cruzi* persistence (51,52), there is minor evidence that commonly described modes of immune regulation control anti-*T. cruzi* $CD8^+$ T cell responses, even during late

chronic (>250 days) *T. cruzi* infection in mice. In our research group, we have eliminated, through blockade or the infection of deficient mice, the contributions of the regulatory cytokines IL-10 and TGF-β (53), along with the actions of Tregs (54), as major regulators of CD8 function during *T. cruzi* infection. Increasing the level of muscle MHC expression, and likely T cell stimulation, makes $CD8^+$ T cells in *T. cruzi* infection resemble those observed in classical models of persistent infection like LCMV and *Plasmodium*. In those systems, T cell regulation via inhibitory receptor engagement compromises function and pathogen elimination (48,55,56).

The ability of short-term MHC overexpression to induce inhibitory receptor-based regulation of $CD8^+$ T cells is even more striking, as PD-1 blockade or infection of PD-1 KO mice (>150 dpi) has no impact on *T. cruzi* levels in mice with normal MHC expression (Chapter 2). We believe that the success of the PD-1 blockade is likely through T cell reinvigoration (57), as we observed an increase in parasite-specific cell frequency and activation potential (CD69 expression). It's also likely that there were changes in T cell function, but additional experiments would be required to confirm this. The success of the anti-PD-1 treatment at countering muscle inflammation and local T cell regulation, is likely reliant on the expression of B7 ligands, upregulated in presence of high IFNγ levels, on muscle cells (50,58-62). These ligands are capable of crosslinking with CTLA-4 and associated receptor family members and support the successful use of immune suppressing therapies in some forms of myositis (62). We are currently working to determine whether $CD8^+$ T cells in induced $H2K^b$-rtTA mice express other inhibitory receptors like LAG3, TIM3, or CTLA-4, which are frequently coexpressed with PD-1 in terminally differentiated T cell populations (63-67).

Although it appears that normal muscle MHC expression fails to induce significant T cell regulation during chronic mouse *T. cruzi* infection, inhibitory receptor (PD-1) expression is

observed on $CD8^+$ T cells in a subset of patients chronically infected (decades) with *T. cruzi* (68). These $CD8^+$ T cells resemble those observed in $H2K^b$-rtTA mice with >30 days of MHC overexpression and fail to robustly respond to *ex vivo* stimulation (68-71). In separate studies, $CD8^+$ T cell dysfunction during human *T. cruzi* infection has been associated with enhanced cardiac disease progression (68-71) and increased HLA expression on myocardial cells and the adjacent endothelial cells was observed in subjects with chronic chagasic cardiomyopathy (72). It would be interesting to know if these two observations are directly related and whether *T. cruzi*-infected subjects with compromised *ex vivo* $CD8^+$ T cell function also had higher than average HLA expression in cardiac tissue. Although unconfirmed at this time, it is not unreasonable to imagine the presence of heightened cardiac HLA levels in patients who develop severe Chagas disease complications and highly regulated T cell responses (80); with most *T. cruzi* infected subjects expressing "average" HLA levels, which support parasite control, retained $CD8^+$ T cell function, and limited disease.

Through the course of this study, we have identified a new system that is capable of rapidly inducing reversible $CD8^+$ T cell exhaustion. This tool may be used in the future to explore the impact of MHC class I expression, and perhaps even antigen stimulation on $CD8^+$ T cell function in non-lymphoid tissues. Normal muscle MHC class I expression appears to be host-protective, by restricting the magnitude of muscle surveillance and the development of debilitating inflammation. In the case of *T. cruzi* infection, increasing muscle $CD8^+$ T cell surveillance and effector activity initially supports improved parasite control; but ultimately results in systemic exhaustion and compromise of a relatively successful immunologic response that limits parasites to extremely low levels.

Figure 3.1. Muscle-specific $H2K^b$ expression is regulatable by doxycycline administration

A) Schematic describes breeding scheme for the generation of a transgenic line capable of doxycycline induced MHC Class I overexpression. Mice expressing a reverse tetracycline controlled transactivator under the skeletal muscle promoter action alpha 1 (HSA-rtTA) were crossed with mice carrying the tetracycline-response element (TRE-H-$2K^b$) transgene, yielding progeny ($H2K^b$-rtTA) that overexpress MHC class I in skeletal muscle myocytes upon doxycycline administration. B) A nearly 2 fold increase in MHC I expression following transgene induction is confirmed by western blot analysis (left) and quantified by comparing the ratio of H2Kb/α-tubulin in skeletal muscle. C) Transgene induction by ad libitum doxycycline administration in drinking water for 19 days results in a nearly two fold in $H2K^b$ expression on the surface of CD45 negative muscle cells when expression was measured via flow cytometry. Data are representative of two independent experiments and are mean + SEM. * indicates groups that are significantly different (**$P \leq 0.01$) between specified groups.

Figure 3.1

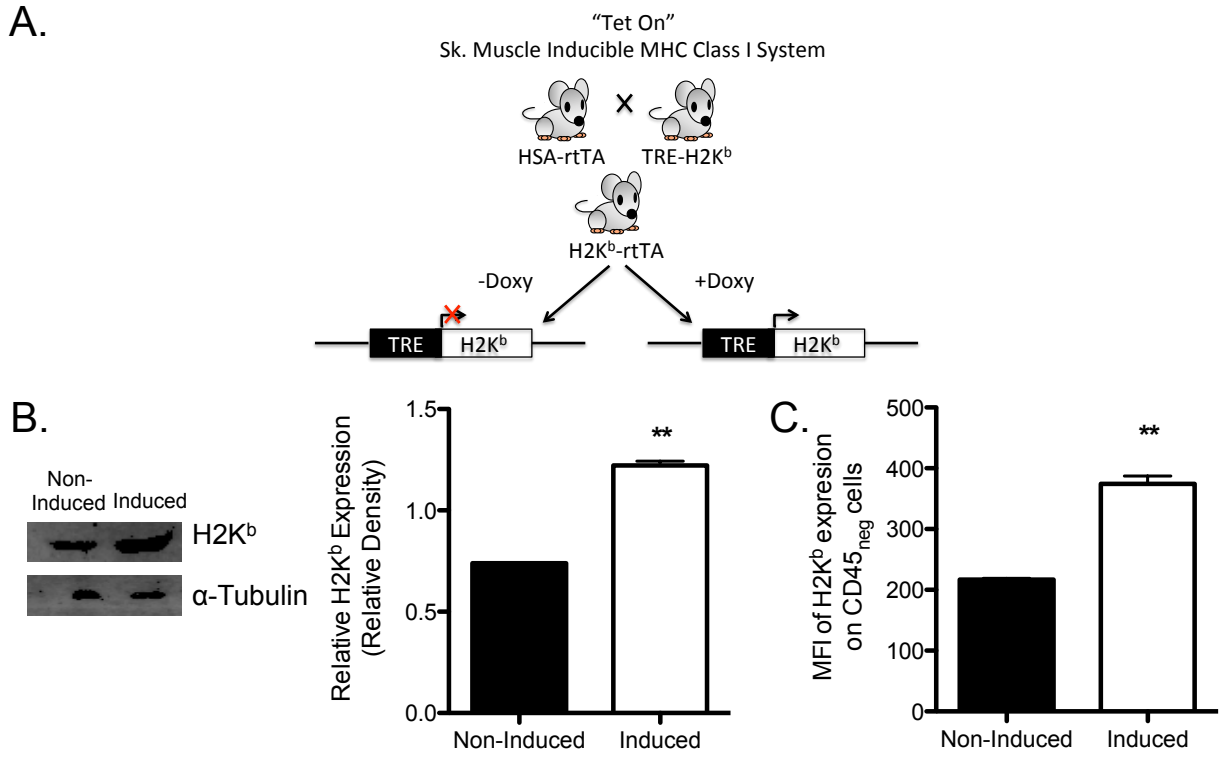

A.
"Tet On"
Sk. Muscle Inducible MHC Class I System
HSA-rtTA
TRE-H2K^b
H2K^b-rtTA
-Doxy
+Doxy
TRE
H2K^b
TRE
H2K^b
B.
Non-Induced
Induced
H2K^b
α-Tubulin
Relative H2K^b Expression (Relative Density)
1.5
1.0
0.5
0.0
**
Non-Induced
Induced
C.
MFI of H2K^b expresion on CD45$_{neg}$ cells
500
400
300
200
100
0
**
Non-Induced
Induced

Figure 3.2. MHC I overexpression during early acute infection results in improved control and fewer highly infected cells

A) Increased antigen presentation at 19 days post infection results in a significant decrease in parasite load in skeletal muscle when assessed by qPCR and when the frequency of infected cells (number of infected cells per 100 fields) is determined by histology analysis (B). C) The percentage of infected cells with 50-100 and 100+ amastigotes per cell was decreased with MHC I overexpression. These numbers are quantified graphically in column format (top) and in pie charts (bottom) following histological analysis at approximately 20 days post infection. D) Visual inspection of H&E histology sections confirms that MHC I overexpression resulted in a decrease in the total number of heavily infected cells. Data are representative of at least 4 experiments with n=3-5. Data are mean + SEM. * indicates percentage levels that are significantly different (* $P \leq 0.05$) between specified groups.

Figure 3.2

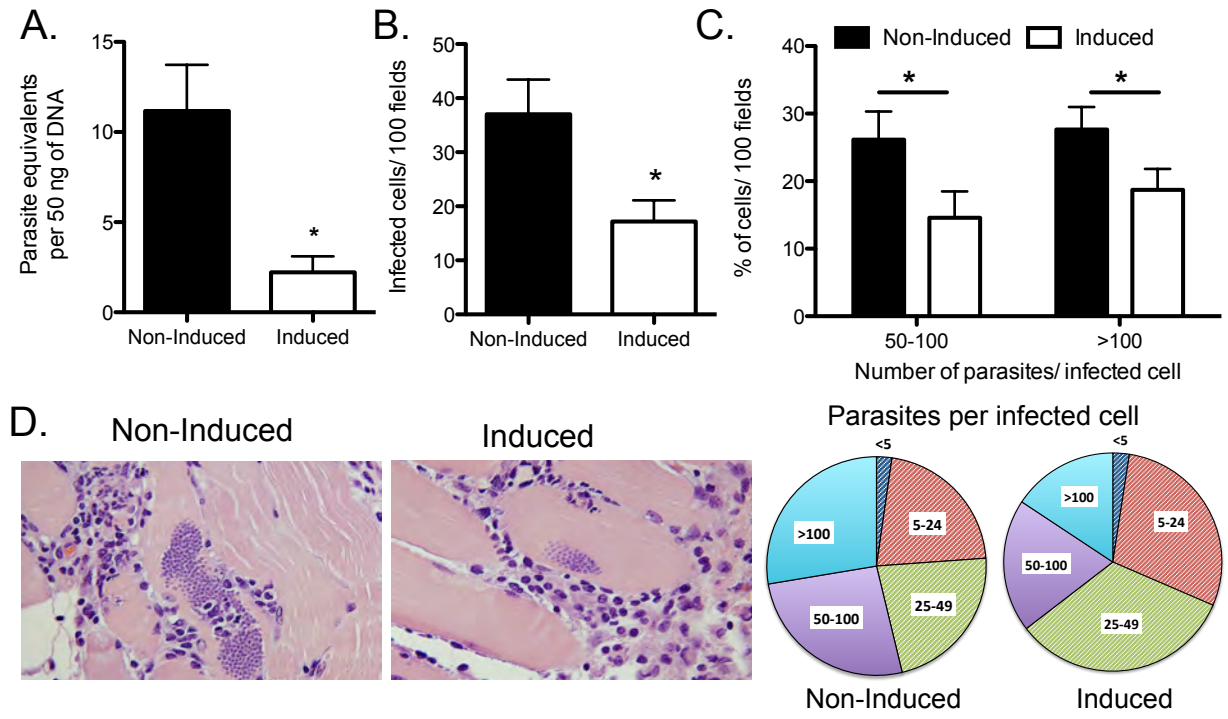

A.
Parasite equivalents per 50 ng of DNA
15
10
5
0
*
Non-Induced
Induced
B.
Infected cells/ 100 fields
50
40
30
20
10
0
*
Non-Induced
Induced
C.
Non-Induced
Induced
% of cells/ 100 fields
40
30
20
10
0
*
*
50-100
>100
Number of parasites/ infected cell
D.
Non-Induced
Induced
Parasites per infected cell
<5
5-24
25-49
50-100
>100
Non-Induced
Induced

Figure 3.3. $CD8^+$ T cells are involved in improved parasite control

A) Schematic describes the treatment schedule use for administration of doxycycline and CD8 depleting antibodies. Briefly, transgene induction was initiated upon infection and continued throughout the course of the experiment, while anti-CD8 monoclonal antibodies were provided every 3d between days 12 to 21 post infection. B) Depletion of $CD8^+$ T cells during MHC I overexpression reverses improvements in the parasite control observed during MHC I overexpression and results in significant increases in parasite number via qPCR and the number of infected cells per 50 histology fields C) in skeletal muscle. Data are representative of one experiment with n=4-6 and are mean + SEM. * indicates parasite levels that are significantly different (* $P \leq 0.05$) between specified groups.

Figure 3.3

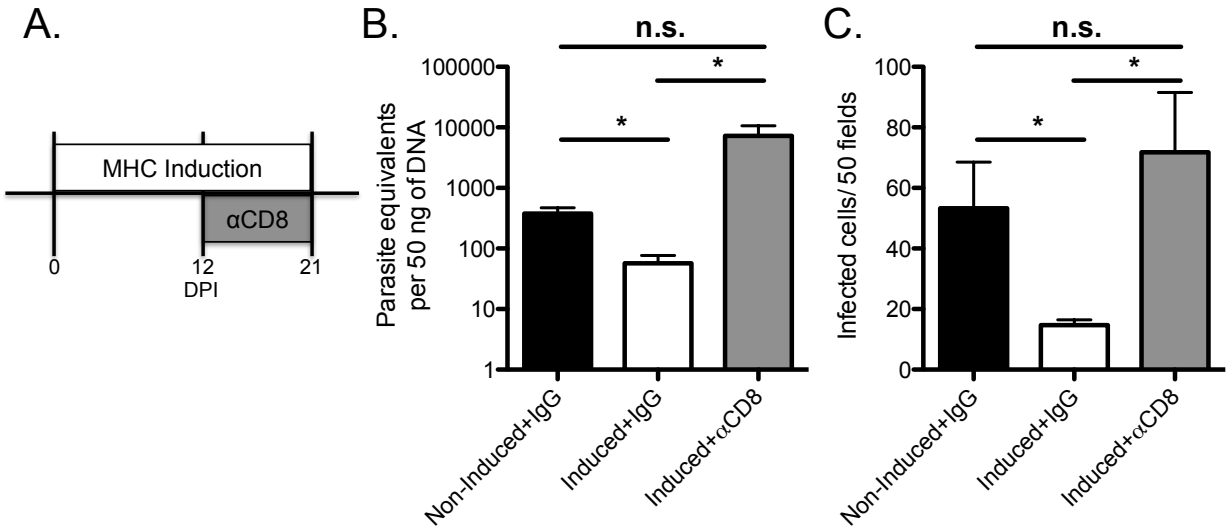

A.
MHC Induction
αCD8
0
12
21
DPI
B.
n.s.
*
*
Parasite equivalents per 50 ng of DNA
100000
10000
1000
100
10
1
Non-Induced+IgG
Induced+IgG
Induced+αCD8
C.
n.s.
*
*
Infected cells/ 50 fields
100
80
60
40
20
0
Non-Induced+IgG
Induced+IgG
Induced+αCD8

Figure 3.4. Increased antigen availability impacts $CD8^+$ T cell activation phenotype and *in vivo* cytokine production in muscle

A) Mice were infected with 10^4 *Listeria monocytogenes* expressing the protein ova to generate a SIINFEKL specific $CD8^+$ T cell population 17 days prior to infection with *T. cruzi* and the start of doxycycline administration for MHC overexpression.

The increased number of tetramer positive T cells during MHC I overexpression in the muscle is antigen dependent. The number of parasite specific ($TSKB20^+$) increased significantly in mice with transgene induction, while the frequency of ova specific ($SIINFEKL^+$) remained unchanged at 20 days post *T. cruzi* infection. B) The frequency of recently activated (CD69+) cells increased significantly in skeletal muscle (right), while splenic (left) CD69 expression remained unchanged following 20 days of transgene induction. C) More muscle $CD8^+$ T cells produced key inflammatory cytokine *in vivo* in mice with MHC I overexpression. Mice were injected with the protein export inhibitor, brefeldin A, 6-8 hours prior to tissue collection. Activated ($CD44^{hi}$) T cells were assayed directly *ex vivo* for IFNγ and TNFα production. The percentage of cytokine positive cells in the total $CD8^+$ (left) and parasite specific population (right) is presented. D) Mice were infected with *Listeria*-ova 17 days prior to infection with *T. cruzi* and the start of doxycycline administration. The number of $TSKB20^+$ T cells producing IFNγ *in situ* increased significantly upon MHC I overexpression, while the numbers of IFNγ+ SIINFEKL+ cells remained unchanged. E) Despite significant increases in the number of $CD8^+$ T cells during MHC I overexpression, the number of $CD4^+$ T cells in the muscle remains unchanged at 20 days post infection. Data are mean + SEM. * indicates averages that are significantly different (* $P \leq 0.05$) between specified groups.

Figure 3.4

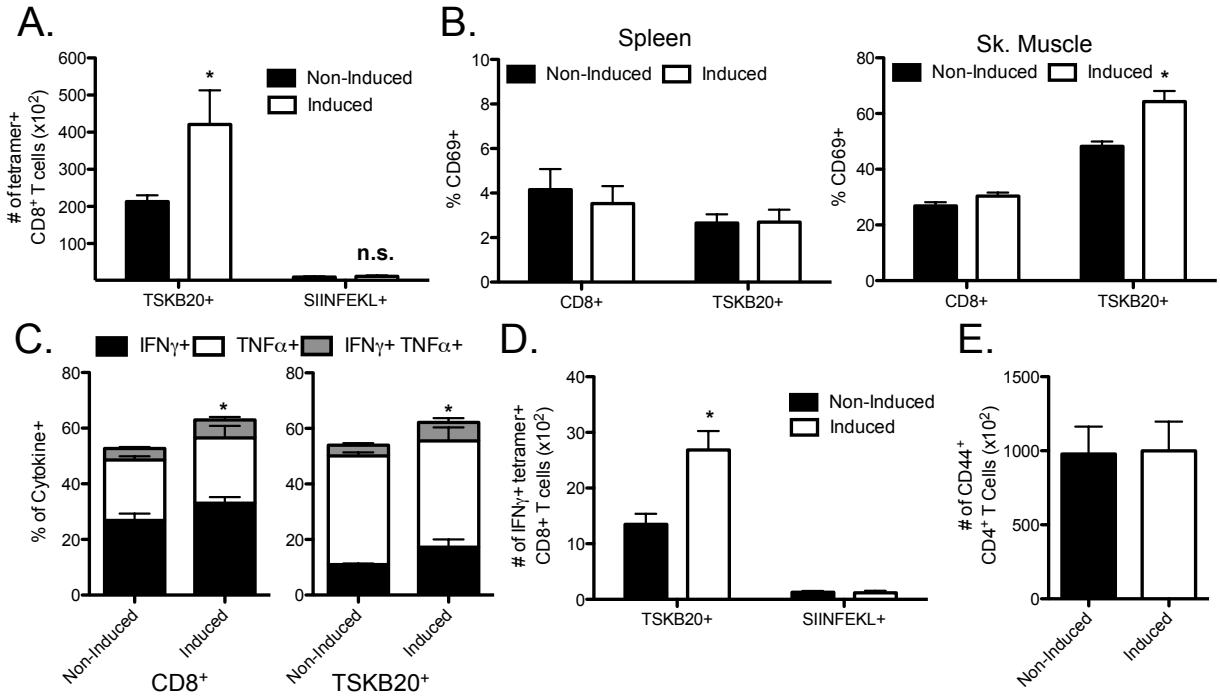

A.
# of tetramer+ CD8+ T cells (x10²)
Non-Induced
Induced
*
n.s.
TSKB20+
SIINFEKL+
B.
Spleen
% CD69+
Non-Induced
Induced
CD8+
TSKB20+
Sk. Muscle
% CD69+
Non-Induced
Induced
*
CD8+
TSKB20+
C.
IFNγ+
TNFα+
IFNγ+ TNFα+
% of Cytokine+
*
*
Non-Induced
Induced
Non-Induced
Induced
CD8+
TSKB20+
D.
# of IFNγ+ tetramer+ CD8+ T cells (x10²)
Non-Induced
Induced
*
TSKB20+
SIINFEKL+
E.
# of CD44+ CD4+ T Cells (x10²)
Non-Induced
Induced

Figure 3.5. Prolonged overexpression results in compromised $CD8^+$ T cell function and parasite control

At 30 days post infection and from the start of transgene induction, mice were sacrificed and the impact of increased antigen availability was assessed. A) Parasite burden in the skeletal muscle was significantly increased in mice with ≥30 days of MHC I overexpression when assessed by qPCR and analysis of histology slides B). C) Histology images reveal that prolonged (≥30 days) MHC overexpression results in severe inflammation and increased parasite levels in skeletal muscle. Black arrows point to *T. cruzi* infected cells. D) MHC I overexpression in skeletal muscle compromises *T. cruzi* control in the hearts of infected mice with prolonged (≥30 days) MHC overexpression. E) Parasite-specific $CD8^+$ T cell IFNγ production *in situ* in muscle is compromised following prolonged overexpression. Mice were injected with the protein export inhibitor brefeldin 6-8 hours before tissue collection. The frequency of IFNγ+ cells in the total and TSKB20+ $CD8^+$ T cell population is depicted. F) Response to *ex vivo* parasite specific peptide stimulation (TSKB20) is compromised in the $CD8^+$ T cells from the spleen (left) and skeletal muscle (right) of mice with prolonged antigen exposure. Data are representative of 2-3 experiments with n=3-5 and bars are mean + SEM. * indicates averages that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) between specified groups.

Figure 3.5

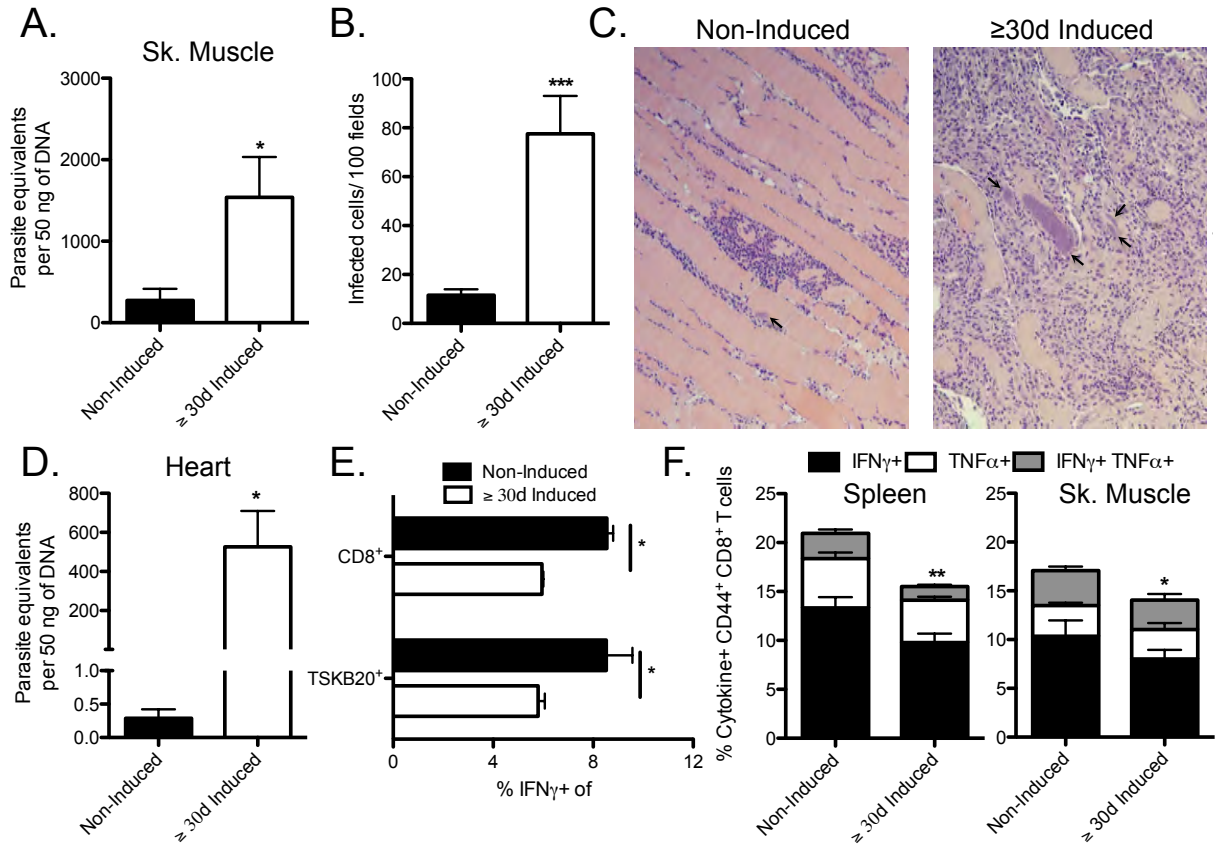

A.
Sk. Muscle
Parasite equivalents per 50 ng of DNA
3000
2000
1000
0
*
Non-Induced
≥ 30d Induced
B.
Infected cells/ 100 fields
100
80
60
40
20
0
***
Non-Induced
≥ 30d Induced
C.
Non-Induced
≥30d Induced
D.
Heart
Parasite equivalents per 50 ng of DNA
800
600
400
200
1.0
0.5
0.0
*
Non-Induced
≥ 30d Induced
E.
Non-Induced
≥ 30d Induced
CD8+
TSKB20+
*
*
0
4
8
12
% IFNγ+ of
F.
IFNγ+
TNFα+
IFNγ+ TNFα+
Spleen
Sk. Muscle
% Cytokine+ CD44+ CD8+ T cells
25
20
15
10
5
0
**
*
Non-Induced
≥ 30d Induced
Non-Induced
≥ 30d Induced

Figure 3.6. Regulation via PD-1 contributes to lost parasite control during prolonged MHC I overexpression

A) An increased frequency of $CD8^+$ and $TSKB20^+$ T cells express PD-1 following MHC I overexpression in the muscle between 25-27 days post infection. B) Schematic summarizes the experimental approach for data presented in panels C-F. Beginning on the 15^{th} day of *T. cruzi* infection and MHC I overexpression (in appropriate groups), PD-L1 antibody was administered every 72 hours for the next 20 days. C) PD-L1 blockade during overexpression increased the frequency of parasite specific $CD8^+$ T cells in spleen and skeletal muscle at 35 days post infection. D) Administration of PD-L1 antibody during overexpression restores the activation potential (indicated by CD69 expression) of CD8 cells in the skeletal muscle at 35 days post infection. E) The presence of muscle inflammation and infection intensity is significant reduced following PD-L1 treatment in H2Kb-rtTA mice with MHC I overexpression at 35 days post infection. F) Control of muscle *T. cruzi* infection, as measured by qPCR, is restored upon PD-L1 blockade in mice with increased MHC I expression on day 35 post infection. Data are representative of two independent experiments with $n \geq 4$. Data are mean + SEM. * indicates averages that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) between specified groups.

Figure 3.6

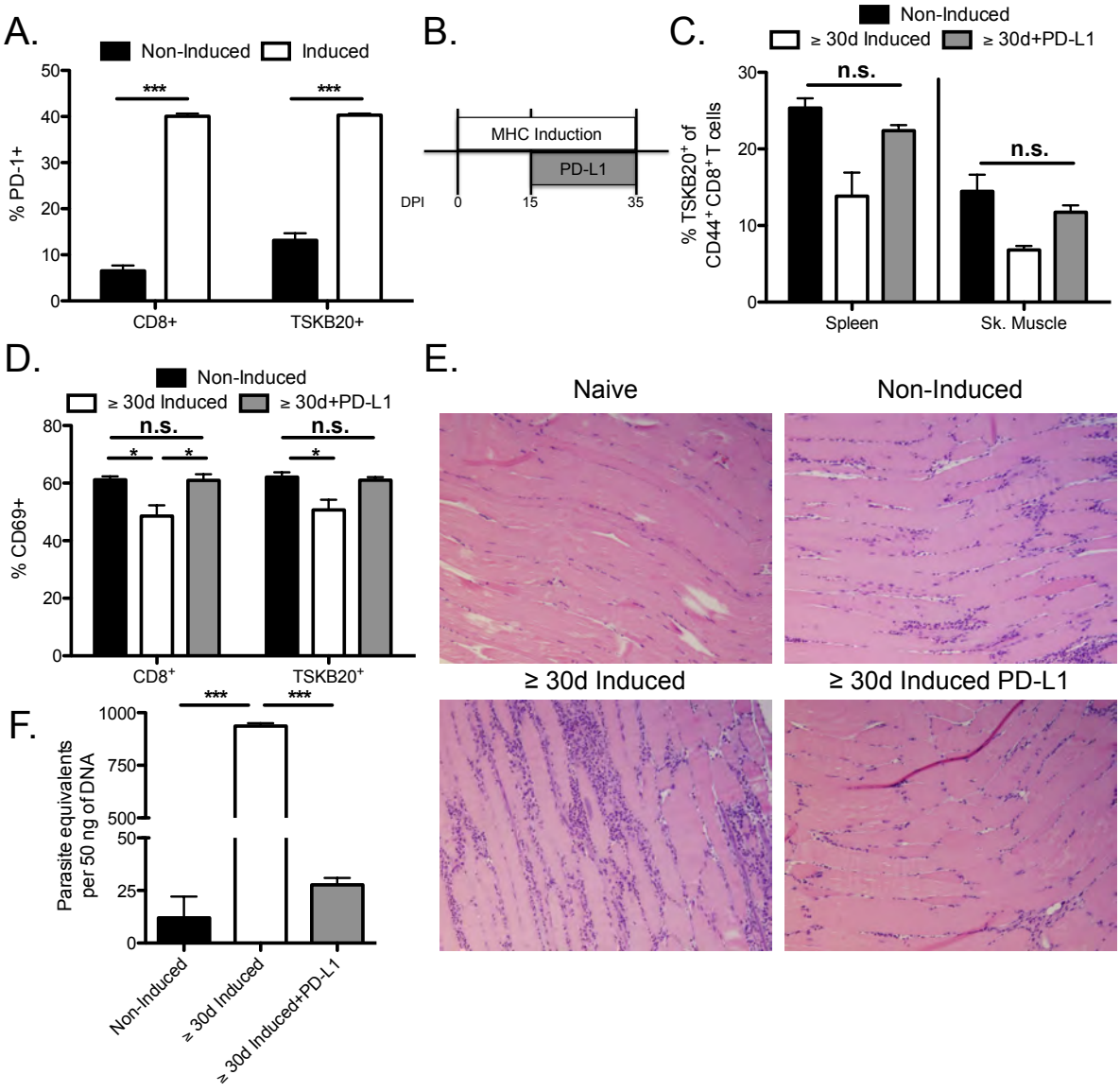
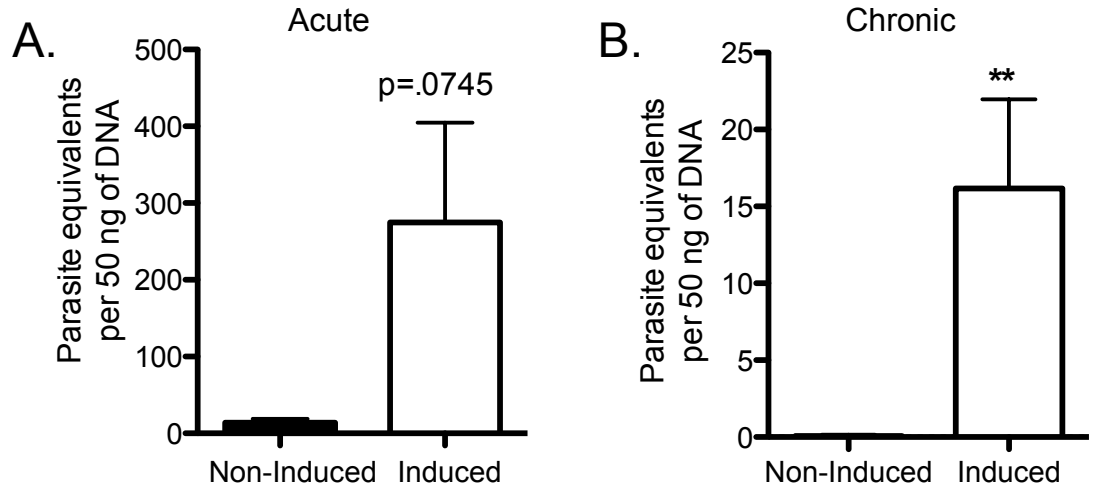

A.
Non-Induced
Induced
% PD-1+
***
***
CD8+
TSKB20+
B.
MHC Induction
PD-L1
DPI
0
15
35
C.
Non-Induced
≥ 30d Induced
≥ 30d+PD-L1
% TSKB20+ of CD44+CD8+ T cells
n.s.
n.s.
Spleen
Sk. Muscle
D.
Non-Induced
≥ 30d Induced
≥ 30d+PD-L1
% CD69+
n.s.
n.s.
CD8+
TSKB20+
E.
Naive
Non-Induced
≥ 30d Induced
≥ 30d Induced PD-L1
F.
Parasite equivalents per 50 ng of DNA
Non-Induced
≥ 30d Induced
≥ 30d Induced+PD-L1

Figure 3.7. MHC I overexpression fails to resolve established T. cruzi infection in muscle

A) Mice were infected with *T. cruzi* for 15 days prior to the start of doxycycline administration to induce MHC overexpression. Parasite burden in muscle was measured by qPCR on 35 days post infection. B) MHC I overexpression was initiated at >120 days post infection and continued for ~20 days. Overexpression failed to improve parasite control when assessed via qPCR. Data are representative of two independent experiments with $n \geq 3$. Data are mean + SEM. * indicates averages that are significantly different (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) between specified groups.

Figure 3.7


A.
Acute
p=.0745
Parasite equivalents per 50 ng of DNA
500
400
300
200
100
0
Non-Induced
Induced
B.
Chronic
**
Parasite equivalents per 50 ng of DNA
25
20
15
10
5
0
Non-Induced
Induced

3.6 References

1. Pan American Health Organization, W. P. o. N. T., and Diseases. (2007) Estimación cuantitativa de la enfermedad de Chagas en les Américas. Pan American Health Organization, Washington

2. Schofield, C. J., Jannin, J., and Salvatella, R. (2006) The future of Chagas disease control. *Trends in parasitology* **22**, 583-588

3. Bern, C., Montgomery, S. P., Herwaldt, B. L., Rassi, A., Jr., Marin-Neto, J. A., Dantas, R. O., Maguire, J. H., Acquatella, H., Morillo, C., Kirchhoff, L. V., Gilman, R. H., Reyes, P. A., Salvatella, R., and Moore, A. C. (2007) Evaluation and treatment of chagas disease in the United States: a systematic review. *JAMA : the journal of the American Medical Association* **298**, 2171-2181

4. Marcon, G. E., de Albuquerque, D. M., Batista, A. M., Andrade, P. D., Almeida, E. A., Guariento, M. E., Teixeira, M. A., and Costa, S. C. (2011) Trypanosoma cruzi: parasite persistence in tissues in chronic chagasic Brazilian patients. *Memorias do Instituto Oswaldo Cruz* **106**, 85-91

5. Caradonna, K. L., and Burleigh, B. A. (2011) Mechanisms of host cell invasion by Trypanosoma cruzi. *Adv Parasitol* **76**, 33-61

6. Melo, R. C., and Brener, Z. (1978) Tissue tropism of different Trypanosoma cruzi strains. *The Journal of parasitology* **64**, 475-482

7. Frontera, W. R., and Ochala, J. (2015) Skeletal muscle: a brief review of structure and function. *Calcif Tissue Int* **96**, 183-195

8. Daar, A. S., Fuggle, S. V., Fabre, J. W., Ting, A., and Morris, P. J. (1984) The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation* **38**, 287-292

9. Appleyard, S. T., Dunn, M. J., Dubowitz, V., and Rose, M. L. (1985) Increased expression of HLA ABC class I antigens by muscle fibres in Duchenne muscular dystrophy, inflammatory myopathy, and other neuromuscular disorders. *Lancet* **1**, 361-363

10. Nagaraju, K., Raben, N., Loeffler, L., Parker, T., Rochon, P. J., Lee, E., Danning, C., Wada, R., Thompson, C., Bahtiyar, G., Craft, J., Hooft Van Huijsduijnen, R., and Plotz, P. (2000) Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 9209-9214

11. Figarella-Branger, D., Lacroix, C., Coquet, M., Gherardi, R., and Pellissier, J. F. (2001) [Idiopathic inflammatory myopathies]. *Ann Pathol* **21**, 279-284

12. Pavlath, G. K. (2002) Regulation of class I MHC expression in skeletal muscle: deleterious effect of aberrant expression on myogenesis. *J Neuroimmunol* **125**, 42-50

13. Civatte, M., Schleinitz, N., Krammer, P., Fernandez, C., Guis, S., Veit, V., Pouget, J., Harle, J. R., Pellissier, J. F., and Figarella-Branger, D. (2003) Class I MHC detection as a diagnostic tool in noninformative muscle biopsies of patients suffering from dermatomyositis (DM). *Neuropathol Appl Neurobiol* **29**, 546-552

14. Wong, P., and Pamer, E. G. (2003) CD8 T cell responses to infectious pathogens. *Annual review of immunology* **21**, 29-70

15. Harty, J. T., Tvinnereim, A. R., and White, D. W. (2000) CD8+ T cell effector mechanisms in resistance to infection. *Annual review of immunology* **18**, 275-308

16. Harty, J. T., and Bevan, M. J. (1999) Responses of CD8(+) T cells to intracellular bacteria. *Current opinion in immunology* **11**, 89-93

17. Halenius, A., Gerke, C., and Hengel, H. (2015) Classical and non-classical MHC I molecule manipulation by human cytomegalovirus: so many targets-but how many arrows in the quiver? *Cell Mol Immunol* **12**, 139-153

18. Neefjes, J., Jongsma, M. L., Paul, P., and Bakke, O. (2011) Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews. Immunology* **11**, 823-836

19. Benz, C., Reusch, U., Muranyi, W., Brune, W., Atalay, R., and Hengel, H. (2001) Efficient downregulation of major histocompatibility complex class I molecules in human epithelial cells infected with cytomegalovirus. *The Journal of general virology* **82**, 2061-2070

20. Petersen, J. L., Morris, C. R., and Solheim, J. C. (2003) Virus evasion of MHC class I molecule presentation. *J Immunol* **171**, 4473-4478

21. Krmpotic, A., Messerle, M., Crnkovic-Mertens, I., Polic, B., Jonjic, S., and Koszinowski, U. H. (1999) The immunoevasive function encoded by the mouse cytomegalovirus gene m152 protects the virus against T cell control in vivo. *The Journal of experimental medicine* **190**, 1285-1296

22. Furman, M. H., Dey, N., Tortorella, D., and Ploegh, H. L. (2002) The human cytomegalovirus US10 gene product delays trafficking of major histocompatibility complex class I molecules. *Journal of virology* **76**, 11753-11756

23. Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H., and Johnson, D. (1995) Herpes simplex virus turns off the TAP to evade host immunity. *Nature* **375**, 411-415

24. Hornef, M. W., Wick, M. J., Rhen, M., and Normark, S. (2002) Bacterial strategies for overcoming host innate and adaptive immune responses. *Nature immunology* **3**, 1033-1040

25. Joly, E., Mucke, L., and Oldstone, M. B. (1991) Viral persistence in neurons explained by lack of major histocompatibility class I expression. *Science* **253**, 1283-1285

26. Cabral, C. M., Tuladhar, S., Dietrich, H. K., Nguyen, E., MacDonald, W. R., Trivedi, T., Devineni, A., and Koshy, A. A. (2016) Neurons are the Primary Target Cell for the Brain-Tropic Intracellular Parasite Toxoplasma gondii. *PLoS pathogens* **12**, e1005447

27. Low, H. P., Santos, M. A., Wizel, B., and Tarleton, R. L. (1998) Amastigote surface proteins of Trypanosoma cruzi are targets for CD8+ CTL. *J Immunol* **160**, 1817-1823

28. Martin, D. L., Weatherly, D. B., Laucella, S. A., Cabinian, M. A., Crim, M. T., Sullivan, S., Heiges, M., Craven, S. H., Rosenberg, C. S., Collins, M. H., Sette, A., Postan, M., and Tarleton, R. L. (2006) CD8+ T-Cell responses to Trypanosoma cruzi are highly focused on strain-variant trans-sialidase epitopes. *PLoS pathogens* **2**, e77

29. Garg, N., Nunes, M. P., and Tarleton, R. L. (1997) Delivery by Trypanosoma cruzi of proteins into the MHC class I antigen processing and presentation pathway. *J Immunol* **158**, 3293-3302

30. Tarleton, R. L., Koller, B. H., Latour, A., and Postan, M. (1992) Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection. *Nature* **356**, 338-340

31. Tarleton, R. L., Grusby, M. J., Postan, M., and Glimcher, L. H. (1996) Trypanosoma cruzi infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *International immunology* **8**, 13-22

32. Wizel, B., Nunes, M., and Tarleton, R. L. (1997) Identification of Trypanosoma cruzi trans-sialidase family members as targets of protective CD8+ TC1 responses. *J Immunol* **159**, 6120-6130

33. Rao, P., and Monks, D. A. (2009) A tetracycline-inducible and skeletal muscle-specific Cre recombinase transgenic mouse. *Developmental neurobiology* **69**, 401-406

34. Masopust, D., Vezys, V., Marzo, A. L., and Lefrancois, L. (2001) Preferential localization of effector memory cells in nonlymphoid tissue. *Science* **291**, 2413-2417

35. Liu, F., and Whitton, J. L. (2005) Cutting edge: re-evaluating the in vivo cytokine responses of CD8+ T cells during primary and secondary viral infections. *J Immunol* **174**, 5936-5940

36. Cummings, K. L., and Tarleton, R. L. (2003) Rapid quantitation of Trypanosoma cruzi in host tissue by real-time PCR. *Molecular and biochemical parasitology* **129**, 53-59

37. Pittella, J. E. (1993) Central nervous system involvement in Chagas' disease. An updating. *Rev Inst Med Trop Sao Paulo* **35**, 111-116

38. Dalakas, M. C., and Hohlfeld, R. (2003) Polymyositis and dermatomyositis. *Lancet* **362**, 971-982

39. Mantegazza, R., Bernasconi, P., Confalonieri, P., and Cornelio, F. (1997) Inflammatory myopathies and systemic disorders: a review of immunopathogenetic mechanisms and clinical features. *J Neurol* **244**, 277-287

40. Goebels, N., Michaelis, D., Engelhardt, M., Huber, S., Bender, A., Pongratz, D., Johnson, M. A., Wekerle, H., Tschopp, J., Jenne, D., and Hohlfeld, R. (1996) Differential expression of perforin in muscle-infiltrating T cells in polymyositis and dermatomyositis. *The Journal of clinical investigation* **97**, 2905-2910

41. Vanneman, M., and Dranoff, G. (2012) Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer* **12**, 237-251

42. Grosso, J. F., Kelleher, C. C., Harris, T. J., Maris, C. H., Hipkiss, E. L., De Marzo, A., Anders, R., Netto, G., Getnet, D., Bruno, T. C., Goldberg, M. V., Pardoll, D. M., and Drake, C. G. (2007) LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *The Journal of clinical investigation* **117**, 3383-3392

43. Haggerty, T. J., Dunn, I. S., Rose, L. B., Newton, E. E., Pandolfi, F., and Kurnick, J. T. (2014) Heat shock protein-90 inhibitors enhance antigen expression on melanomas and increase T cell recognition of tumor cells. *PloS one* **9**, e114506

44. Stevenson, P. G., May, J. S., Smith, X. G., Marques, S., Adler, H., Koszinowski, U. H., Simas, J. P., and Efstathiou, S. (2002) K3-mediated evasion of CD8(+) T cells aids amplification of a latent gamma-herpesvirus. *Nature immunology* **3**, 733-740

45. Lybarger, L., Wang, X., Harris, M. R., Virgin, H. W. t., and Hansen, T. H. (2003) Virus subversion of the MHC class I peptide-loading complex. *Immunity* **18**, 121-130

46. Utzschneider, D. T., Alfei, F., Roelli, P., Barras, D., Chennupati, V., Darbre, S., Delorenzi, M., Pinschewer, D. D., and Zehn, D. (2016) High antigen levels induce an exhausted phenotype in a chronic infection without impairing T cell expansion and survival. *The Journal of experimental medicine*

47. Richter, K., Brocker, T., and Oxenius, A. (2012) Antigen amount dictates CD8(+) T-cell exhaustion during chronic viral infection irrespective of the type of antigen presenting cell. *European journal of immunology*

48. Wherry, E. J., Blattman, J. N., Murali-Krishna, K., van der Most, R., and Ahmed, R. (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *Journal of virology* **77**, 4911-4927

49. Kahan, S. M., Wherry, E. J., and Zajac, A. J. (2015) T cell exhaustion during persistent viral infections. *Virology* **479-480**, 180-193

50. Nishio, J., Suzuki, M., Miyasaka, N., and Kohsaka, H. (2001) Clonal biases of peripheral CD8 T cell repertoire directly reflect local inflammation in polymyositis. *J Immunol* **167**, 4051-4058

51. Leavey, J. K., and Tarleton, R. L. (2003) Cutting edge: dysfunctional CD8+ T cells reside in nonlymphoid tissues during chronic Trypanosoma cruzi infection. *J Immunol* **170**, 2264-2268

52. Laucella, S. A., Postan, M., Martin, D., Hubby Fralish, B., Albareda, M. C., Alvarez, M. G., Lococo, B., Barbieri, G., Viotti, R. J., and Tarleton, R. L. (2004) Frequency of interferon- gamma -producing T cells specific for Trypanosoma cruzi inversely correlates with disease severity in chronic human Chagas disease. *The Journal of infectious diseases* **189**, 909-918

53. Martin, D. L., Postan, M., Lucas, P., Gress, R., and Tarleton, R. L. (2007) TGF-beta regulates pathology but not tissue CD8+ T cell dysfunction during experimental Trypanosoma cruzi infection. *European journal of immunology* **37**, 2764-2771

54. Kotner, J., and Tarleton, R. (2007) Endogenous CD4(+) CD25(+) regulatory T cells have a limited role in the control of Trypanosoma cruzi infection in mice. *Infection and immunity* **75**, 861-869

55. Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., Freeman, G. J., and Ahmed, R. (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* **439**, 682-687

56. Horne-Debets, J. M., Faleiro, R., Karunarathne, D. S., Liu, X. Q., Lineburg, K. E., Poh, C. M., Grotenbreg, G. M., Hill, G. R., MacDonald, K. P., Good, M. F., Renia, L., Ahmed, R., Sharpe, A. H., and Wykes, M. N. (2013) PD-1 dependent exhaustion of CD8+ T cells drives chronic malaria. *Cell reports* **5**, 1204-1213

57. Freeman, G. J., Wherry, E. J., Ahmed, R., and Sharpe, A. H. (2006) Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *The Journal of experimental medicine* **203**, 2223-2227

58. Murata, K., and Dalakas, M. C. (1999) Expression of the costimulatory molecule BB-1, the ligands CTLA-4 and CD28, and their mRNA in inflammatory myopathies. *The American journal of pathology* **155**, 453-460

59. Behrens, L., Kerschensteiner, M., Misgeld, T., Goebels, N., Wekerle, H., and Hohlfeld, R. (1998) Human muscle cells express a functional costimulatory molecule distinct from B7.1 (CD80) and B7.2 (CD86) in vitro and in inflammatory lesions. *J Immunol* **161**, 5943-5951

60. Nagaraju, K., Raben, N., Merritt, G., Loeffler, L., Kirk, K., and Plotz, P. (1998) A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli. *Clin Exp Immunol* **113**, 407-414

61. Hoogendijk, J. E., Amato, A. A., Lecky, B. R., Choy, E. H., Lundberg, I. E., Rose, M. R., Vencovsky, J., de Visser, M., and Hughes, R. A. (2004) 119th ENMC international workshop: trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis, 10-12 October 2003, Naarden, The Netherlands. *Neuromuscul Disord* **14**, 337-345

62. Wiendl, H., Mitsdoerffer, M., Schneider, D., Chen, L., Lochmuller, H., Melms, A., and Weller, M. (2003) Human muscle cells express a B7-related molecule, B7-H1, with strong negative immune regulatory potential: a novel mechanism of counterbalancing the immune attack in idiopathic inflammatory myopathies. *Faseb J* **17**, 1892-1894

63. Legat, A., Speiser, D. E., Pircher, H., Zehn, D., and Fuertes Marraco, S. A. (2013) Inhibitory Receptor Expression Depends More Dominantly on Differentiation and Activation than "Exhaustion" of Human CD8 T Cells. *Front Immunol* **4**, 455

64. Crawford, A., and Wherry, E. J. (2009) The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Current opinion in immunology* **21**, 179-186

65. Odorizzi, P. M., and Wherry, E. J. (2012) Inhibitory receptors on lymphocytes: insights from infections. *J Immunol* **188**, 2957-2965

66. Blackburn, S. D., Shin, H., Haining, W. N., Zou, T., Workman, C. J., Polley, A., Betts, M. R., Freeman, G. J., Vignali, D. A., and Wherry, E. J. (2009) Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nature immunology* **10**, 29-37

67. Koyama, S., Akbay, E. A., Li, Y. Y., Herter-Sprie, G. S., Buczkowski, K. A., Richards, W. G., Gandhi, L., Redig, A. J., Rodig, S. J., Asahina, H., Jones, R. E., Kulkarni, M. M., Kuraguchi, M., Palakurthi, S., Fecci, P. E., Johnson, B. E., Janne, P. A., Engelman, J. A., Gangadharan, S. P., Costa, D. B., Freeman, G. J., Bueno, R., Hodi, F. S., Dranoff, G., Wong, K. K., and Hammerman, P. S. (2016) Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat Commun* **7**, 10501

68. Arguello, R. J., Albareda, M. C., Alvarez, M. G., Bertocchi, G., Armenti, A. H., Vigliano, C., Meckert, P. C., Tarleton, R. L., and Laucella, S. A. (2012) Inhibitory receptors are expressed by Trypanosoma cruzi-specific effector T cells and in hearts of subjects with chronic Chagas disease. *PloS one* **7**, e35966

69. Lasso, P., Mateus, J., Pavia, P., Rosas, F., Roa, N., Thomas, M. C., Lopez, M. C., Gonzalez, J. M., Puerta, C. J., and Cuellar, A. (2015) Inhibitory Receptor Expression on CD8+ T Cells Is Linked to Functional Responses against Trypanosoma cruzi Antigens in Chronic Chagasic Patients. *J Immunol* **195**, 3748-3758

70. Mateus, J., Lasso, P., Pavia, P., Rosas, F., Roa, N., Valencia-Hernandez, C. A., Gonzalez, J. M., Puerta, C. J., and Cuellar, A. (2015) Low frequency of circulating CD8+ T stem cell memory cells in chronic chagasic patients with severe forms of the disease. *PLoS neglected tropical diseases* **9**, e3432

71. Albareda, M. C., Laucella, S. A., Alvarez, M. G., Armenti, A. H., Bertochi, G., Tarleton, R. L., and Postan, M. (2006) Trypanosoma cruzi modulates the profile of memory CD8+ T cells in chronic Chagas' disease patients. *International immunology* **18**, 465-471

72. Reis, D. D., Jones, E. M., Tostes, S., Lopes, E. R., Chapadeiro, E., Gazzinelli, G., Colley, D. G., and McCurley, T. L. (1993) Expression of major histocompatibility complex antigens and adhesion molecules in hearts of patients with chronic Chagas' disease. *The American journal of tropical medicine and hygiene* **49**, 192-200

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

Infection by the parasite *Trypanosoma cruzi* continues for the lifetime of most infected hosts. Understanding the factors, host or parasite related, that enable the establishment of *T. cruzi* persistence, is critical for the development of intervention strategies for *T. cruzi* elimination in infected subjects.

Before this study, very little was known about the functional contribution of $CD8^+$ T cells in *T. cruzi* control beyond the acute phase of infection. Despite the essential contribution of $CD8^+$ T cells in control and survival of early infection (1,2), the quality of anti-*T. cruzi* $CD8^+$ T cell responses during the chronic phase was in question. These doubts were largely based upon the observation of *ex vivo* compromise upon polyclonal stimulation from muscle-derived cells during chronic infection (3,4) and reports in the literature highlighting failed immunity as the cause of infection persistence in model systems (5-7). The first aim of this project was to assess whether $CD8^+$ T cells contribute to anti-*T. cruzi* immunity during chronic infection. I chose to include the responses of $CD8^+$ T cells in the muscle in these analyses, as this population was the most likely to experience functional compromise as a consequence of continued stimulation by *T. cruzi* infected cells. The results support my hypothesis that $CD8^+$ T cell function, even in a site of parasite persistence (muscle), is retained beyond the acute phase of infection. This is demonstrated by the detection of effectors that express markers of recent activation, indicative of their continued engagement with infected cells during chronic infection. Even more striking, is the direct observation of *in situ* effector function, in the forms of IFNγ, TNFα, and granzyme B production, by $CD8^+$ T cells in tissues of parasite persistence. The function of *T. cruzi*-specific $CD8^+$ T cells does not appear to be inhibited by the regulatory cytokine IL-10 or the inhibitory

receptor PD-1, as inactivation of these molecules has no impact on chronic *T. cruzi* control. In addition, the quality of T. *cruzi* control during chronic infection is dependent on the presence of these effectors, as CD8 depletion lead to significant parasite expansion in tissues commonly associated with persistence. These results suggest that *T. cruzi* is a unique model of persistent infection, where highly regulated $CD8^+$ T cell immunity fails to explain pathogen chronicity; a stark contrast to the observations made in several viral and protozoal infections (8-11).

Functional *T. cruzi*-specific $CD8^+$ T cells are present in skeletal muscle, but parasites are not completely eliminated from the tissue. The second aim of this work was to evaluate the factors in the muscle environment that selectively permit *T. cruzi* survival there. Basal MHC class I expression is extremely low and is highly regulated in muscle (12,13). I hypothesized that this normal host regulation might impact the surveillance potential of muscle, allowing a selective survival advantage for *T. cruzi* in that site. Through the use of a mouse model where skeletal muscle MHC I expression is conditionally upregulated by doxycycline administration, I determined that normal muscle MHC expression is likely insufficient to facilitate *T. cruzi* clearance. When MHC I levels were increased, I initially observed significant improvements in *T. cruzi* control, that were mediated by IFNγ and TNFα producing $CD8^+$ T cells in the muscle. These enhancements in control were likely reflective of improved tissue surveillance, as the frequency of highly infected cells decreased significantly with overexpression. I had anticipated that continued muscle MHC class I overexpression would lead to *T. cruzi* infection resolution. Instead, $CD8^+$ T cells in the skeletal muscle received excessive stimulation, forcing the induction of regulation via PD-1. This regulation compromised *T. cruzi* control and $CD8^+$ T cell function in skeletal muscle and systemically, although this lost control was reversible and restored by PD-1 blockade.

This study suggests that "normal" levels of MHC expression and the resulting T cell stimulation, likely preserve $CD8^+$ T cell function and parasite control during *T. cruzi* infection. This is most clearly demonstrated during MHC I overexpression during chronic infection. The parasite burden during the chronic phase in skeletal muscle is normally extremely low, but expands exponentially when $CD8^+$ T cells receive additional stimulation through MHC I overexpression. This loss of control coincides with increased PD-1 expression on muscle $CD8^+$ T cells. These data also highlight the quality of the $CD8^+$ T responses normally present during chronic *T. cruzi* infection, as discussed in the first study.

Together, these studies suggest that $CD8^+$ T function is tuned to allow *T. cruzi* control without significant compromise by regulation, even during chronic mouse infection. It is possible for *T. cruzi*-specific CD8 responses to mirror the highly regulated states observed in persistent viral infections (14). This occurs when $CD8^+$ T cells receive excessive stimulation (MHC overexpression) or following extremely long (decades) infection in humans (15,16). These data also suggest that simply providing additional stimulation for immunodominant $CD8^+$ T cells is insufficient for T. cruzi infection resolution; an important bit of information to consider during the development of immunotherapies or vaccines. It is possible that different responses, i.e. the presence of effector $CD8^+$ T cells that are capable of earlier infected cell recognition (17) or of differing specificities, may improve the rate of spontaneous cure during *T. cruzi* infection. Conversely, these studies highlight the need for caution against the overzealous modulation of cytotoxic T cell responses. Excessive myocyte lysis and the resulting irreversible pathology in valuable host tissues like the heart, in support of improved *T. cruzi* control, is not a viable option.

4.2 References

1. Tarleton, R. L. (1990) Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with Trypanosoma cruzi. *J Immunol* **144**, 717-724

2. Tarleton, R. L., Sun, J., Zhang, L., and Postan, M. (1994) Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. *Infection and immunity* **62**, 1820-1829

3. Leavey, J. K., and Tarleton, R. L. (2003) Cutting edge: dysfunctional CD8+ T cells reside in nonlymphoid tissues during chronic Trypanosoma cruzi infection. *J Immunol* **170**, 2264-2268

4. Kotner, J., and Tarleton, R. (2007) Endogenous CD4(+) CD25(+) regulatory T cells have a limited role in the control of Trypanosoma cruzi infection in mice. *Infection and immunity* **75**, 861-869

5. Bhadra, R., Gigley, J. P., and Khan, I. A. (2012) PD-1-mediated attrition of polyfunctional memory CD8+ T cells in chronic toxoplasma infection. *The Journal of infectious diseases* **206**, 125-134

6. Blattman, J. N., Wherry, E. J., Ha, S. J., van der Most, R. G., and Ahmed, R. (2009) Impact of epitope escape on PD-1 expression and CD8 T-cell exhaustion during chronic infection. *Journal of virology* **83**, 4386-4394

7. Horne-Debets, J. M., Faleiro, R., Karunarathne, D. S., Liu, X. Q., Lineburg, K. E., Poh, C. M., Grotenbreg, G. M., Hill, G. R., MacDonald, K. P., Good, M. F., Renia, L., Ahmed, R., Sharpe, A. H., and Wykes, M. N. (2013) PD-1 dependent exhaustion of CD8+ T cells drives chronic malaria. *Cell reports* **5**, 1204-1213

8. Iwai, Y., Terawaki, S., Ikegawa, M., Okazaki, T., and Honjo, T. (2003) PD-1 inhibits antiviral immunity at the effector phase in the liver. *The Journal of experimental medicine* **198**, 39-50

9. Penaloza-MacMaster, P., Kamphorst, A. O., Wieland, A., Araki, K., Iyer, S. S., West, E. E., O'Mara, L., Yang, S., Konieczny, B. T., Sharpe, A. H., Freeman, G. J., Rudensky, A. Y., and Ahmed, R. (2014) Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. *The Journal of experimental medicine* **211**, 1905-1918

10. Petrovas, C., Casazza, J. P., Brenchley, J. M., Price, D. A., Gostick, E., Adams, W. C., Precopio, M. L., Schacker, T., Roederer, M., Douek, D. C., and Koup, R. A. (2006) PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *The Journal of experimental medicine* **203**, 2281-2292

11. Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., Vanderford, T. H., Chennareddi, L., Silvestri, G., Freeman, G. J., Ahmed, R., and Amara, R. R. (2009) Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* **458**, 206-210

12. Civatte, M., Schleinitz, N., Krammer, P., Fernandez, C., Guis, S., Veit, V., Pouget, J., Harle, J. R., Pellissier, J. F., and Figarella-Branger, D. (2003) Class I MHC detection as a diagnostic tool in noninformative muscle biopsies of patients suffering from dermatomyositis (DM). *Neuropathol Appl Neurobiol* **29**, 546-552

13. Daar, A. S., Fuggle, S. V., Fabre, J. W., Ting, A., and Morris, P. J. (1984) The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation* **38**, 287-292

14. Kahan, S. M., Wherry, E. J., and Zajac, A. J. (2015) T cell exhaustion during persistent viral infections. *Virology* **479-480**, 180-193

15. Albareda, M. C., De Rissio, A. M., Tomas, G., Serjan, A., Alvarez, M. G., Viotti, R., Fichera, L. E., Esteva, M. I., Potente, D., Armenti, A., Tarleton, R. L., and Laucella, S. A. (2013) Polyfunctional T cell responses in children in early stages of chronic Trypanosoma cruzi infection contrast with monofunctional responses of long-term infected adults. *PLoS neglected tropical diseases* **7**, e2575

16. Utzschneider, D. T., Alfei, F., Roelli, P., Barras, D., Chennupati, V., Darbre, S., Delorenzi, M., Pinschewer, D. D., and Zehn, D. (2016) High antigen levels induce an exhausted phenotype in a chronic infection without impairing T cell expansion and survival. *The Journal of experimental medicine*

17. Kurup, S. P., and Tarleton, R. L. (2014) The Trypanosoma cruzi Flagellum Is Discarded via Asymmetric Cell Division following Invasion and Provides Early Targets for Protective CD8(+) T Cells. *Cell host & microbe* **16**, 439-449