NORTHWESTERN UNIVERSITY

The Regulation of Infection-Induced Cardiac Autoimmunity and Natural Dissemination of *Trypanosoma cruzi* in Experimental Chagas Heart Disease

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Field of Integrated Graduate Program in the Life Sciences

By

Kenneth V. Hyland

EVANSTON, ILLINOIS

December 2008

ABSTRACT

The Regulation of Infection-Induced Cardiac Autoimmunity and Natural Dissemination of *Trypanosoma cruzi* in Experimental Chagas Heart Disease

Kenneth V. Hyland

Chagas heart disease, caused by infection with the protozoan parasite *Trypanosoma cruzi*, is still regarded as a major public health problem in Central and South America. The finding of cardiac specific autoimmunity during infection in both humans and experimental animals has provided a basis for investigation of its potential role in disease pathogenesis for many years. However, the complex nature of Chagas disease has left questions of the true mechanisms of cardiac inflammation, in addition to an understanding of the natural course of infection, largely unanswered. My thesis research consisted of investigating aspects of parasite infection-induced cardiac autoimmune responses and the spatiotemporal dissemintation of *T. cruzi* in an experimental model of Chagas heart disease. The infection of A/J mice with the Brazil strain of *T. cruzi* results in the development of acute myocarditis with both humoral and cellular autoreactivity by 21 days post infection. To test the hypothesis that the magnitude of the autoimmune response is directly proportional to the amount of damage elicited by the parasite, I administered a trypanocidal drug, benznidazole, to mice to reduce the number of viable parasites following infection and determined that treatment not only decreased disease severity and eliminated mortality, but also significantly reduced cardiac myosin-specific DTH and antibody production. With a strong enough secondary cardiac insult, the autoreactivity and myocarditis could be restored, indicating the reestablishment of self-tolerance after the eradication of the

parasite was not permanent in our disease model. Overall, an important link between the levels of live parasite and the presence of autoimmunity was provided, suggesting that treatments designed, such as vaccines, to specifically target the parasite will likely reduce or eliminate the induction of autoimmunity as well. To further understand the natural course of infection, I engineered firefly luciferase-expressing *T. cruzi* in order to non-invasively monitor the dissemination of parasites in mice over time using bioluminescence imaging technology. For a more in-depth analysis of parasite tropism during infection, the tissue distribution of *T. cruzi* was determined by imaging heart, spleen, skeletal muscle, lungs, kidneys, liver and intestines *ex vivo*. This novel parasite line has already provided interesting results illustrating the natural dissemination of *T. cruzi* during infection and will continue to serve as a tool for studying a number of aspects of Chagas disease. In conclusion, these results not only provide encouragement for the future exploration of parasite-specific therapeutic strategies for Chagas disease by showing that elimination of *T. cruzi* is effective at reducing or eliminating autoimmunity, but also illustrate a novel tool that could be easily applied to the screening of such therapeutical agents via bioluminescence.

Abstract approved: 

David M. Engman, MD, PhD
Dissertation Director

Professor of Pathology
Title and Department

July, 14, 2008
Date

ACKNOWLEDGEMENTS

The completion of anything in life that requires a surplus of patience, insurmountable persistence and outright determination simply cannot be achieved in the absence of genuine support, comradery and unconditional understanding from those closest to you. Although perhaps not the case for some, the journey of acquiring a PhD was, for me, the very definition of such a task. Without unwavering encouragement and steadfast support from many individuals I would certainly not be composing the final section of my doctoral thesis. To those individuals, I owe an enormous debt of gratitude. While each individual encountered along the way undoubtedly fuels the excitement of the journey, a select few evolve into more than mere acquaintances and truly push you to the finish line. To those encounters that became true friends, somehow capable of keeping the finish line in sight, I also owe an enormous debt of gratitude.

I must first acknowledge the best "salesman/scientist" I've ever known and surely ever will know. My advisor, Dr. David Engman, somehow convinced me to stay the course with graduate school on more than one occasion. Without his persuasive persona and continual belief that I really could finish what I started, I would have promptly departed from Northwestern after one year…and again after three years…without question. Dr. Engman provided an environment of independence, fostering improvement in my abilities to communicate in both written word and speech, critically evaluate the ideas of my peers, and acquire the knowledge needed to troubleshoot virtually any type of problem. He sold me on the value of obtaining a PhD and,

without his constant belief that I could indeed acquire the degree I would have surely stopped short of completion of this journey. I sincerely thank Dr. Engman for his patience with me when he could have just as easily sent me on my way several years ago. My committee members, Drs. Melissa Brown, Lou Laimins, and Susan Winandy also displayed immense amounts of patience as I repeatedly called meetings to discuss piles of failed experiments and redirections of research. All members were very helpful in their thoughtful and critical analyses of my research and insightful on the best ways to proceed with subsequent steps. In particular, I thank Susan Winandy for her graciousness in composing several critical letters of recommendation for funded (and a couple of denied) predoctoral grant applications.

The laboratory underwent its own metamorphosis within the first few months of my joining. I was fortunate enough to interact with some individuals before their departures, others from my start to finish, and some as they arrived years later. Juan Leon opened my eyes to the appeal of immunology and forced me to think critically about every single aspect of every experiment performed. He became a consultant and a great friend as I adapted to life in graduate school and continues to serve as a great mentor as I move forward in my career. Kegiang (John) Wang served as a tremendous assistance and taught me nearly every animal procedure I needed to continue my research after his retirement. It would be difficult to rival his wealth of experience in any other technician and I consider myself fortunate to have had the opportunity to work alongside him throughout his final years in the Engman lab. The starck contrast of optimism and cynicism of Sofya Asfaw and Alina Fridberg made getting to know these two individuals very entertaining during my time in the lab. Both have since moved on, one after

persevering through obtaining her PhD, and the other in pursuit of her medical degree. It was a privilege getting to know and working alongside both of these labmates prior to their departures.

Melvin Daniels was there from day one and continues his relentless pursuit of his PhD still. I can say with utmost certainty I would not be completing this dissertation without having the good fortune of having him by my side (or literally in the chair behind me) each and every step of the way. He is the very definition of a comrade, a constant throught the good days and the frustrating days…always willing to lend a hand. He is the type of person that others can't help but get to know by just passing him in the hall or waiting for an elevator next to him but until you sit next to him day in and day out for five years, you don't get the true experience of knowing Melvin Daniels. I have never, and seriously doubt that I will ever meet someone with the breadth of stories and enthusiasm for telling them as Melvin. His genuine zest for life and constant belief that "it has to work" made doing experiment after experiment possible, knowing that it most likely wouldn't really work. Without Melvin, dealing with the failed experiments would've been much harder and celebrating the rare instances of success much more dull. He became a great friend over the course of my time in graduate school and if nothing else, that alone made the journey worthwhile. I owe Melvin my PhD and if I could give it to him…well, I probably wouldn't, but only because he'll earn one himself in a short time. Cheryl Olson also served as a constant from start to finish. Her love of science and overall patience with maintaining a continually changing laboratory environment is truly inspiring. She takes a very active role in the early development of graduate students in the lab as they get acclimated to the environment and the projects. Cheryl made the transition into the lab feasible and became a good friend over the course of my time there. Despite being largely responsible for keeping the

Engman lab operational on a day-to-day basis, Cheryl was always willing to answer numbers of types of questions and generously offered a helping hand in my research on countless occasions. I thank her so much for her encouragement, instruction and kindness over the last five years.

Some individuals arrived a bit later in the lab. Brian Emmer is one of the smartest, most diligent guys I've ever known. He is undoubtedly going to become a renowned physician or discover something incredibly important someday. If not, I have no doubt he could do just as well in life as an NBA analyst, Fantasy sports professional or Las Vegas bookie. Brian's capability of being critical of any experiment or idea, no matter how detached he is from it, while frustrating at times, was always helpful in thinking more carefully about a strategy. He is the type of person that knows something about nearly everything that makes you aspire to learn more than you already know about your research…and about everything else for that matter. His love of sports and laid-back demeanor were a welcome addition to the lab. Some of my best memories of the lab will forever be Melvin, Brian and I (and an occasional rotation student) making lunch trips to the west-side for pizza or just hanging out watching a game. Despite his frustration with science that every other PhD student experiences at some point, the deep-down passion he has for it will propel Brian into a fruitful career in the future. I admire him for his patience and perseverance and thank him for his friendship and comradery through the time we shared in the Engman lab. Conrad Epting, also an incredibly smart and critical mind from which I was fortunate to benefit, "joined the lab" as he began to develop his own niche and space within the department. For me, the timing of his arrival could not have been better. Conrad truly instilled in me the concept of "creating inertia" which I readily applied to getting the ball rolling on a path to graduation that proved to be a complete success in the end. I owe him so much for

the hours he spent listening to far too many details about my quest to graduate and became a terrific mentor and friend in the process. I thank him so much for his insight and suggestions that helped pave the way to completion of the PhD program. I've been fortunate to work with or mentor a number of other students or technicians that have been great to know and have only made my time in pursuit of the PhD worth so much more; Kevin Bonney, Danijela Maric, Chrissy Souther, Maureen Abbott (Diaz), Yaw Bediako, Shivani Shah, Teresa Schuessler.

I met Patrick Yoon during IGP orientation and developed an instant friendship that continues today. We grinded out the core course requirements together, went our separate ways into rotations and eventual permanent labs but always kept in touch…not only about science but mostly about the next step and our desires to move on with life. I was fortunate enough to see Patrick's wedding, and celebrated with him as he landed a lucrative position in pharmaceutical industry and had a baby boy all within a few months. Patrick is one of the genuinely nicest people I've ever met and I'm very lucky to have him as a friend. Other friends, from years before graduate school was even a blip on the radar, were also essential to the completion of this journey. Rob Messmer, Matt Ours and Dustin Smith became even better friends to me thoughout this process by listening to my complaints when they clearly had little idea of what I was talking about the majority of the time. Many trips were made up to Chicago throughout the years where many good times were had, many memories made and several traditions begun. These guys have been like brothers to me when I needed them most and I thank them all for their outpourings of support to me in obtaining this degree.

My second family (i.e., "in-laws") has been supportive to both myself and perhaps more importantly to my wife throughout this process. Having their daughter and sister away from

home for the first time was certainly a challenging adjustment, but they were continually supportive and made the transition to Chicago much easier. I thank Al and Anita Rust for accepting me as their 4th son and encouraging me to stick with this process til the end. I thank David for understanding what it's like, as he continues to pursue his own PhD, Jason for always telling me how lucky I was to be in graduate school and Ryan for the countless trips he made to wherever we happened to be living in the Chicagoland area; many times to actually help us move and others just to spend a weekend here and there.

My own family, who has been a pillar of support not only through this particular stepping stone, but through every goal I've set out to achieve in the past. I thank my sister, Krysta and brother-in-law Mel for their encouragement despite never really truly understanding what in the world I was working on. My little sister, Sara, with whom I had and continue to have numerous philosophical discussions about why exactly we do the things we do in life. I always say we only became closer because my wife came into the picture, but whatever the reason, I'm grateful for the relationship we've developed over the years as we've both grown up and thank her for believing I could carry this goal to completion. My parents deserve much more acknowledgement than I could ever manage to put into words. In a world where so many individuals grow up in broken homes or bad environments in which parents neglect them, my parents have always gone above and beyond the obligatory responsibilities of parenthood. This dissertation would truly not have been written without the constant encouragement, involvement and love they have provided me my entire life. They have supported me in every endeavor, without pressure or expectation, and listened carefully everytime I had a need to get something off of my chest. My parents continue even now to serve as mentors and advocates in the face of

critical decisions. I cannot begin to thank them both enough for the many trips they made, the helping hands they offered in times of need, and the open ears they had during the both the rough patches and the periods of joy. I thank them for instilling values and virtues that led me to the man I've become and owe them both an enormous debt of gratitude for never doubting that I would complete what I began.

Finally, I save the best for last in every sense of the phrase. My wife, Kara Michele, has been more instrumental in this achievement than anyone else. I began graduate school a month after walking down the isle and never would have dreamed that someone could be so incredibly strong and consistenly supportive in the face of frequently unprecedented pessimism. Kara began dating me when I intended to attend medical school following college graduation, and upon changing my direction, stuck with me without question. When I expressed extreme discontent with graduate school after the 1st year and again many times after, Kara was again nothing but supportive in whatever I chose to do. She provided unconditional love at times when I was at the end of my rope and always believed that things would work out for the best. Kara literally put her life on hold for me to pursue a degree that I claimed on several occasions was not really even that important to me. Looking back on the disappointments, doubt and disbelief in myself I had over the last several years, I truly cannot imagine making it to the end without her right by my side pushing me the entire way. Kara is without question my best friend, biggest fan and the very reason why I am composing the closing section of this doctoral thesis. I've asked for more understanding, patience and cooperation from her in the past five years than anyone has a right to over the course of an entire marriage. I thank her from the bottom of my heart for being the person she is and for being my wife…living up to the vows of "good times

and bad" much stronger than many would ever strive to maintain. As a testimony to the phrase, "good things come to those who wait", our baby boy, Noah Vincent, is living proof. Born nearly a month prior to my completion of graduate school, he was the storybook ending to a journey full of ups and downs.

Phillipians 4:13 states, "I can do all things through Christ who strengthens me." You see this verse on the backs of many marathon runners and it has always resonated in my mind through many things I've accomplished in life. While it unquestionably holds true for this circumstance as well, the absence of any individual acknowledged herein would have greatly hindered my ability to push forward to the end or, more importantly, left me missing priceless memories and relationships that I will cherish from this point forward. I sincerely thank each and every one of you.

PREFACE

This dissertation contains two published manuscripts bounded by an Introduction and a Conclusion. The Introduction contains material from reviews published in the journals *Autoimmunity* in 2006 and *Trends in Parasitology* in 2006. Chapters one and two constitute the following articles: chapter one, Hyland *et al.* **Modulation of autoimmunity by treatment of an infectious disease**. Infection & Immunity (2007) 75, 3641-50; and chapter two, Hyland *et al.* **Bioluminescent imaging of *Trypanosoma cruzi* infection**. International Journal for Parasitology (2008) (in press). The Conclusion consists of a discussion of selected unpublished studies, future directions and the overall significance of completed research.

TABLE OF CONTENTS

ABSTRACT **3**

ACKNOWLEDGEMENTS **5**

PREFACE **13**

LIST OF TABLES **17**

LIST OF FIGURES **18**

1. INTRODUCTION **20**

A. Chagas heart disease – history, impact, and epidemiology **20**

B. *Trypanosoma cruzi* – life cycle and transmission **21**

C. Acute and chronic Chagas disease **25**

D. Experimental Chagas heart disease **27**

E. Proposed mechanisms of Chagas disease pathogenesis **28**

F. Infection-induced autoimmunity **31**

G. The autoimmune hypothesis for Chagas heart disease **32**

H. Evidence for autoimmunity in Chagas heart disease **34**

I. Proposed mechanisms of *T. cruzi*-induced cardiac autoimmunity **38**

J. Criticisms of the autoimmune hypothesis for Chagas heart disease 44

K. Conclusions and overall significance 46

2. MODULATION OF AUTOIMMUNITY BY TREATMENT OF AN INFECTIOUS DISEASE 48

A. Abstract 49

B. Introduction 50

C. Materials and Methods 55

D. Results 59

E. Discussion 84

3. BIOLUMINESCENT IMAGING OF *Trypanosoma cruzi* INFECTION 91

A. Abstract 92

B. Introduction 93

C. Materials and Methods 96

D. Results 100

E. Discussion 118

4. CONCLUSION 127

A. Discussion of selected unpublished studies 127

i. Fluorescent parasite-based adoptive transfer strategy 127

ii. The pathogenic potential of *T. cruzi* protein lysate immunization..... 137

iii. Impact of benznidazole therapy on *T. cruzi* infection-induced autoimmunity 142

B. Summary and Future directions..... 147

C. Final remarks 155

REFERENCES..... 158

CURRICULUM VITAE..... 180

LIST OF TABLES

Table 1. Host proteins to which autoimmunity develops during *T. cruzi* infection 36

Table 2. Molecular mimicry during *T. cruzi* infection or after immunization 43

LIST OF FIGURES

Figure 1. *Trypanosoma cruzi* life cycle and transmission 23

Figure 2. Potential mechanisms of Chagas disease pathogenesis 29

Figure 3. Mechanisms of autoimmunity induction in *T. cruzi* infection 39

Figure 4. Mechanism of action for trypanocidal drug, benznidazole 52

Figure 5. Schematic of benznidazole experimental regimen..... 60

Figure 6. Early benznidazole treatment reduces myosin-specific DTH and inflammation and eliminates parasitosis in acute *T. cruzi* infection..... 62

Figure 7. Early benznidazole treatment reduces anti-myosin and *T. cruzi*-specific antibody production in acute *T. cruzi* infection..... 64

Figure 8. Initiating benznidazole treatment within the first week of infection eliminates myosin- and *T. cruzi*-specific DTH at 60 d.p.i. 68

Figure 9. Initiating benznidazole treatment within the first week of infection has no significant effect on IgG production at 60 d.p.i..... 70

Figure 10. Initiating benznidazole treatment within 2 weeks of infection results in an absence of myosin-specific and a reduction of *T. cruzi*-specific DTH at 90 d.p.i. 72

Figure 11. Initiating benznidazole treatment within 2 weeks of infection reduces myosin-specific but has no significant effect on *T. cruzi*-specific IgG production at 90 d.p.i..... 74

Figure 12. *T. cruzi* reinfection or myosin immunization of infected, benznidazole-treated mice restores cardiac inflammation but not parasitosis..... 77

Figure 13. *T. cruzi* reinfection or myosin immunization of infected, benznidazole-treated mice restores myosin-specific cellular autoimmunity 79

Figure 14. *T. cruzi* reinfection or myosin immunization of infected, benznidazole-treated mice restores myosin-specific humoral autoimmunity 81

Figure 15. Generation and epimastigote analysis of luminescent *T. cruzi* 101

Figure 16. Southern blot analysis of luciferase gene integration into *T. cruzi* genome 103

Figure 17. Bioluminescent imaging of *T. cruzi*-infected myoblasts *in vitro* 105

Figure 18. Luminescent *T. cruzi* imaged at various times post infection in A/J mice with an IVIS imaging system 108

Figure 19. Whole-body parasite burden quantification from infection of A/J mice with multiple dosages of luminescent or wild-type *T. cruzi* trypomastigotes 110

Figure 20. Course of parasite dissemination in a mouse model of experimental Chagas heart disease 113

Figure 21. Detection of luminescent *T. cruzi* in the internal organs of infected A/J mice . 116

Figure 22. Imaging of luminescent *T. cruzi* subjected to benznidazole *in vitro* 124

Figure 23. Schematic of proposed adoptive transfer strategy using fluorescent *T. cruzi* .. 129

Figure 24. Fluorescence microscopy of mCherry *T. cruzi* 131

Figure 25. Flow cytometric analysis of mCherry *T. cruzi* fluorescence distribution 133

Figure 26. Re-distribution of fluorescence intensity from single cell clone isolated from "high-expressing" gated population 135

1. INTRODUCTION

A. Chagas heart disease — History, Impact and Epidemiology

In 1908, untold numbers of slaves and laborers working the railroads connecting Rio, Brazil to the heart of the Amazon succumbed to malaria, yellow fever and other mysterious, undiagnosed illnesses. Having been previously successful at reducing malarial disease transmission in the Santos shipping industry four years earlier, Carlos Chagas was appointed the challenge of alleviating the infectious disease burden being faced in the Brazilian interior. Upon relocating to the undeveloped, rural area of Lassance, he encountered droves of individuals complaining about irregular heartbeats, atypical arrythmias, cardiac insufficiencies and inexplicable cases of sudden death. Chagas had received training in fields of public health and parasitology from renowned physician, Oswaldo Cruz, and wisely deduced a link between the endemicity of myocardial failure and the triatomine bug. While unheard of along the more developed Brazilian coast, these large black insects would often emerge from cracked mud walls and thatch roofs to feed on the blood of inhabitants throughout the night. They were often referred to as “kissing bugs” for the trademark swollen bite sites often left near the eyelids and lips of their victims. Upon dissection of the triatomine bug, Chagas discovered a eukaryotic, flagellated protozoan similar to *Trypanosoma brucei*, earlier identified as the agent of African sleeping sickness. After finding this parasite in the bloodstream of young girl who had experienced fever, lymphadenopathy, hepatosplenomegaly and heart failure prior to death, after being been bitten by the reduvvid bug, Chagas confirmed the link between his novel trypanosome discovery and disease by infecting

monkeys with triatomine droppings and observing identical clinical symptoms. Chagas named the protozoan after his mentor, *Trypanosoma cruzi*, and the associated disease eventually bore his own name.

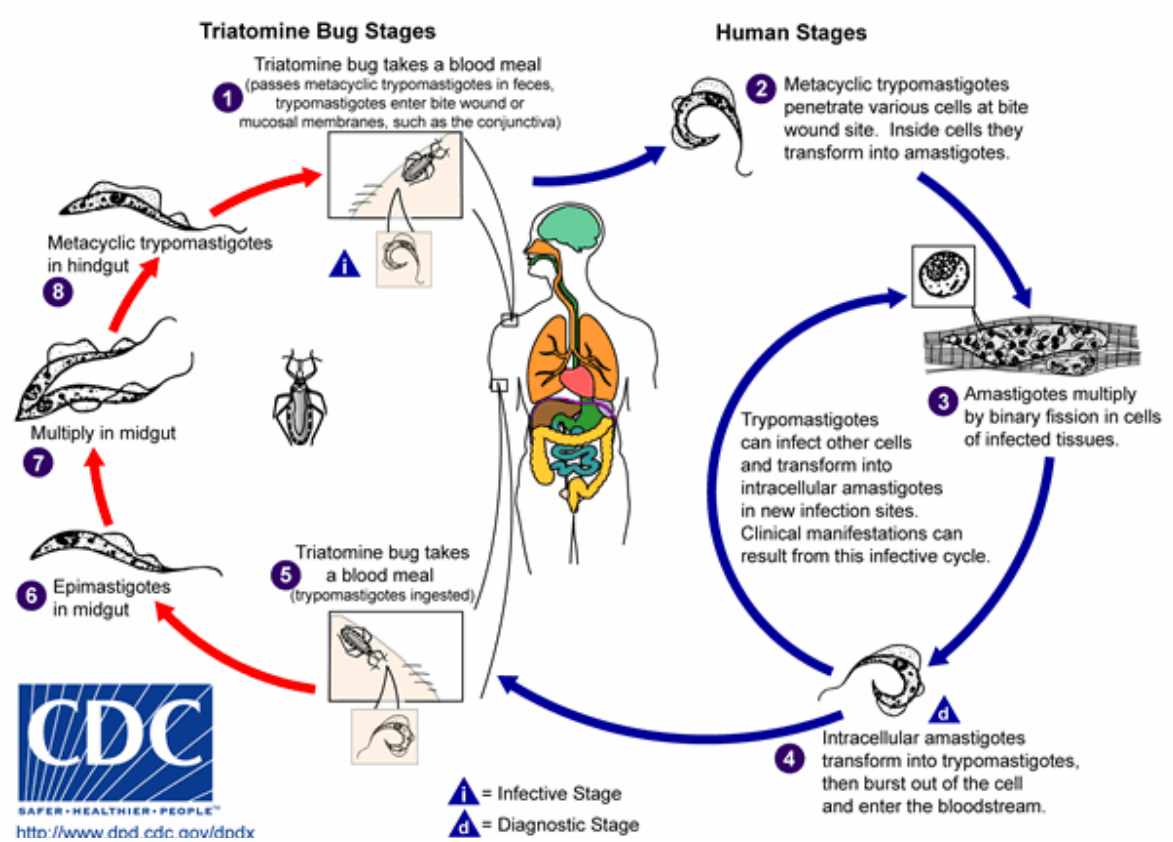
After nearly a century of its identification, Chagas disease remains a significant public health issue and a major cause of suffering and death in Latin America. The Centers for Disease Control estimates that 8 – 11 million people in Mexico, Central and South America have Chagas disease and many are unaware they are even infected (http://www.cdc.gov/chagas/factsheet.html). The large numbers of currently infected individuals, along with the estimated 100 million at risk in 21 countries and approximate 50,000 annual fatalities, make *T. cruzi* infection one of the leading causes of heart disease and cardiovascular-related deaths in endemic areas (1-3). Public health efforts geared toward limiting vectorborne transmission have significantly reduced the number of newly infected individuals, but the cases now being identified outside of the typical endemic regions from increasing incidences of blood transmission (4) and organ transplantation (5) still make Chagas one of the most important diseases to understand due to its history of morbidity and mortality (6). Despite its obvious clinical importance and the efforts of many investigators, the pathogenesis of Chagas heart disease is still elusive due to the complex nature of the host-parasite interrelationship and numerous pathogenic mechanisms that have been proposed over the last century of research.

B. *Trypanosoma cruzi* — Life cycle and Transmission

The life cycle of *T. cruzi* involves two intermediate hosts (triatomine insects and mammals) and three well-defined morphological and functional developmental stages: epimastigotes, trypomastigotes and amastigotes (7). As illustrated in Figure 1, the epimastigote forms replicate in the midgut of the reduviid bug insect vector and develop into nonreplicative metacyclic trypomastigote forms residing in the vector hindgut. When the insects feed on blood, they release their excreta containing metacyclic trypomastigotes that subsequently penetrate the mammalian host through either scratching of the bite wound or permissive mucosa or conjunctival membranes and initiate cellular invasion. Trypomastigotes survive the acidic parasitophorous vacuole and freely enter the host-cell cytoplasm where they differentiate into the replicative amastigote form. Following many rounds of multiplication by binary fission, the cell cytosol fills with amastigotes which ultimately transform into bloodform trypomastigotes. A fully parasitized cell will then rupture, releasing trypomastigotes to the blood stream where they can either infect adjacent cells, disseminate through the blood, or be taken up by a new reduviid bug, thus completing the cycle. A less common, yet increasingly significant, route of parasite transmission is through transfusion of blood products (8-10). As such, Chagas disease has become a potential problem associated with migration of infected individuals from endemic areas to the United States, Canada, Eastern Europe, Australia and Japan (11). Fortunately, the appropriate selection of blood donors, the use of more sensitive and accurate advanced molecular diagnostic tests and the application of a mandatory quality assurance system have improved the safety of blood banks in Latin American and have reduced the overall risk of acquisition of blood-borne Chagas disease (12, 13). Although reported in Latin American countries where Chagas disease is prevalent in those of reproductive age, congenital transmission of *T. cruzi* is

Figure 1. ***Trypanosoma cruzi* life cycle and transmission.**

The transmission and life cycle of *T. cruzi* starts when a triatomine vector, or reduvvid bug, defecates onto the skin of a mammalian host while taking a blood meal. Either through the bite wound itself, or through mucous membranes, metacyclic trypomastigotes present in the bug feces enter the host. Following the invasion of a host cell, trypomastigotes transform to amastigotes capable of mulitple rounds of replication via binary fission. After about 5 days of reproduction, amastigotes differentiate back into trypomastigotes which rupture the cell membrane. This release back into the host bloodstream enables the infection of adjacent cells or ingestion by the triatomine vector from a bloodmeal. Once inside the midgut of the vector, ingested trypomastigotes differentiate into epimastigotes capable of replication. The epimastigotes gradually transform into metacyclic trypomastigotes which are transmitted to the mammalian host. Illustration adapted from Centers for Disease Control (http://www.dpd.cdc.gov/dpdx).


Triatomine Bug Stages
Human Stages
Triatomine bug takes a blood meal
(passes metacyclic trypomastigotes in feces, trypomastigotes enter bite wound or mucosal membranes, such as the conjunctiva)
1
2
Metacyclic trypomastigotes penetrate various cells at bite wound site. Inside cells they transform into amastigotes.
Metacyclic trypomastigotes in hindgut
8
i
3
Amastigotes multiply by binary fission in cells of infected tissues.
Multiply in midgut
7
Trypomastigotes can infect other cells and transform into intracellular amastigotes in new infection sites. Clinical manifestations can result from this infective cycle.
Triatomine bug takes a blood meal
(trypomastigotes ingested)
5
6
Epimastigotes in midgut
d
4
Intracellular amastigotes transform into trypomastigotes, then burst out of the cell and enter the bloodstream.
CDC
SAFER·HEALTHIER·PEOPLE™
http://www.dpd.cdc.gov/dpdx
i = Infective Stage
d = Diagnostic Stage

categorized as an uncommon route of transmission, along with organ transplantation (14), oral transmission (15), laboratory accidents and accidental self-inoculation by hospital personel.

C. Acute and chronic Chagas disease

There are typically two stages of infection in human Chagas heart disease: the acute stage which occurs shortly after the infection and the chronic stage which appears after a silent period that may last many years. The acute stage of the disease, generally seen in children, is characterized by fever, lymphadenopathy and hepatosplenomegaly, muscle and joint pains, malaise, respiratory disturbances and local inflammation at the site of infection. Focal cardiac inflammation and heart enlargement, attributed to mononuclear cell, mast cell and neutrophil infiltration, has also been observed (16). In nearly 95% of cases, clinical symptoms are either absent or mild and non-specific (6), making it difficult to diagnose disease in the acute stage of infection. In instances when symptoms manifest, less than 5% of individuals can succumb to infection, typically of either myocarditis or meningoencephalitis.

More commonly, acute cases with or without symptoms progress to a chronic stage, where *T. cruzi* establishes a lifelong, low-grade infection which can present in any age group (6). Interestingly, two thirds of individuals harboring chronic parasite infection, often termed “indeterminate”, fail to demonstrate any detectable clinical signs and do not die of Chagas disease. However, in about one-third of cases (17), a chronic form of disease develops, causing irreversible damage to the heart, esophagus and colon, with dilatation and disorders of nerve conduction of these organs. The infiltrate in chronic Chagas heart disease primarily consists of lymphocytes with lower numbers of macrophages, eosinophils, plasma cells, neutrophils and

mast cells (18). While studies on myocardial biopsy fragments from chronic Chagasic patients indicate a predominance of CD8+ over CD4+ T cells (19, 20), *T. cruzi* infection also causes a decrease in expression of lymphocyte surface molecules including CD3, CD8, and CD4 in order to circumvent host immunity (21). Questions remain pertaining to the cytokine environment produced during chronic infection. While some argue that heart-infiltrating T cells yield only a significant production of IFN-γ and TNF-α, contributing to IL-12 synthesis and control of the infection (22), others claim that macrophage IL-10 production facilitates the replication and survival of the pathogen (23, 24). Interestingly, parasites are rarely found in the hearts of chronic Chagasic patients, yet parasite DNA can be detected in some inflammatory lesions (25). Through an uncertain mechanism, myocyte destruction continues throughout the course of disease, causing the gradual accumulation of fibrosis and decreased contractility of the heart. The diminished muscle mass, rhythm irregularity (arrhythmia or ventricular tachycardia) (26), and ultimate heart failure is the leading cause of death in chronic Chagas patients (27). In fact, 10% of all *T. cruzi* infected patients will die from refractory, end-stage heart failure or severe arrhythmia (26, 27), giving chronic Chagas disease patients a shorter survival and worse prognosis than cardiomyopathies of non-inflammatory etiology (28).

Current chemotherapeutic approaches for the specific treatment of Chagas disease are considered to be unsatisfactory because of frequent toxic side effects and overall limited efficacy, particularly in the chronic form of the disease. In fact, the irreversible nature of the diminished cardiac contractility observed in the chronic phase of Chagas makes heart transplantation the only viable therapeutic option (29). The frequent side effects of currently accepted treatments, benznidazole and nifurtimox, likely result from bystander reductive or

oxidative damage in mammalian tissues that is intended to specifically exploit the deficiency of detoxification mechanisms in *T. cruzi*. While the use of these nitroderivatives has had limited success in the treatment of acute infection, physicians have been hesitant to prescribe such treatment since complete eradication of *T. cruzi* is uncommon using such measures (30). When employed for the treatment of chronic Chagas disease, these therapies were unable to prevent lesions of the heart and digestive tract and had no impact on mortality after 10 years of administration (31). Unfortunately, rather than prescribing what is clearly an insufficient treatment for chronic Chagas, physicians are forced to treat symptoms as they appear instead of the disease itself.

D. Experimental Chagas heart disease

A variety of animal models of Chagas disease have been employed in order to address a number of issues including mortality, immune function, cardiac pathology, chemotherapeutic agents and autoimmunity. Among the animals analyzed are dogs (32, 33), monkeys, rabbits (34), hamsters (35-37) and more commonly rats (38-43) and mice (44-52). A number of parasite strains and clones (*e.g.*, Silvio, Brazil, Tulahuen, Y, Colombian, Corpus Christ, etc.) have been used to infect a variety of strains of mice (*e.g.*, BALB/c, C3H, A/J, DBA/2, etc.). While no single parasite-mouse combination recapitulates the entire spectrum of human infection—for instance, experimentally infected mice seldom develop end-stage heart failure during chronic infection—each combination does seem to reflect some particular aspect of the disease, including acute or chronic myocarditis and in some cases the indeterminate, or asymptomatic, infection. As such, interpretations can then be made based on the aspect of interest, rather than Chagas disease as a

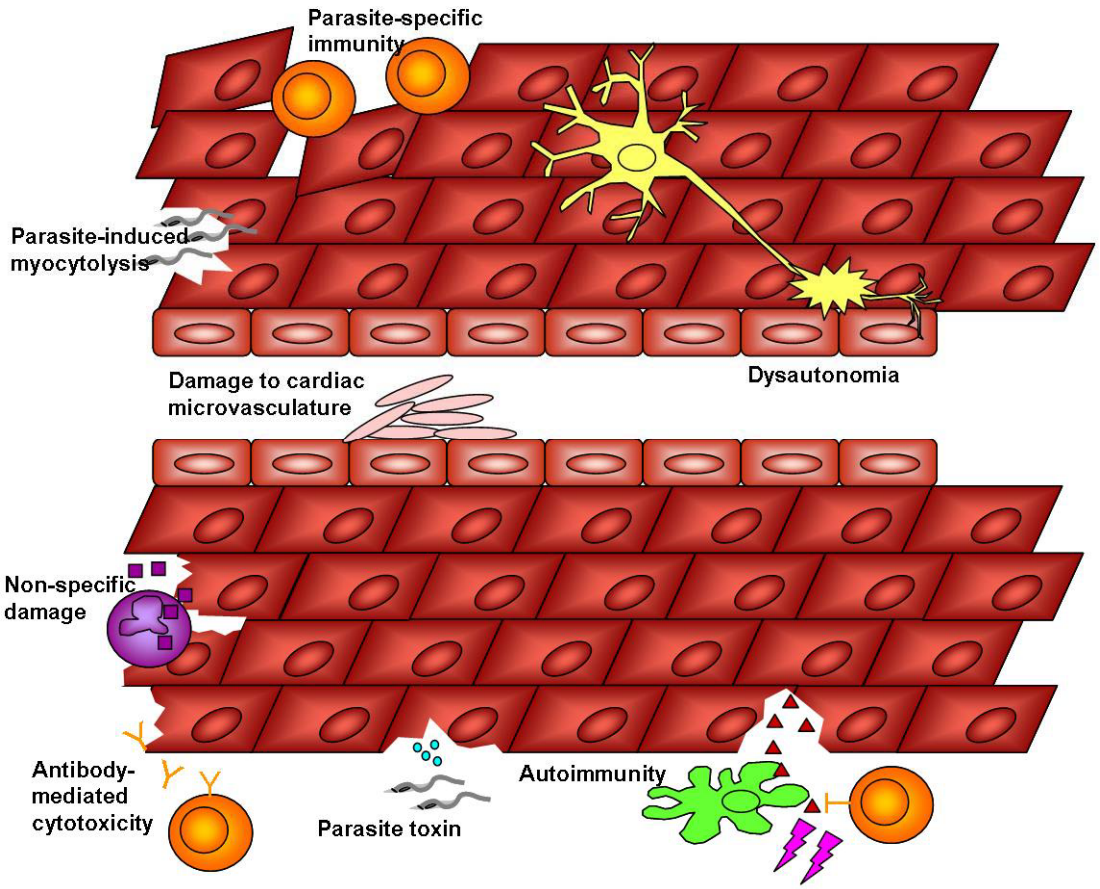
whole. One of the critical points in validating any aspect of Chagas disease is the use of animal models that reproduce most components of the human disease. The most widely used models of *T. cruzi* infection-induced myocarditis today are (i) BALB/c mice infected with the Colombian strain for a 150-240 days (17) for the chronic phase of infection, (ii) C3H mice infected with the Silvio X-10/4 clone (53, 54) and (iii) A/J mice infected with the Brazil strain for 7-30 days (55, 56) for the acute phase, though *T. cruzi* related pathogenesis has been investigated in other systems as well. Although the term "acute" is given to the A/J-Brazil model, it is based solely on the amount of time taken to observe disease and does not necessarily reflect a direct model of acute human infection any more than the BALB/c-Colombian model represents chronic human Chagas disease. In fact, the relevance of animal models to human disease is an issue for nearly all experimental models. Nevertheless, the consistent development of parasite-induced cardiac autoimmunity and the short term of disease development make the "acute" model very attractive for addressing specific potential mechanisms of *T. cruzi*-induced Chagas disease.

E. **Proposed mechanisms of Chagas disease pathogenesis**

Biventricular enlargement, apical aneurysm and diffuse interstitial fibrosis, lymphocytic infiltrate and myocytolysis of cardiac tissue are common observations made in the examinations of patients who die of Chagas disease-induced heart failure. A variety of explanations have been proposed for this damage observed in Chagas heart disease, as illustrated in Figure 2. The nature of *T. cruzi* infection makes *parasite-induced myocytolysis* the most obvious explanation for tissue damage since host cells burst to release bloodform trypomastigotes into circulation.

Figure 2. **Potential mechanisms of Chagas disease pathogenesis**.

There are several proposed mechanisms for Chagas disease pathogenesis. Chagas may be caused by *parasite-induced myocytolysis* from the repeated process of cardiomyocyte invasion, replictation and release, *damage to the cardiac microvasculature* via impaired blood perfusion and subsequent platelet aggregation, *dysautonomia* of cardiac tissue consisting of overactivation of sympathetic and neurohormonal pathways, *cardiotoxin* (hemolysin) *secretion by the parasite, non-specific damage* induced by infiltrating neutrophils and/or eosinophils, cardiac-specific *antibody-mediated cytotoxicity*, or immune responses generated against the *parasite* or *host* (*autoimmunity*). These potential mechanisms are not mutually exclusive and pathogenesis likely occurs as a result of a combination of several. Illustration adapted from (57).


Parasite-specific immunity
Parasite-induced myocytolysis
Damage to cardiac microvasculature
Dysautonomia
Non-specific damage
Antibody-mediated cytotoxicity
Parasite toxin
Autoimmunity

Mechanisms of *microvascular disturbances,* where hypoperfusion in areas of altered cardiac wall motion results in heart tissue damage (58-61), *destruction of heart neuronal tissue,* briefly reviewed in (62), and *toxin secretion by the parasite* (63) have also been suggested to play a role in Chagas pathogenesis. From a more immunological perspective, mechanisms of *antibody-mediated cytotoxicity* and *nonspecific neutrophil- and eosinophil-induced damage* (64, 65), *parasite-specific immune responses to T. cruzi antigens persistent in the heart tissue* (66-70), and *autoimmunity* have all received experimental support. Although generally controversial among investigators (65, 71-79), this last mechanism suggests that cardiac damage, regardless of its initial cause, leads to a breakdown in self-tolerance and autoimmunity induction. Importantly, none of these proposed mechanisms can be considered to be mutually exclusive and it is very likely that the combination of viable parasite-induced myocardial damage, parasite antigen-specific immunity *and* autoimmunity contribute to the inflammation and heart failure in Chagas disease in a coordinated manner.

F. Infection-induced autoimmunity

Autoimmune diseases are frequently considered to be the consequence of aberrant immune responses against pathogens (80, 81) when the immune system continues to elicit damage to self-proteins rather than properly shutting down. Immune responses against harmless antigens can cause allergic diseases, including rhinitis, atopic dermatitis and allergic asthma. However, in several instances the immune system can initiate severe autoimmune disease in response to a pathogen. Persistent infection with Theiler's murine encephalomyelitis virus (TMEV) induces a chronic demyelinating disease of the central nervous system (CNS) characteristic of human

multiple sclerosis (82, 83). Other viruses, including coxsackievirus types B3 (84) and B4 (85, 86) and encephalomyocarditis virus (ECMV) (87), have been shown to persist in their target organs (either the viral RNA or infectious virus) whereby autoimmune disorders of myocarditis or diabetes gradually occur. Both streptococcal and borrelia bacterial strains have also been implicated in the development of rheumatic fever or heart disease (88-90) and Lyme arthritis (91), respectively. Despite animal models showing direct evidence of infection-induced autoimmunity, including those established for *T. cruzi*-induced autoimmunity, there is no real understanding of the underlying mechanism(s).

G. The autoimmune hypothesis for Chagas heart disease

In its simplest terms *autoimmunity* can be defined as an immune reaction directed against an organism's own proteins. Specifically, a healthy body is equipped with a powerful set of tools for resisting the onslaught of invading microorganisms (such as viruses, bacteria, and parasites). Through a number of potential mechanisms, the immune system occasionally goes awry and attacks the body itself, resulting in the production of autoantibodies or T lymphocytes reactive with host antigens. The concept of autoimmunity as the cause of human illness in particular is relatively new, and it was not accepted into the mainstream of medical thinking until the 1950s and 1960s. *Autoimmune diseases* are defined as diseases in which the progression from *benign* autoimmunity to *pathogenic* autoimmunity occurs.

The concept of Chagas disease having an autoimmune etiology, officially put forth in the mid-1970s (92), stemmed from the consistent observations, made over many years of investigation, of cardiac pathology, including inflammation and fibrosis, in the virtual absence of

T. cruzi. Early discoveries of parasite and host crossreactive antibodies in chronic Chagas patients, along with the ability to block host-specific antibodies with *T. cruzi* antigens (93, 94) strongly encouraged further exploration of the basis of autoimmunity for the disease (95). Since this early finding of autoantibodies in Chagas disease, many studies have identified cellular aspects of cardiac autoimmunity. In addition, the notion of parasites being absent from chronic Chagasic tissue lesions have been repeatedly challenged by investigators claiming a more vital importance for parasite persistence and its derived immunity in the pathogenesis of disease. Both perspectives, with corresponding criticisms, and the possible involvement of other relevant mechanisms have been nicely summarized in several reviews (24, 65, 75, 78, 79, 96-100).

Original postulates set forth by Witebsky state that (i) an autoimmune response be recognized in the form of autoantibody- or cell-mediated immunity, (ii) the corresponding antigen be identified and (iii) an analogous autoimmune response be induced in an experimental animal (101). While these criteria for autoimmunity have been clearly fulfilled via several experimental models and human disease, they do not account for the delineation between benign and pathogenic types. Accordingly, Rose and Bona later expanded on these principles by establishing criteria for defining a disease as 'autoimmune'. They include (i) <u>direct evidence</u> from transfer of pathogenic antibodies or T cells, (ii) <u>indirect evidence</u> based on reproduction of the autoimmune disease in experimental animals and (iii) <u>circumstantial evidence</u> from clinical clues (*e.g.*, mononuclear cell infiltrate in affected tissue/organ, high serum IgG antibody levels) (102). Again, both indirect and circumstantial evidence of pathogenic autoimmunity in Chagas heart disease have been clearly illustrated by a number of investigators, but direct evidence in this context is lacking, rendering the autoimmune hypothesis still largely hypothetical.

The overall clinical significance and potential implications of Chagas heart disease being an autoimmune disease are serious. If the autoimmune responses typically observed in Chagas patients were the undeniable root cause of pathogenesis, the development of *T. cruzi*-specific chemotherapy would be insufficient, since the self-propogated immune response to host tissue would be unblocked. From a preventative standpoint, any attempts to develop an effective vaccine against *T. cruzi* would need to acknowledge that the selected antigens were incapable of eliciting any anti-host responses due to crossreactivity. Even if shown to be negative for the induction of such responses, regulatory healthcare authorities would surely continue to be hesistant to approve a vaccine with the mere potential of inducing chronic myocarditis in what could be millions of recipients. As such, the determination of the importance of autoimmunity in the context of *T. cruzi* infection, whether it be simply a benign artifact or a potent contributor to pathogenesis, is critical to the advancement of Chagas treatment.

H. Evidence for autoimmunity in Chagas heart disease

The hypothesis that Chagas disease is an autoimmune disease requires that (i) *T. cruzi* infection induces autoimmunity and (ii) this autoimmunity is pathogenic. While the first condition has been extensively proven using multiple experimental models (17, 55, 73, 75, 77), the second is considered unsubstantiated and continues to exist as a matter of debate (65, 72, 99). Autoantibodies against a variety of heart, skeletal muscle and nervous tissue, including ribosomal P proteins (103, 104), cardiac myosin (105, 106), β1 adrenergic receptor (107, 108), cytoskeletal microtubule associated proteins (109), LIST neuronal proteins (110, 111), and a novel mammalian protein termed Cha (112) have been identified in *T. cruzi* infections (see Table

1). However, little evidence adequately shows these antibodies are more prevalent in patients with Chagas disease than in asymptomatic, infected individuals. From a cellular perspective, T cell clones from chronic Chagasic patients have been shown to proliferate in culture with either human cardiac myosin peptide or the B13 parasite antigen (113), suggestive of molecular mimicry. Despite the wide array of immunological findings, the clinical relevance of humoral and cellular immunity is unclear.

As one potential component of Chagas pathogenesis, investigators have examined the contribution of autoantibodies to disease. *T. cruzi*-induced autoantibodies alter the contraction and cell signaling of cardiac myocytes and lyse myocytes through an antibody dependent cytotoxicity mechanism *in vitro* (114, 115). Immunization of mice with *T. cruzi* antigen, cruzipain, also induced autoantibodies to myosin thought to be pathogenic due to conduction abnormalities observed in both the mother and pups (116). However, transfer of autoantibodies from an infected donor to naïve recipient has failed to induce disease in animal models. It is also noteworthy that levels of anti-myosin antibodies are significantly increased in patients with heart damage resulting from causes other than *T. cruzi* infection such as myocardial infarction, coronary artery bypass, heart valve surgery, and viral myocarditis (117, 118). Based on these reports, it would not seem unreasonable to infer that cardiac tissue damage resulting from tissue infection, possibly through a bystander effect, could cause the level of anti-myosin immunity to rise in Chagas heart disease.

Several lines of evidence also support a role for cellular autoimmunity in Chagas heart disease. Splenocytes harvested from chronically infected mice elicit lysis of syngeneic myoblasts *in vitro* and induce electrocardiographic abnormalities when transferred to a naïve

Table 1. Host proteins to which autoimmunity develops during *T. cruzi* infection.

Cell, molecule or substance	Host*	Immune mediator
Cardiac myosin	M	CD4+ T cells
Cardiac myosin, p150	M	Serum IgG
Heart homogenate	M	T cells
43 kDa muscle glycoprotein	M	Serum IgG
Nervous tissue, heart, skeletal muscle	M	Serum IgG
2nd extracellular loop, M2 cholinergic receptor	M	Serum IgG
2nd extracellular loop, beta1 adrenergic receptor	M	Serum IgG
M2 cholinergic receptor	H	Serum IgG
M2 cholinergic receptor	H	Serum IgG
M2 muscarinic acethylcoline receptor	H	Serum IgG
2nd extracellular loop, M2 cholinergic receptor	H	Serum IgG
Neurons	H	Serum IgG
Sciatic nerve homogenate	H	Serum IgG
Small nuclear ribonucleoprotein	H	Serum IgG
Cardiac myocytes	H	Complement (C5-C9 complex)
Heart homogenate	H	T cells
Cardiac myocytes	H	T cells
Cardiac myocytes	Rb	T cells

* M, mouse; H, human; Rb, rabbit

recipient (49). Perhaps the most compelling, yet controversial, finding from Ribiero dos Santos was that CD4+ T cells from chronically infected mice mediated the rejection of normal syngeneic newborn hearts transplanted into the ear of recipients (119). In theory, the syngeneic hearts should not have been targeted if there were not autoreactive cells specific to cardiomyocytes. However, the main criticism of this finding came later when an analogous model system was used for a similar analysis. In this model, Tarleton's group showed that *T. cruzi* was systematically present in the heart grafts undergoing rejection (25), raising questions of whether the rejection was strictly CD4+ T cell-mediated. His group claimed that it was the infiltration of live parasites, and subsequent immune responses against the pathogen that was responsible for the rejection rather than an autoimmune response. In addition, inconsistent results are observed when the parasite-mouse strain combination is changed. Ribiero dos Santos utilizes the considerably more invasive and persistent parasite strains (Y and Colombian) (119) while Tarleton employs the less virulent Sylvio X10/4 strain (25). The conflicting nature of these results may be explained by differences in the ability of individual *T. cruzi* strains to induce autoimmunity or differences in the susceptibility of particular mouse strains to develop autoimmunity. In an attempt to provide additional evidence for a role of CD4+ autoreactive T cells in Chagas pathogenesis, Pontes de Carvalho and colleagues assessed inflammation and fibrosis in the hearts of mice subjected to treatment with both a cardiac myosin-rich antigen emulsified in complete Freund's adjuvant (CFA) and an anti-CD4 cell monoclonal antibody (17) prior to infection. The purpose of the treatment was to induce immunological tolerance to cardiac myosin. Microscopic examination of heart tissue from *T. cruzi*-infected mice given this tolerization regimen displayed less intense inflammation than control mice receiving only anti-

CD4 treatment without myosin. However, tolerized mice produced unaltered levels of anti-cardiac myosin IgG. Since isotype switching is a T helper (CD4)-dependent event, the efficacy of tolerization became a matter of question, making the experimental results inconclusive. Finally, transfer of splenic T cells from chronically infected mice to naïve syngeneic recipients was found to induce heart lesions similar to those resulting from *T. cruzi* infection (120). Treatment was administered for the depletion of B cells, IgM, IgG and macrophages, but appropriate controls indicating an absence of parasitic contamination in the transfer were not performed, providing a basis for skepticism of the study.

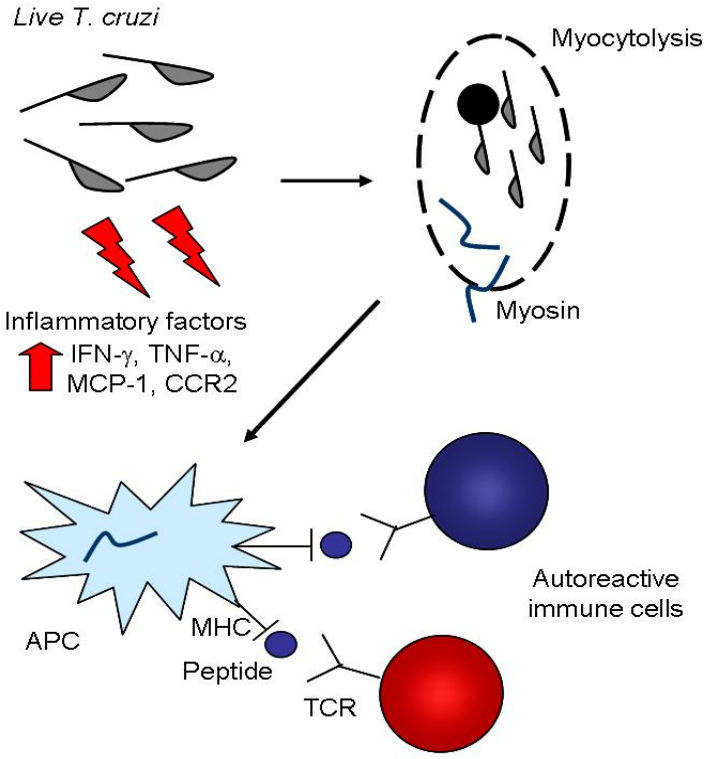
I. Proposed mechanisms of *T. cruzi*-induced cardiac autoimmunity

Although several mechanisms thought to play a potential role in the induction of autoimmunity following *T. cruzi* infection exist, *bystander activation* and *molecular mimicry* are considered the most widely investigated and supported (see Figure 3). Bystander activation requires the presence of viable parasites that are thought to elicit destruction of heart tissue and cause release of host antigens (121). The induction of autoimmunity in this instance is thought to be dependent on both the myocardial cytolysis and subsequent antigen release from infected host tissue, in addition to the inflammatory environment resulting from the presence of a pathogen (122). Ordinarily maintained, self-tolerance is breached as the excess of host antigen is released into a predisposed proinflammatory environment rich in cytokines, chemokines, and nitric oxide resulting from infection. Although not completely characterized, the inflammatory infiltrate in Chagas disease is known to consist of a predominance of CD8+ T cells that express granzyme, CD4+ T cells to a lesser extent and an elevated macrophage population (123). As a result of this

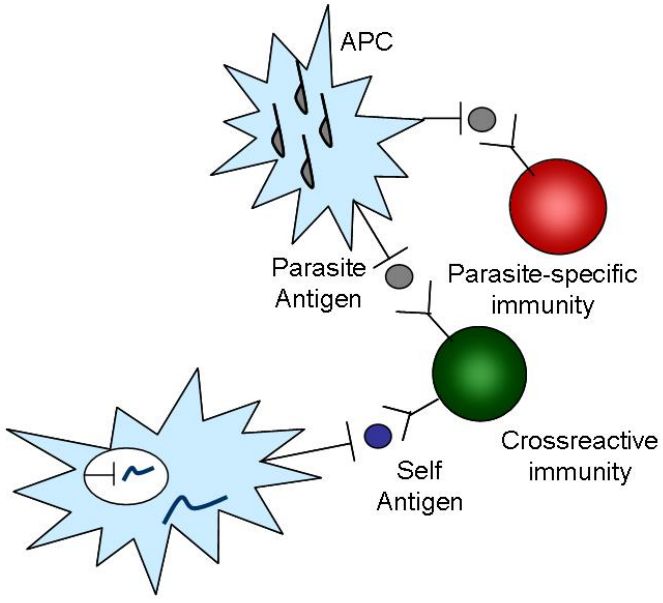
Figure 3. **Mechanisms of autoimmunity induction in *T. cruzi* infection**.

During *T. cruzi* infection, the lysis of cardiac myocytes causes the release of cardiac antigens, one of which is known to be myosin. The presence of a live infection induces elevated production of inflammatory factors such as chemokines (*e.g.* MCP-1, CCR2) and cytokines (e.g. IFN-γ, TNF-α, IL-10), contributing to the creation of a proinflammatory environment. *Bystander activation* states that live parasite-induced myocytolysis and antigen release into a predisposed proinflammatory environment can promote the activation of autoreactive T cells encountering self-peptide in the context of MHC. *Molecular mimicry* states that *T. cruzi* derived peptides immunologically resemble host peptides and initiate a cross-reactive T cell response leading to the activation of autoreactive T cells (regardless of proinflammatory environment or presence of live parasite). Although not the only potential mechanisms responsible for the induction of autoimmunity following *T. cruzi* infection, these are the most widely investigated.

Bystander Activation



Molecular Mimicry



infiltration, enhanced expression of adhesion molecules (human leukocyte antigen class I and II), chemokines and chemokine receptors (membrane cofactor protein (MCP)-1, CCR2 and CXCR3) and cytokines (IFN-γ, TNF-α, IL-6 and IL-10) has been reported (124). In fact, the high expression of inflammatory cytokines, particularly IFN-γ and TNF-α, has been closely associated with progressively severe cardiac disease (125, 126) and the differential production of these cytokines versus the more regulatory-type IL-10 is thought to be responsible for the pathogenic or asymptomatic outcomes observed after infection, respectively (124, 127). In support of bystander activation, there are several examples of autoimmunity occuring after cardiac damage including cardiac surgery (117), cardiac transplant rejection (128) and infection with viruses (129).

Molecular mimicry suggests that an infectious agent (parasite, bacteria or virus) displays epitopes immunologically resembling host determinants and due to minor antigenic differences between the two, the pathogen epitope is able to induce an immune response that breaks tolerance to the host epitope, resulting in autoimmunity (130). Though this mechanism has been attributed to a variety of cases of infection-induced autoimmunity, including streptococcus-induced myocarditis (90, 131) and even *T. cruzi*-induced Chagas disease (77, 113), the criteria set forth for claiming "true" molecular mimicry are rather stringent and often difficult to fulfill (76, 132). The criteria established to distinguish molecular mimicry from bystander activation can be summarized as: (i) a relationship between a specific microbial infection and a specific inflammatory state, (ii) the identification of responsible microbial and self-epitope capable of eliciting crossreactive T cell responses and (iii) a causal relationship between the existence of T cells elicited by the microbe and responsive to both microbe and self-epitopes and the particular

autoimmune disease. A number of crossreactive antigens with both cardiac and non-cardiac specificity have been identified, primarily by serologic approaches, in experimental models of Chagas disease (Table 2). In particular, the A/J mice infected with the Brazil *T. cruzi* strain has consistently provided evidence of cardiac myosin-specific autoimmunity in forms of both autoantibody production as well as cellular immunity (55, 56). Infection with live parasites or immunization of mice with *T. cruzi* lysate induced myosin-specific autoantibody production and delayed-type hypersensitivity even though this immunization repeatedly failed to induce heart inflammation. Interestingly, however, mice immunized with cardiac myosin develop *T. cruzi*-specific DTH and antibodies and myosin tolerization effectively suppresses *T. cruzi* DTH. Conversely, tolerization to *T. cruzi* (using parasite lysate) suppresses myosin DTH responses (133, 134). This induction of bidirectional, crossreactive immunity between *T. cruzi* and cardiac myosin was shown to be specific since such crossreactivity did not occur in *Leishmania* protein extract or skeletal myosin immunizations. A number of cases of immunological crossreactivity/antigenic mimicry between defined *T. cruzi* and host self-antigens have been described for human Chagas disease (reviewed in (122)). Regarding the importance of finding cardiac myosin-specific autoimmunity in the context of Chagas disease, it is important to reiterate that levels of anti-myosin antibodies are also found to be significantly increased in patients with heart damage from infectious and non-infectious causes (117, 118). Antigen exposure in a damaged, proinflammatory environment could cause the level of anti-myosin immunity to elevate in Chagas disease. However, the numerous and compelling findings of molecular mimicry indicate a potentially vital mechanism by which ongoing autoimmunity could propogate and contribute to cardiac pathogenesis.

Table 2. Molecular mimicry during *T. cruzi* infection or after immunization.

Cell, molecule or substance	*T. cruzi* antigen	Host*	Immune mediator§
Neurons, liver, kidney, testis	?	M, Rb	mAb
Neurons	?	Rb	mAb
Neurons	Sulphated glycolipids	H	mAb
Heart tissue	?	M	Serum IgG
Heart and skeletal muscle	Microsomal fraction	H	mAb
Human cardiac myosin heavy chain	B13 Protein	H	Ab, T cell
Human cardiac myosin heavy chain	Cruzipain	M	Ab
Cardiac myosin	*T. cruzi* lysate	M	Ab
95 kDa myosin tail	*T. cruzi* cytoskeleton	M	mAb
Smooth and striated muscle	150 kDa protein	H, M	Serum IgG
Glycosphingolipids	Glycosphingolipids	H, M	Serum IgG
MAP (Brain)	MAP	H, M	rDNA
Myelin basic protein	*T. cruzi* soluble extract	M	Serum IgG, T cell
28 kDa lymphocyte membrane protein	55 kDa membrane protein	H, M	mAb
47 kDa neuron protein	FL-160	H	rDNA
23 kDa ribosomal protein	23 kDa ribosomal protein	H	Ab
Ribosomal P protein	Ribosomal P protein	H	rDNA, Ab
Beta1-Adrenoreceptor, M2 muscarinic	Ribosomal P0 protein	H	rDNA, Ab
Beta1-Adrenoreceptor, M2 cholinergic	150 kDa protein	H, M	mAb
38 kDa heart antigen	R13 peptide from ribosomal P1, P2	M	IgG1, IgG2
Cardiac muscarinic acetylcholine receptor	?	H	Ab
Cha antigen	SAPA, 36 kDa TENU2845	M	Ab, T cell

*M, mouse; H, human; Rb, rabbit § rDNA, recombinant DNA, mAb, monclonal antibody; Ig, immunoglobulin

Other proposed mechanisms have included *cryptic epitope* and *polyclonal activation.* In the first instance, either parasite-induced damage leads to the release of previously sequestered epitopes or the inflammatory environment resulting from infection stimulates the immune processing and presentation of novel self epitopes. Immunity ensues against these *novel* epitopes because the host immune system has not previously established tolerance (135). In the case of *polyclonal activation*, specific *T. cruzi* strains stimulate proliferation of both T and B cells, regardless of antigen specificity and, in some cases, by interacting with surface molecules other than antigen receptors (136). Importantly, these mechanisms are certainly not mutually exclusive and are likely cooperatively functional. Once autoimmunity is initiated, other models of organ-specific inflammation have cited epitope spreading as a mechanism of autoimmunity propagation (137-142) which may also play a role in *T. cruzi*-induced autoimmunity.

J. Criticisms of the autoimmune hypothesis for Chagas heart disease

Several reasons have been cited for the skepticism regarding Chagas disease having an autoimmune etiology. Although the obvious presence of autoreactivity cannot be legitimately disputed, in light of substantial evidence that has accumulated throughout decades of study, the issue of referring to autoreactivity as pathogenic is frequently considered by many investigators questionable at best. In general, some investigators refuse to believe findings largely generated from murine models of Chagas disease. Although certainly a very common means of testing hypotheses, experimental murine models are not viewed as reliable recapitulations of pathological characteristics of human Chagas patients by those who largely study the human disease (143). In addition to this unavoidable shortcoming, a number of specific criticisms have

been cited regarding the interpretation of inconclusive findings. For example, one typical criticism pertaining to the adoptive transfer of autoimmunity to naïve recipients from infected donors is that appropriate measures to insure a complete absence of parasite material in the transferred population have not been adequately taken (65, 96). This would suggest that any potential parasitic contamination could be responsible for the induction of myocarditis, rather than the damage initiated by the autoreactive lymphocytes. Another common issue encountered is the frequent inability to reproduce *in vivo* what is observed in an *in vitro* system. While certain anti-cardiomyocyte antibodies have been capable of mediating antibody-dependent cellular cytotoxicity *in vitro*, these antibodies fail to cause *in vivo* lesions characteristic of chronically infected mice (65, 115). It is these reproducibility issues, insufficient or unconvincing data, and conflicts about T cell specificity or whether *T. cruzi* plays a role in the outcomes of transfer-based experiments that continue to contribute to the ongoing debates of the relevance of Chagas-related autoimmunity.

Proponents of the autoimmunity hypothesis for Chagas disease have traditionally garnered support from the observation that *T. cruzi* is virtually absent in tissue lesions from chronically infected individuals. However, as technology has become more advanced with enhanced sensitivity, many investigators have reported findings of both parasite antigen and DNA from infected tissues and even specific inflammatory lesions (144-148). Despite these findings, some investigators maintain that no correlation between the intensity of inflammation and parasitism has been adequately established (149, 150). The fact that chronic Chagasic patients can have parasites identified in other organs, free from significant inflammation, with concurrent myocardial inflammation and fibrosis (151) suggests that other immunological

mechanisms (such as organ specific autoreactivity) are likely involved in cardiac pathogenesis. Some proponents of the parasite persistence hypothesis will concede that other mechanisms, including autoimmunity, are probably contributors but importantly note that the absence of a direct correlation between the amount of *T. cruzi* antigen and the intensity of inflammation does not discount the obvious association between the presence of parasite antigen and severe or moderate inflammation (24). This clear association has been established in studies where the detection of *T. cruzi* antigen in 100% of hearts from chronic Chagasic patients that died of heart failure was made (24, 150). Still, other studies have shown that only a fraction of patients with progressive Chagas heart lesions displayed signs of detectable parasites (152). Some investigators will combine two theories of pathogenic mechanisms by suggesting that *T. cruzi* might serve as a necessary adjuvant for a persistent immunological crossreaction between common parasitic and myocardial antigens (153), in accordance with the molecular mimicry hypothesis of infection-induced autoimmunity.

K. Conclusions and overall significance

Why is it important to resolve the controversy surrounding autoimmunity as a relevant mechanism of pathogenesis in Chagas disease? If immune responses to *T. cruzi* antigens cross-react with epitopes of key host tissues, drugs targeted solely at eliminating the parasite would not necessarily suppress initial immune responsiveness to parasite antigen and subsequent host antigen. Alternatively, if autoimmunity proves not to be a root cause of pathogenesis in Chagas disease, attempts to develop chemotherapy and vaccines would merit encouragement and support. Although it seems obvious to assume that the presence of the parasite antigen in tissue

leads to the development of parasite-specific immunity, consequent tissue inflammation and heart failure, this has not been formally proven. Similarly, although *T. cruzi*-induced autoimmunity is a well established and widely investigated subject matter, its role in disease pathogenesis and the mechanism(s) by which it occurs remain obscure issues. The major obstacle of infection-induced models of organ-specific inflammation is the ability to attribute one specific mechanism to the overall process. The array of potential mechanisms responsible for myocarditis has brought about controversy regarding appropriate treatment for *T. cruzi* infection. While maintaining an open-minded approach to the investigation of autoimmunity in experimental Chagas disease, it is important to consider that pathogenesis likely results from a mixture of viable parasite-induced damage, parasite-specific immunity, *and* autoimmunity. The ultimate goal of any research endeavor should always be to gain further understanding of a specific process while ideally being able to apply discoveries to other related scientific questions. As such, the following chapters describe research designed to not only supplement the current knowledge of Chagas heart disease, but also to hopefully provide gradual insight into mechanisms of other similar infection-induced cardiomyopathies.

2. MODULATION OF AUTOIMMUNITY BY TREATMENT OF AN INFECTIOUS DISEASE

Kenneth V. Hyland,[1] Juan S. Leon,[2] Melvin D. Daniels,[1] Nick Giafis,[1] LaKitta M. Woods,[1] Thomas J. Bahk,[3] Kegiang Wang,[1] and David M. Engman[1*]

Departments of Microbiology-Immunology and Pathology and Feinberg Cardiovascular Research Institute, Northwestern University Feinberg School of Medicine, Chicago, Illinois [1]; *Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia* [2]; *Pediatric Critical Care of Arizona, Phoenix, Arizona* [3]

*Corresponding author. Mailing address: Northwestern University, Department of Pathology, Ward Building 6-175, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-1288. Fax: (312) 503-1265. E-mail: d-engman@northwestern.edu.

Running title: Regulation of cardiac autoimmunity through control of infection

A. Abstract

Chagas heart disease (CHD), caused by the parasite *Trypanosoma cruzi*, is the most common form of myocarditis in Central and South America. Some humans and experimental animals develop both humoral and cell-mediated, cardiac-specific, autoimmunity during infection. Benznidazole, a trypanocidal drug, is effective at reducing parasite load and decreasing the severity of myocarditis in acutely-infected patients. We hypothesized that the magnitude of autoimmunity that develops following *T. cruzi* infection is directly proportional to the amount of damage caused by the parasite. To test this hypothesis, we used benznidazole to reduce the number of parasites in an experimental model of CHD and determined whether this treatment altered the autoimmune response. Infection of A/J mice with the Brazil strain of *T. cruzi* leads to the development of severe inflammation, fibrosis, necrosis, and parasitosis in the heart accompanied by vigorous cardiac myosin-specific delayed-type hypersensitivity (DTH) and antibody production twenty one days post-infection. Mice succumb to infection within a month if untreated. Treatment of infected mice with benznidazole eliminated mortality and decreased disease severity. Treatment also reduced cardiac myosin-specific DTH and antibody production. Reinfection of treated mice with a heart-derived, virulent strain of *T. cruzi* or immunization with myosin led to the redevelopment of myosin-specific autoimmune responses and inflammation. These results provide a direct link between the levels of *T. cruzi* and the presence of autoimmunity and suggest that elimination of the parasite may result in the reduction or elimination of autoimmunity in the chronic phase of infection.

B. Introduction

Chagas disease is one of several diseases, along with *Leishmania*sis and African sleeping sickness, caused by parasites of the family *Trypanosomatidae*. Endemic to Central and South America, the parasite that causes Chagas disease, *Trypanosoma cruzi*, is the world's leading cause of myocarditis (1). The World Health Organization estimates that 16–18 million people are infected with *T. cruzi*, with about 100 million people at risk in 21 countries (2, 3). In spite of recent advances in the control of the vectorial and transfusional transmission of *T. cruzi* (154), Chagas disease remains a serious infectious disease in Latin America due to its prevalence, morbidity and mortality (6). Despite its obvious clinical importance and the efforts of many investigators, the pathogenesis of CHD is still incompletely understood.

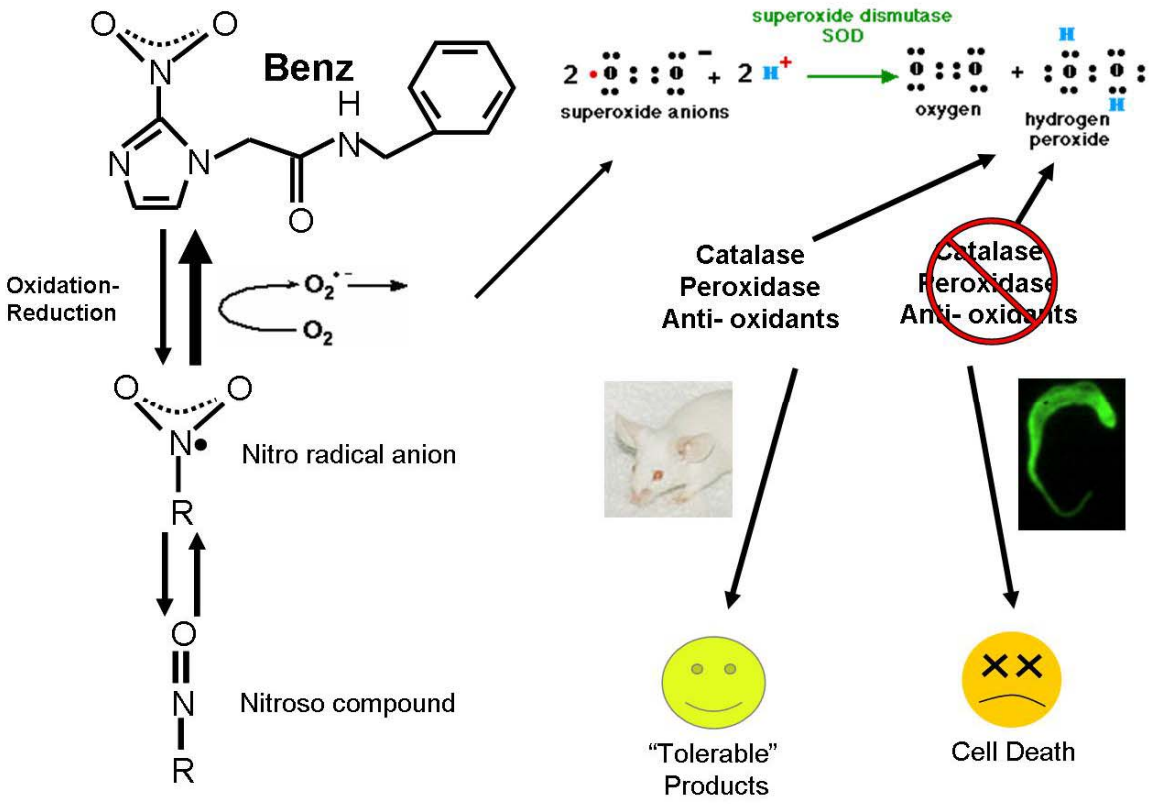
A variety of explanations have been proposed for the damage observed in CHD. Potential mechanisms include (i) toxin secretion by the parasite (63), (ii) damage to cardiac microvasculature (58), (iii) destruction of heart neuronal tissue (62), (iv) parasite-specific immune responses to *T. cruzi* antigens persistent in the heart tissue (66-68, 70, 155-158), (v) antibody-mediated cytotoxicity and nonspecific neutrophil- and eosinophil-induced damage (64, 65) and (vi) autoimmunity (55, 75, 98-100, 133). Although autoimmunity is defined simply as an immune reaction against an organism's own proteins, the progression from *benign* to *pathogenic* autoimmunity, resulting in disease, is a distinction often overlooked. It is this distinction which has spurred controversy among investigators (65, 71-77, 98-100, 159), about the significance of autoimmunity in disease pathogenesis. This mechanism suggests that *T. cruzi*-induced cardiac damage and/or molecular mimicry between parasite and host leads to a breakdown in self-

tolerance resulting in eventual autoimmune tissue damage. Our overall hypothesis is that the combination of viable parasite-induced myocardial damage, parasite antigen-specific immunity *and* autoimmunity contribute to the inflammation and heart failure in CHD.

Although *T. cruzi*-induced autoimmunity has been a matter of considerable investigation (55, 75, 103-113, 143), its role in disease pathogenesis and the mechanism(s) by which it develops remain unclear. Here, we address the role of viable parasites in the induction and persistence of autoimmunity in A/J mice by the administration of benznidazole, a nitroheterocyclic drug employed in the chemotherapy of human Chagas disease (160). Although the mechanism of drug action is not entirely understood, it is thought that when *T. cruzi* metabolizes benznidazole, its lack of catalase and peroxidase enzymes hinder its ability to dispose of newly generated free radicals (161) (Figure 4). The presence of a full repertoire of antioxidant enzymes provides mammals the ability to cope with the drug more effectively, though not without detrimental side effects including abdominal pain, diarrhea, nausea and vomiting. Benznidazole exerts a number of effects on the host immune response to *T. cruzi* infection, including the enhancement of macrophage-associated phagocytosis and proinflammatory cytokine production (162), the selective expansion of effector and memory CD8+ T lymphocytes (163) and the decrease of both P-selectin and vascular cell adhesion molecule-1 levels (164). In addition, host immune factors including interleukin 12 (165) and interferon-γ (166) are important for maximum efficacy of benznidazole therapy during infection. The proven trypanocidal activity of benznidazole, along with its ease of administration in the drinking water, enabled us to efficiently reduce the number of parasites at various times during infection.

Figure 4. Mechanism of action for trypanocidal drug, benznidazole.

As the nitroheterocyclic compound, benznidazole, is metabolized by both the host and parasite, a series of oxidation-reduction reactions lead to the production of nitro radical and superoxide anions. Both the host and parasite contain enzymes (superoxide dismutases) capable of converting these anions to oxygen and hydrogen peroxide, but while the mammalian host has peroxidases and catalases to further process these compounds to tolerable products (not without substantial side effects), the parasite lacks these enzymes, ultimately resulting in their death.


Benz
Oxidation-
Reduction
Nitro radical anion
Nitroso compound
superoxide dismutase
SOD
superoxide anions
oxygen
hydrogen
peroxide
Catalase
Peroxidase
Anti- oxidants
Catalase
Peroxidase
Anti- oxidants
"Tolerable"
Products
Cell Death

Benznidazole treatment administered within the first week of infection reduced the magnitude of myosin-specific cellular and humoral immunity compared to untreated controls 21 days post-infection (d.p.i.) with *T. cruzi*. Since mice succumb to disease 30 d.p.i. in our experimental CHD model, we were unable to include infected-untreated controls for long-term experiments. However, by comparing immune responses to baseline uninfected-treated animals we could make conclusions pertaining to the change in myosin-specific autoreactivity at later time points. Using this basis of comparison we observed that the initiation of treatment within the first or second week of infection eliminated myosin-specific cellular autoimmunity at 60 or 90 days d.p.i., respectively. All stages of disease displayed an overall decrease in inflammation and complete absence of parasites in the heart tissue. Finally, after terminating drug treatment, reinfection with *T. cruzi* or immunization with cardiac myosin led to the restoration of strong myosin-specific immunity and inflammation in mice, indicating that cardiac autoimmunity can be regulated indirectly by modulating the levels of the parasite.

C. Materials and Methods

Experimental animals and *T. cruzi* infections.

Four- to six-week old male A/J mice (Jackson Laboratories, Bar Harbor, ME) were housed under specific pathogen-free conditions. Mice were infected by intraperitoneal injection of 1×10^4 Brazil strain *T. cruzi* trypomastigotes derived from infection of tissue culture H9C2 rat myoblasts (American Type Culture Collection, Manassas, VA). A cardiotropic substrain of the Brazil strain of *T. cruzi* was isolated from the heart of an infected mouse and propagated in H9C2 rat myoblasts to generate trypomastigotes for use in reinfection experiments. This strain has since been maintained and termed "Brazil heart" strain. Parasitemias were measured by hemacytometry from tailbleeds. Uninfected controls received intraperitoneal injections of Dulbecco's phosphate buffered saline (GibcoBRL, Grand Island, NY) of equal volume. Mice were anesthetized by a single intraperitoneal injection of sodium pentobarbital (60 mg/kg) for each experimental manipulation. The use and care of mice were conducted in accordance with the guidelines of the Center for Comparative Medicine at Northwestern University.

Preparation of myosin and *T. cruzi* antigen.

Cardiac myosin heavy chains and *T. cruzi* antigen were prepared as described previously (55). Briefly, hearts were rinsed in ice cold saline, minced, homogenized and stirred for 90 minutes at 4^oC. Muscle residue was removed by centrifugation for 10 minutes at $12{,}000 \times g$. The supernatant was then centrifuged at $140{,}000 \times g$ for 4 hours. This supernatant was added to cold water and precipitate was allowed to settle overnight. The cloudy precipitate was centrifuged for

15 minutes at 12,000 × *g*, after which the myosin pellet was homogenized and redissolved. Actin contamination was removed by centrifugation for 30 minutes at 43,000 × *g* and myosin was reprecipitated overnight in cold water. This procedure was repeated to remove actomyosin and myosin was finally redissolved in a glycerol buffer. The final myosin concentration was determined by Bradford assay and SDS-PAGE analysis.

Induction of autoimmune myocarditis.

Mice were immunized with purified cardiac myosin (300 μg) in an emulsion of complete Freund's adjuvant (CFA, Difco, Detroit, MI) in a total volume of 0.1 ml. Three subcutaneous sites in the dorsal flank were injected with equal amounts. Seven days later, mice were boosted in an identical manner.

Drug treatment.

Benznidazole (Roche Chemical and Pharmaceutical Products, South America, Sao Paulo, Brazil) was administered in the drinking water of *T. cruzi* infected, myosin-immunized or saline-injected mice at a concentration of 100 mg/kg/day as described (163, 167). Treatment was initiated and terminated at various time points post-infection.

Histopathology.

Hearts were removed, rinsed with saline, and fixed for 24 hours in 10% buffered formalin. Fixed hearts were embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined by light microscopy. Two sections were taken from each heart, one including both atria and the

other both ventricles. Each section was examined for evidence of mononuclear and polymorphonuclear cell infiltration, necrosis and mineralization, *T. cruzi* pseudocysts (parasitosis), and fibrosis and was assigned a histologic score of between 0 (no involvement noted) to 4 (100% involvement), with 1, 2, 3 representing 25, 50, and 75% involvement of the histologic section (134).

Serologic analysis.

Levels of cardiac myosin-specific and *T. cruzi*-specific IgG were determined by ELISA as described (55). Endpoint dilution titers for total IgG were defined as the highest serum dilution that resulted in an absorbance value (OD_{450}) of two standard deviations above the mean of a negative control sample (pooled sera from uninfected mice) included on every plate.

Delayed-type hypersensitivity (DTH).

Myosin-specific and *T. cruzi*-specific (DTH) was quantified using a standard ear swelling assay (55). Antigen-induced ear swelling was the result of mononuclear cell infiltration and exhibited typical DTH kinetics (*i.e.*, minimal swelling at 4 hours, maximal swelling at 24-48 hours post-injection).

Statistical analyses.

DTH values were $\log_{10}$ transformed prior to statistical analyses if they were not normally distributed. For comparison of two groups, the significance of DTH values was analyzed by Student's t-test. For comparison of multiple groups to a control, the significance of DTH values

was analyzed by a one-way ANOVA followed by adjustment for multiple comparisons by the Dunnett test (post-hoc analysis). The control group for comparison is specified in each figure legend. Antibody values were not normally distributed and so were analyzed for significance by the Mann-Whitney U test. Values of $p < 0.05$ were considered significant unless otherwise specified.

D. Results

Early parasiticidal drug treatment reduces cardiac myosin-specific autoimmunity and cardiac inflammation, and reduces tissue parasitosis in acute *T. cruzi* infection.

Autoimmunity is only one mechanism of Chagas disease pathogenesis which may develop as a result of antigenic molecular mimicry or bystander activation after parasite-induced tissue destruction. In either case, levels of the parasite might correlate with the presence of autoimmunity. The goal of the present study was to explore this relationship. To test the association of autoimmunity and levels of *T. cruzi*, we employed our experimental model of CHD in which A/J mice are infected with the Brazil strain of *T. cruzi*, leading to the development of cardiac inflammation, fibrosis, necrosis, and parasitosis accompanied by vigorous cardiac myosin-specific DTH and antibody production 21 d.p.i. and mortality within 30 d.p.i. (55). We administered the trypanocidal drug benznidazole (100 mg/kg/day) to infected mice at different times post-infection to ascertain the association between levels of parasite, myocarditis, and autoimmunity. All of the experiments included in this paper are diagrammed in Figure 5. Groups of infected/untreated and saline-injected/benznidazole-treated mice were included as controls. The results that follow are representative of three separate experiments.

Treatment initiated during the first week of infection (*i.e.*, at 0, 2 or 7 d.p.i.) significantly reduced myosin-specific DTH and antibody titers (Figures 6A and 7). In general, levels of myosin-specific DTH were lower when benznidazole administration was begun earlier. Histopathologic examination of heart sections from these animals revealed reduction of inflammation and absence of parasitosis compared to untreated control animals (Figure 6B). The

Figure 5. Schematic of benznidazole experimental regimen.

Experimental regimens used in this chapter. *T. cruzi* infected, myosin-immunized, or PBS immunized mice were treated with a curative dose of 100 mg/kg/day of benznidazole in the drinking water for the course of disease indicated (d21, d60, d90). Times during which benznidazole was administered are indicated with a crosshatched line and those when no treatment was given are indicated with a simple line. Untreated, control mice (infected or immunized) received only water. Days post-infection (d.p.i.) when benznidazole treatment was initiated or mice were sacrificed for analysis (black circle containing white A) are indicated. Analyses included DTH, antibody assays, and cardiac histology. A large group of infected mice treated with benznidazole beginning at 14 d.p.i. was divided into three subgroups at 90 d.p.i. One subgroup was reinfected with *T. cruzi*, another was immunized with PBS, and a third was immunized with myosin. Additional control groups were included as indicated. Single dagger associated with the infected, untreated group indicates that the mice were sacrificed because they do not survive past 30 d.p.i.

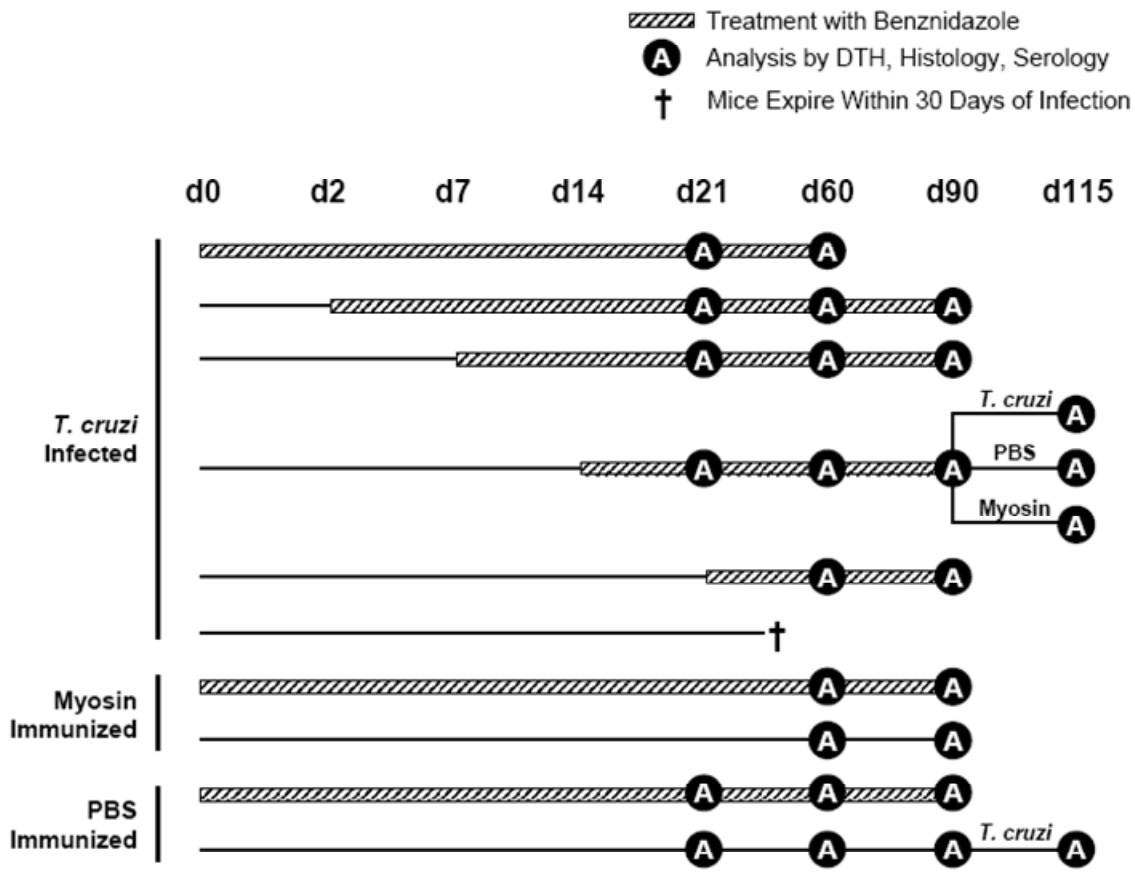

Treatment with Benznidazole
Analysis by DTH, Histology, Serology
Mice Expire Within 30 Days of Infection
d0
d2
d7
d14
d21
d60
d90
d115
T. cruzi Infected
T. cruzi
PBS
Myosin
Myosin Immunized
PBS Immunized
T. cruzi

Figure 6. Early benznidazole treatment reduces myosin-specific DTH and inflammation and eliminates parasitosis in acute *T. cruzi* infection.

T. cruzi infected and saline-injected A/J mice were treated with a curative dose of benznidazole (at d.p.i. indicated) for the course of disease. Untreated, infected mice were also included (Untreated). (A) At 21 d.p.i. myosin-specific and *T. cruzi*-specific DTH responses were measured by a 24-hour ear swelling assay (168). Error bars indicate standard errors of the mean for a minimum of three animals. (B) Inflammation and parasitosis were assessed by analyzing hematoxylin and eosin stained heart sections. Each dot corresponds to one animal. * $p < .05$ relative to untreated control group. See Figure 5 for schematic of experimental design.

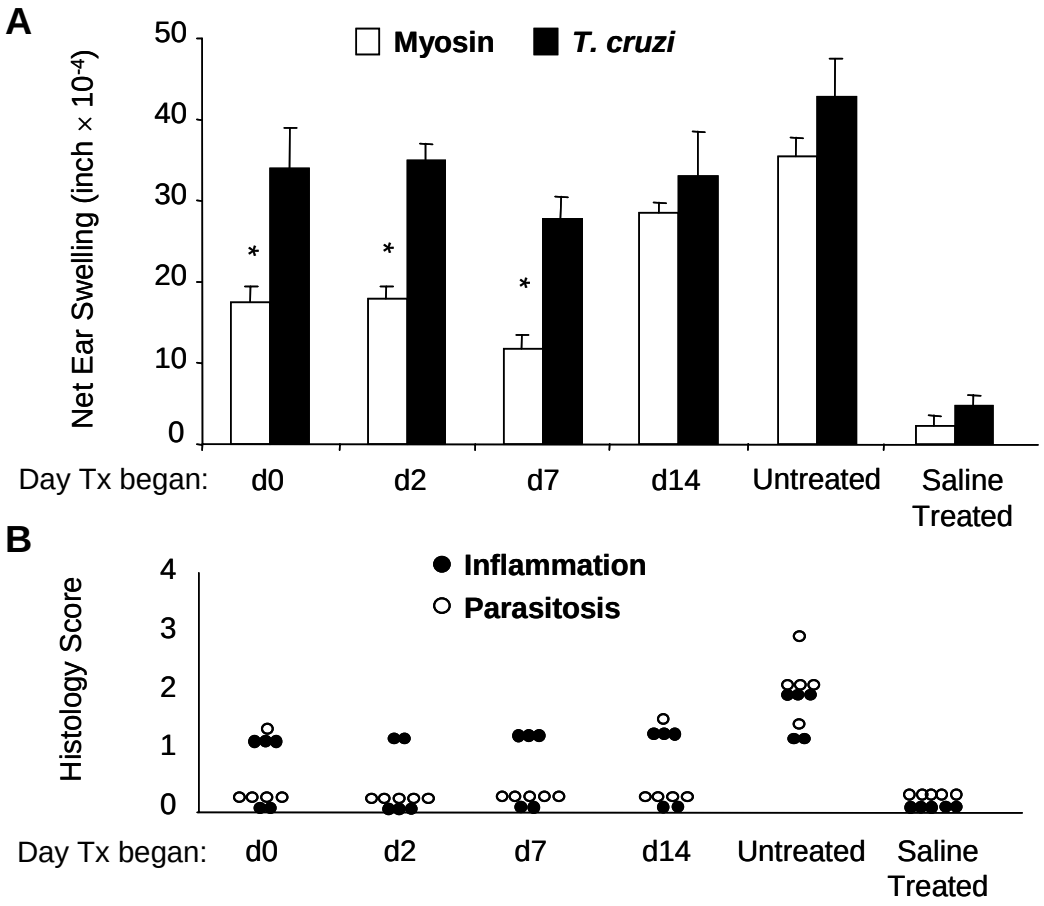
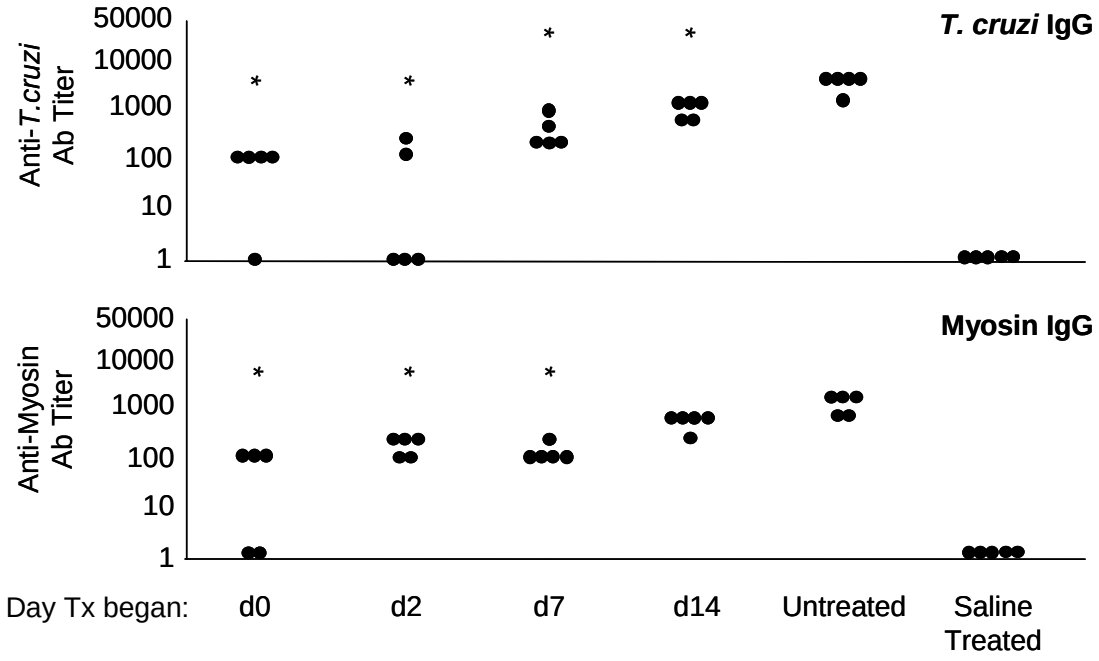

A
Myosin
T. cruzi
Net Ear Swelling (inch × 10-4)
50
40
30
20
10
0
*
*
*
Day Tx began:
d0
d2
d7
d14
Untreated
Saline Treated
B
Inflammation
Parasitosis
Histology Score
4
3
2
1
0
Day Tx began:
d0
d2
d7
d14
Untreated
Saline Treated

Figure 7. Early benznidazole treatment reduces anti-myosin and *T. cruzi*-specific antibody production in acute *T. cruzi* infection.

T. cruzi infected and saline-injected A/J mice were treated with a curative dose of benznidazole (at d.p.i. indicated) for the course of disease. Untreated, infected mice were also included (Untreated). At 21 d.p.i. myosin-specific and *T. cruzi*-specific IgG antibody titer values were determined by ELISA. Each dot corresponds to one animal. * $p < .05$ relative to untreated control group. See Figure 5 for schematic of experimental design.


T. cruzi IgG
Anti-T.cruzi Ab Titer
50000
10000
1000
100
10
1
Myosin IgG
Anti-Myosin Ab Titer
50000
10000
1000
100
10
1
Day Tx began:
d0
d2
d7
d14
Untreated
Saline Treated

cardiac inflammation in all benznidazole-treated animals, however, was consistent despite clear differences in cardiac autoimmunity. As expected, the number of parasites found in the blood was also significantly lower in animals in which treatment was begun within the first week of infection (data not shown). Interestingly, benznidazole treatment caused a significant reduction in parasite-specific antibody production, yet had no significant impact on *T. cruzi*-specific cellular immunity (Figures 7 and 6).

Drug treatment administered into the chronic phase of *T. cruzi* infection (60 or 90 d.p.i.) results in the elimination of myosin-specific cellular autoimmunity.

For the purpose of this study, we use the term “acute” to refer to disease present at 21 d.p.i. of A/J mice with the Brazil strain of *T. cruzi* and “chronic” to refer to disease present after animals reach 60 d.p.i. For such long term experiments, infected, untreated controls cannot be used, since A/J mice do not survive past 30 d.p.i. with the Brazil strain of *T. cruzi*. To control for the effects of benznidazole on myosin-specific DTH and antibody production, myosin immunized mice treated with benznidazole were examined to determine the possible effects of the drug on myosin autoimmunity (Figures 8-11). There was no effect, indicating that any effect of the drug on autoimmunity in infected mice would be related to its antiparasitic activity and not to an immunomodulatory action.

Drug treatment in infected mice was initiated at time points indicated (Figure 5) for all infected animals and maintained until 60 or 90 d.p.i., at which time DTH and antibody levels were ascertained and cardiac histopathology was assessed. Since we were unable to maintain viable infected, untreated control animals at 60 and 90 d.p.i. time points we established a

baseline (negative control) using uninfected, healthy animals expected to yield negligible, if not a complete absence of, autoreactivity. If our experimental group produced autoimmunity not significantly different (using appropriate statistical parameters) from negative controls, we referred to the autoimmunity associated with this particular group as “eliminated.” We observed that the myosin-specific DTH in infected mice at 60 d.p.i. was similar to that observed at 21 d.p.i. (Figures 8 and 6A). In both cases, earlier initiation of benznidazole treatment led to lower myosin-specific DTH than did later benznidazole treatment. However, to our surprise, we found that myosin-specific DTH was eliminated in infected/treated mice (Figure 8). In other words, myosin-specific DTH levels in mice treated within the first week of infection were not significantly different from those in saline controls. At 60 d.p.i., myosin-specific antibodies were present at low levels, which did not differ significantly with the timing of benznidazole initiation (Figure 9). Interestingly, earlier initiation of benznidazole treatment also led to lower levels of *T. cruzi*-specific DTH than did later initiation. *T. cruzi*-specific antibody levels were high and did not differ among the benznidazole-treated groups. At 60 d.p.i., both cardiac inflammation and tissue parasitosis were eliminated in all infected, treated animals (data not shown). We also observed *T. cruzi*-specific DTH in myosin-immunized mice, in agreement with previous findings (133).

Similar to our findings at 60 d.p.i, we found that myosin-specific DTH was absent at 90 d.p.i. in mice that had been treated within the first two weeks of infection (Figure 10). Myosin-specific antibody levels were also low and were absent in some animals in which treatment was begun early (Figure 11; d7 and d14). We also observed that *T. cruzi*-specific DTH levels were lower than those seen at 21 d.p.i., although *T. cruzi*-specific antibody levels were high and did

Figure 8. Initiating benznidazole treatment within the first week of infection eliminates myosin- and *T. cruzi*-specific DTH at 60 d.p.i.

T. cruzi- infected and saline-injected A/J mice were treated with a curative dose of benznidazole beginning at the indicated d.p.i.. Mice immunized with myosin/CFA, with or without benznidazole treatment, were also included (right). Infected, untreated control animals were not included since they expire within 30 d.p.i. At 60 d.p.i., myosin-specific and *T. cruzi*-specific DTH responses were measured by a 24-hour ear swelling assay (168). Error bars indicate standard errors of the mean for a minimum of three animals . * $p < .05$ relative to saline-injected, treated control animals. See Figure 5 for schematic of experimental design.

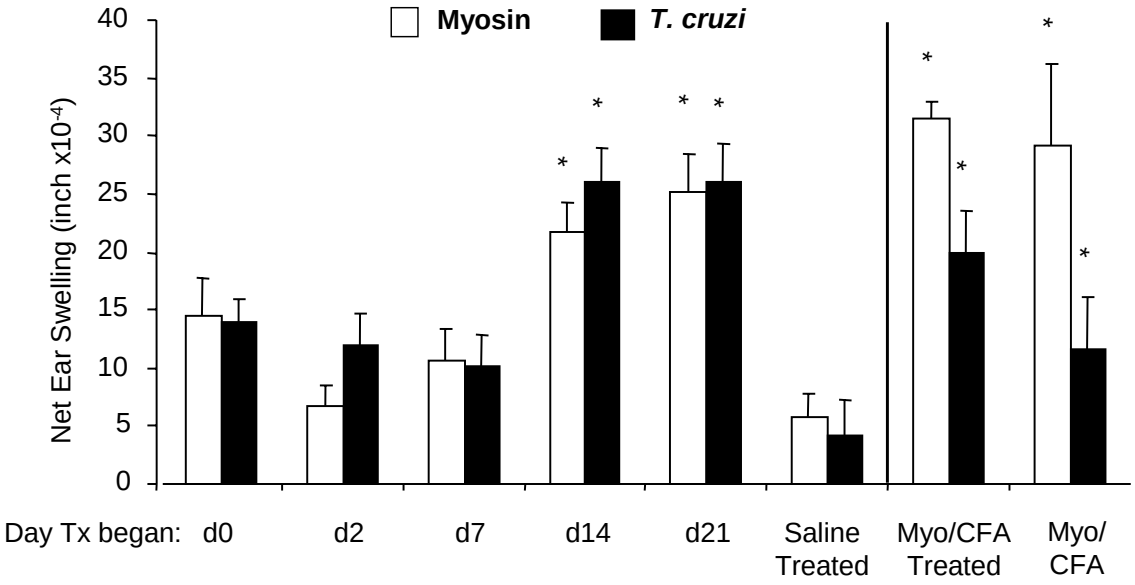

Myosin
T. cruzi
Net Ear Swelling (inch x10-4)
40
35
30
25
20
15
10
5
0
Day Tx began:
d0
d2
d7
d14
d21
Saline Treated
Myo/CFA Treated
Myo/ CFA

Figure 9. Initiating benznidazole treatment within the first week of infection has no significant effect on IgG production at 60 d.p.i.

T. cruzi- infected and saline-injected A/J mice were treated with a curative dose of benznidazole beginning at the indicated d.p.i.. Mice immunized with myosin/CFA, with or without benznidazole treatment, were also included (right). Infected, untreated control animals were not included since they expire within 30 d.p.i. At 60 d.p.i., *T. cruzi*-specific and myosin-specific IgG titers were determined by ELISA. Each dot corresponds to one animal. * $p < .05$ relative to saline-injected, treated control animals. See Figure 5 for schematic of experimental design.

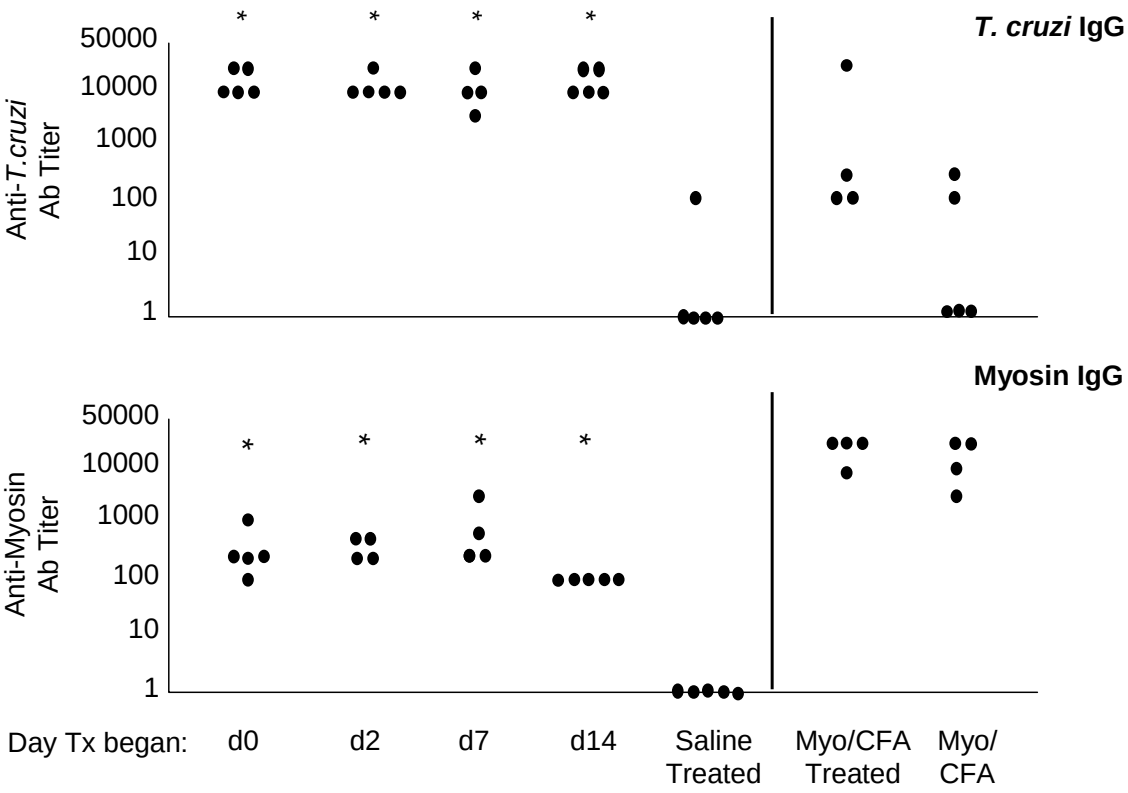

T. cruzi IgG
Anti-T.cruzi Ab Titer
50000
10000
1000
100
10
1
Myosin IgG
Anti-Myosin Ab Titer
Day Tx began:
d0
d2
d7
d14
Saline Treated
Myo/CFA Treated
Myo/ CFA

Figure 10. Initiating benznidazole treatment within 2 weeks of infection results in an absence of myosin-specific and a reduction of *T. cruzi*-specific DTH at 90 d.p.i.

T. cruzi infected and saline-injected A/J mice were treated with a curative dose of benznidazole beginning at the indicated d.p.i.. Mice immunized with myosin/CFA, with or without benznidazole treatment, were also included (right). Infected, untreated control animals were not included since they expire within 30 d.p.i. At 90 d.p.i., myosin-specific and *T. cruzi*-specific DTH responses were measured by a 24-hour ear swelling assay. Error bars indicate standard errors of the mean for a minimum of three animals.. * $p < .05$ relative to saline-injected, treated control animals. # no statistical significance since only two mice involved. See Figure 5 for schematic of experimental design.

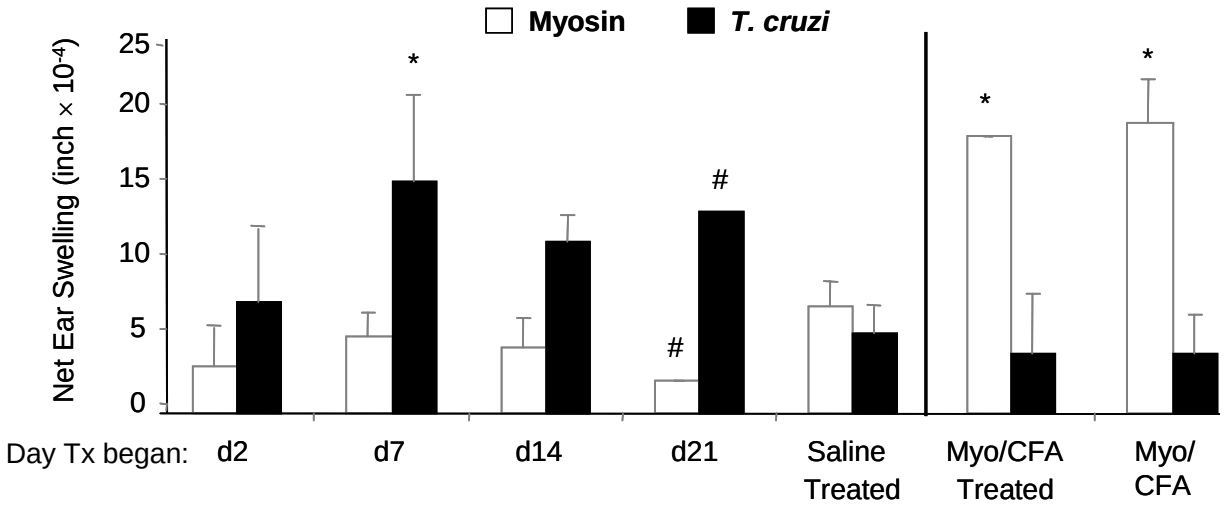
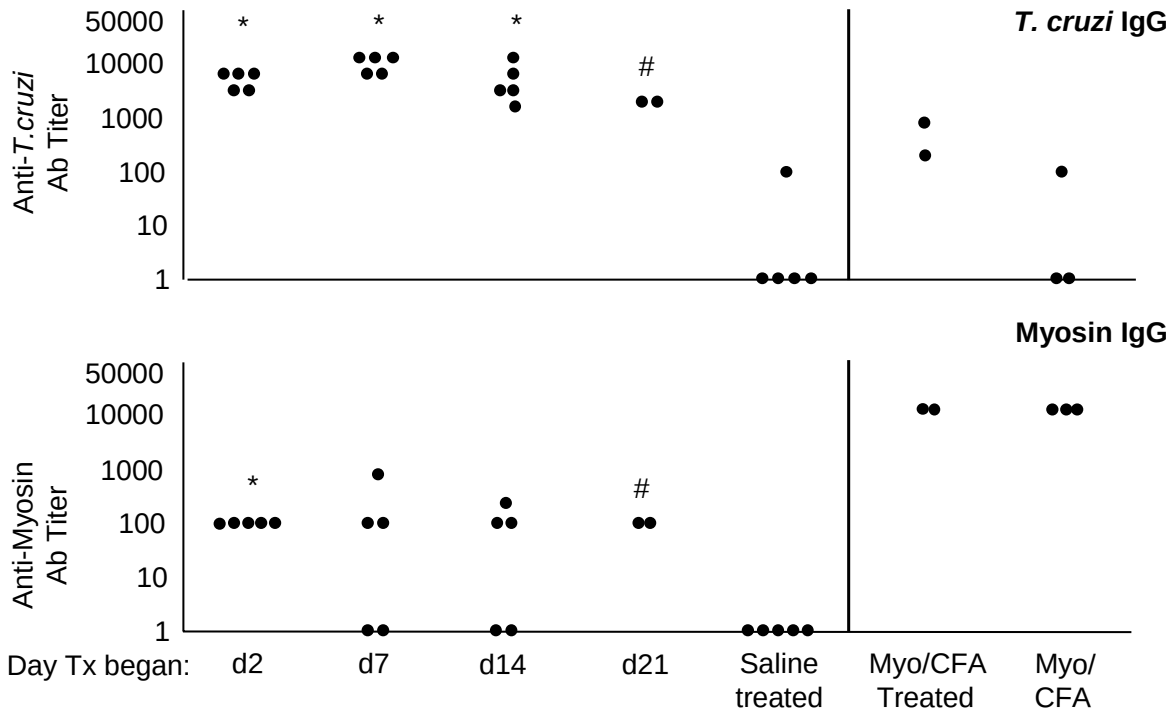

Myosin
T. cruzi
Net Ear Swelling (inch × 10-4)
25
20
15
10
5
0
*
*
*
#
#
Day Tx began:
d2
d7
d14
d21
Saline Treated
Myo/CFA Treated
Myo/ CFA

Figure 11. Initiating benznidazole treatment within 2 weeks of infection reduces myosin-specific, but has no significant effect on *T. cruzi*-specific IgG production at 90 d.p.i.

T. cruzi infected and saline-injected A/J mice were treated with a curative dose of benznidazole beginning at the indicated d.p.i.. Mice immunized with myosin/CFA, with or without benznidazole treatment, were also included (right). Infected, untreated control animals were not included since they expire within 30 d.p.i. At 90 d.p.i., *T. cruzi*-specific and myosin-specific IgG titers were determined by ELISA. Each dot corresponds to one animal. * $p < .05$ relative to saline-injected, treated control animals. # no statistical significance since only two mice involved. See Figure 5 for schematic of experimental design.


T. cruzi IgG
Anti-T.cruzi Ab Titer
50000
10000
1000
100
10
1
*
*
*
#
Myosin IgG
Anti-Myosin Ab Titer
50000
10000
1000
100
10
1
*
#
Day Tx began:
d2
d7
d14
d21
Saline treated
Myo/CFA Treated
Myo/ CFA

not differ among treatment groups. At 90 d.p.i., cardiac inflammation and parasitosis were absent from all infected, treated animals (data not shown). In contrast to what was observed at 60 d.p.i., *T. cruzi*-specific DTH was eliminated in myosin immunized mice.

Secondary *T. cruzi* infection or myosin-immunization induces cardiac autoimmunity and myocarditis in benznidazole-treated mice.

Finally, we investigated the ability of a secondary *T. cruzi* infection or a noninfectious cardiac insult to initiate a cardiac autoimmune response and/or myocarditis in animals that had been treated with benznidazole and no longer exhibit myosin-specific DTH. Since we observed the complete disappearance of inflammation, parasitosis and myosin-specific DTH in *T. cruzi*-infected mice that had received long-term treatment with benznidazole, we began with these animals for the experiment. After continuous daily treatment of infected mice from 14 d.p.i. to 90 d.p.i., we terminated drug treatment and either re-infected mice with a virulent, heart-derived substrain of the *T. cruzi* Brazil strain, immunized mice with cardiac myosin, or injected mice with saline. As an additional control, we also infected naïve mice that had received benznidazole for 90 days. Twenty-five days later (115 d.p.i.) we found that re-infection or myosin-immunization of these mice resulted in the restoration of both cellular and humoral cardiac autoimmunity and mild myocarditis (Figures 12-14). The magnitude of autoimmunity and the severity of myocarditis in re-infected animals were not as great as seen in animals infected for the first time (Figures 6 and 7). Despite the absence of parasitosis observed after re-infection, parasite-specific DTH and antibody levels were induced to high levels (Figures 13 and 14). Interestingly, in a separate experiment, treated mice reinfected with the original Brazil *T. cruzi*

Figure 12. *T. cruzi* reinfection or myosin immunization of infected, benznidazole-treated mice restores cardiac inflammation but not parasitosis.

Mice were infected with *T. cruzi* or injected with saline and treated with benzinidazole from 14 days d.p.i. through 90 d.p.i. At 90 d.p.i., treatment was terminated and groups of animals were immunized with saline, immunized with myosin, primarily infected with *T. cruzi*, or re-infected with *T. cruzi* as indicated. (A) Twenty-five days later inflammation and parasitosis were assessed by analyzing hematoxylin and eosin stained heart sections. Each dot corresponds to one animal. (B) Representative cardiac sections from histologic analysis of mice at 115 d.p.i. Mice primarily infected with *T. cruzi* after PBS injection and no drug treatment until day 90 developed myocardial inflammation and necrosis with the presence of parasite pseudocysts (right). Mice infected with *T. cruzi* and treated with benznidazole until 90 d.p.i. and then immunized with myosin developed severe myocarditis and fibrosis (center). Mice infected with *T. cruzi* and treated with benznidazole until 90 d.p.i. and then immunized with PBS had normal cardiac histology (left). See Figure 5 for schematic of experimental design.

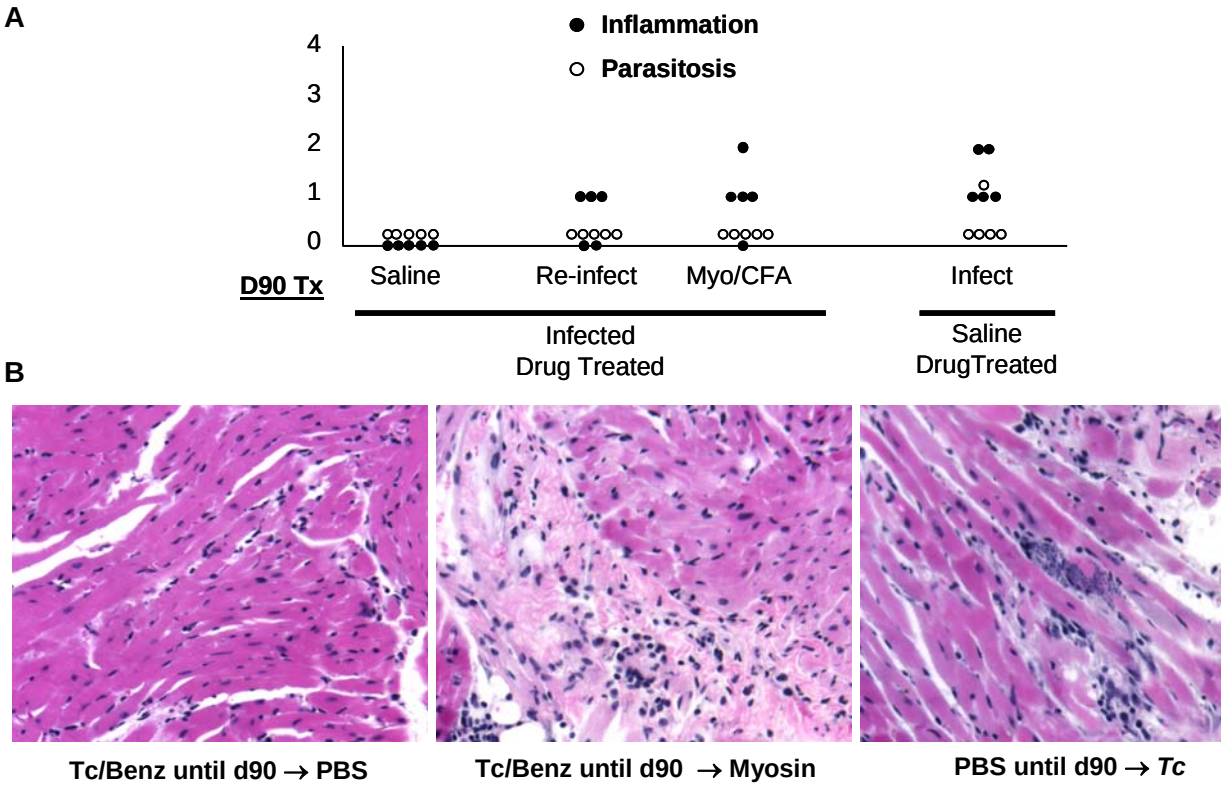

A
Inflammation
Parasitosis
4
3
2
1
0
D90 Tx
Saline
Re-infect
Myo/CFA
Infect
Infected
Drug Treated
Saline
DrugTreated
B
Tc/Benz until d90 → PBS
Tc/Benz until d90 → Myosin
PBS until d90 → Tc

Figure 13. *T. cruzi* reinfection or myosin immunization of infected, benznidazole-treated mice restores myosin-specific cellular autoimmunity.

Mice were infected with *T. cruzi* or injected with saline and treated with benzinidazole from 14 days d.p.i. through 90 d.p.i. At 90 d.p.i., treatment was terminated and groups of animals were immunized with saline, immunized with myosin, primarily infected with *T. cruzi*, or re-infected with *T. cruzi* as indicated. Twenty-five days later (115 days after the initiation of the experiment), myosin-specific and *T. cruzi*-specific DTH responses were measured by a 24-hour ear swelling assay. Error bars indicate standard errors of the means for a minimum of three animals. Statistics are provided relative to the infected, treated, group immunized with saline at 90 d.p.i. (* $p < .05$, ¥ $p = .057$). See Figure 5 for schematic of experimental design.

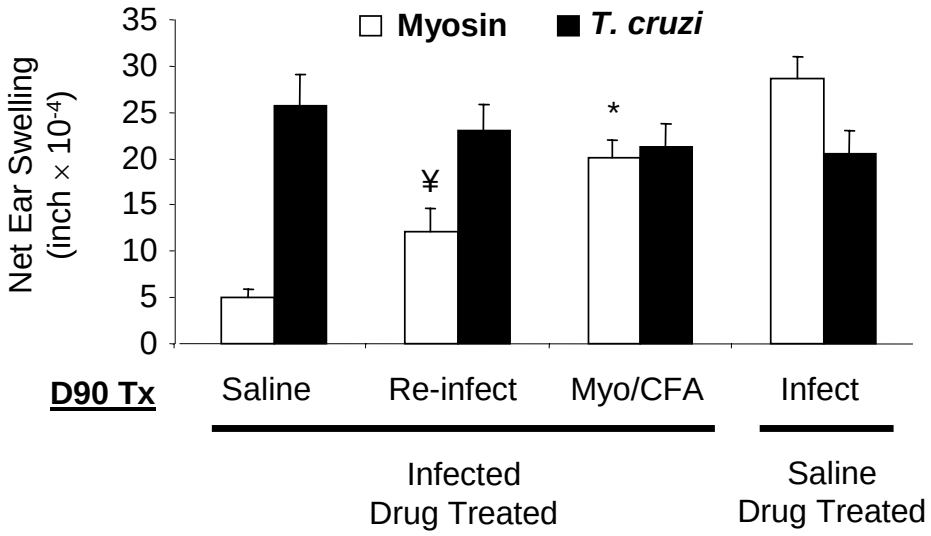
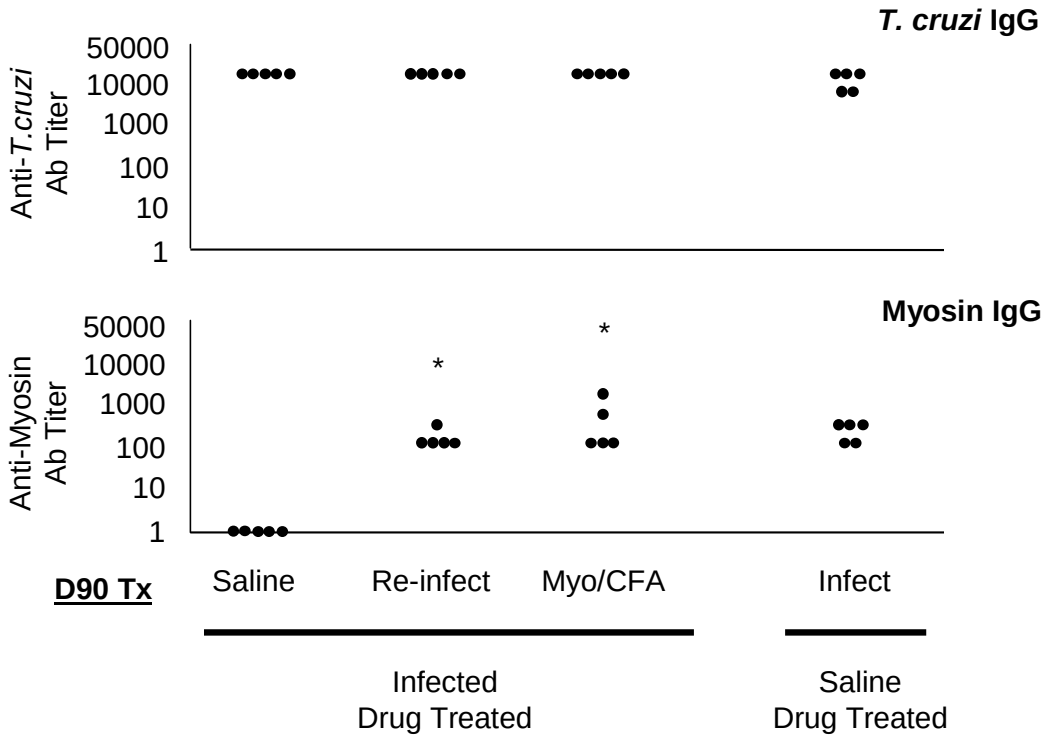

Myosin
T. cruzi
Net Ear Swelling
(inch × 10-4)
35
30
25
20
15
10
5
0
¥
*
D90 Tx
Saline
Re-infect
Myo/CFA
Infect
Infected
Drug Treated
Saline
Drug Treated

Figure 14. *T. cruzi* reinfection or myosin immunization of infected, benznidazole-treated mice restores myosin-specific humoral autoimmunity.

Mice were infected with *T. cruzi* or injected with saline and treated with benzinidazole from 14 days d.p.i. through 90 d.p.i. At 90 d.p.i., treatment was terminated and groups of animals were immunized with saline, immunized with myosin, primarily infected with *T. cruzi*, or re-infected with *T. cruzi* as indicated. Twenty-five days later (115 days after the initiation of the experiment), myosin-specific and *T. cruzi*-specific IgG titer values were determined by ELISA. Each dot corresponds to one animal. Statistics are provided relative to the infected, treated, group immunized with saline at 90 d.p.i. (* $p < .05$, ¥ $p = .057$). See Figure 5 for schematic of experimental design.


T. cruzi IgG
Anti-T.cruzi Ab Titer
50000
10000
1000
100
10
1
Myosin IgG
Anti-Myosin Ab Titer
50000
10000
1000
100
10
1
*
*
D90 Tx
Saline
Re-infect
Myo/CFA
Infect
Infected Drug Treated
Saline Drug Treated

strain displayed no signs of myocarditis and maintained the absence of myosin-specific DTH (data not shown).

E. Discussion

Autoimmunity develops in some humans and experimental animals as a result of *T. cruzi* infection. However, the role of autoimmunity in disease pathogenesis and the mechanism(s) by which it is induced remain obscure issues. In this study, we addressed the importance of live *T. cruzi*-induced damage to the induction and persistence of cardiac autoimmunity by reducing the number of parasites in infected animals with the trypanocidal drug, benznidazole. Infection of A/J mice with the Brazil strain of *T. cruzi* typically leads to the development of severe inflammation and parasitosis in the heart along with strong cardiac myosin-specific cellular and humoral immunity 21 d.p.i. and mortality by 30 d.p.i. The early administration of benznidazole decreased the severity of myocarditis, eliminated mortality in infected animals, and permitted us to conduct long term experiments (60 and 90 d.p.i.). Unfortunately, the early mortality associated with our disease model prevented us from analyzing infected, untreated controls for the duration of the experiment (specifically at 60 or 90 d.p.i.). However, because autoimmunity persists throughout the course of long-term infection when other strains of parasite and mouse are employed (17, 119, 143, 169), it is highly unlikely that autoimmunity in A/J mice infected with the Brazil strain would spontaneously resolve within several months of infection. Further, our finding that autoimmunity can be “restored” by infection or immunization of treated-cured mice at 90 d.p.i. shows that these animals retain their autoimmune potential upon treatment. Finally, it should be noted that autoimmunity persists in some humans with chronic *T. cruzi* infection (77, 97, 170, 171).

Depending on the time of drug treatment initiation, myosin-specific immunity, measured by DTH and antibody production, was significantly reduced or eliminated in both acute (21 d.p.i.) and chronic (60 and 90 d.p.i.) phases of experimental CHD. Elimination of both cardiac parasitosis and parasitemia (data not shown) illustrated the effective reduction of the parasite in all drug-treated, infected animals. Additionally, strong myosin-specific immune responses observed in myosin-immunized animals treated with drug clearly showed that benznidazole has no inherent impact on the development of autoimmunity.

The mechanism of bystander activation posits that viable parasites destroy heart tissue, causing release of host antigens (121) and subsequent stimulation of autoreactive cells. Bystander activation has also been invoked to explain the presence of myosin-specific T cells in patients after myocardial infarction (172). In other words, any cause of cardiac myocyte damage, including parasite-induced cytolysis, can cause bystander autoimmunity. Molecular mimicry, on the other hand, states that an infectious agent (parasite, bacteria or virus) possesses epitopes that are immunologically similar to host determinants and due to minor antigenic differences between the two, the pathogen epitope is able to induce an immune response that breaks tolerance to the host peptide (130). This mechanism has been attributed to a variety of cases of infection-induced autoimmunity, including streptococcus-induced myocarditis (90, 131) and even *T. cruzi*-induced Chagas disease (77, 113).

We found that the reduction of infectious parasites drastically lessens or eliminates myosin-specific immunity, lending strong support to the bystander activation mechanism. However, benznidazole treatment also caused significant reductions in *T. cruzi*-specific antibody titers (Figure 7) as well as parasite-specific DTH (Figure 8). Furthermore, the myosin-

immunized animals displayed significant parasite-specific immunity (Figures 8 and 9), consistent with previous results (55) showing cross-reactive immune responses. These results suggest the possibility that reduction of parasite load can eliminate cardiac autoimmunity (i) by attenuating the extent of cardiomyocyte damage and (ii) by lowering the number of pathogenic mimic epitopes (*T. cruzi* antigens) to which cross-reactive T cells can respond. The development of strong myosin-specific immunity and an absence of detectable cardiac damage observed upon immunization with *T. cruzi* protein extract (133) suggests that molecular mimicry is a likely mechanism of autoimmunity during infection, although bystander activation may be required for development of myocarditis. Considered together with previous findings, the results of the current study pointing to bystander activation as vital to autoimmunity in CHD lead us to hypothesize that viable parasites together with parasite specific immunity cooperatively contribute to the onset and maintenance of cardiac autoimmunity.

We observed that levels of both *T. cruzi* and myosin-specific antibody levels were higher the later benznidazole treatment was initiated (Figure 7). One hypothesis to explain this result is that the levels of antigen-specific antibodies may be associated with the severity of myocarditis and level of *T. cruzi*. These, in turn, are related to the day of benznidazole treatment initiation. This hypothesis may also explain why myosin-specific DTH levels increases the later the day of initial benznidazole treatment. However, it does not explain how *T. cruzi*-specific DTH is high regardless of when benznidazole was administered (Figure 6A). The maintenance of this parasite-specific immunity along with low, residual levels of autoreactivity could account for the mild inflammation observed in all drug-treated animals in the acute phase of disease (Figure 6B).

The negligible parasitosis observed in the treated animals at this stage also suggests a potential role for minor parasite-mediated damage, resulting in mild inflammation.

Treatment of infected mice with benznidazole resulted in the elimination of myosin-specific DTH and a decrease in *T. cruzi*-specific DTH by 60 and 90 d.p.i. (Figures 8 and 10). The decrease in antigen-specific DTH, months after removal of the antigenic stimulus, may suggest that levels of antigen-specific memory T cells decrease over time, especially in the absence of continuous antigen stimulation, but this remains to be investigated. This theory is controversial; some contend that antigen persistence is not required for the maintenance of long-lived memory (173) while others have found that antigen persistence is linked to the persistence of T cell memory in cases of Plasmodium exposure (174, 175) and coronavirus-induced encephalitis (176). This hypothesis may also explain why *T. cruzi*-specific DTH levels are eliminated in myosin immunized mice over time (Figures 8 and 10). We also observed a decrease in myosin-specific antibodies in treated mice over time, which may support the hypothesis of a decrease in the autoimmune memory response over time. We did not observe a similar decrease in *T. cruzi*-specific antibody levels over time. The high levels of *T. cruzi* antibodies, seemingly unaffected by benznidazole initiation, may be explained by (i) the insensitivity of our IgG assay in detecting differences in *T. cruzi*-specific IgG at high titers, (ii) persistence of *T. cruzi*-specific memory B cells (177) or (iii) the long half life of *T. cruzi*-specific IgG (178).

The elimination of cardiac autoimmunity observed in the late stage of disease prompted us to investigate whether this re-establishment of self-tolerance could be maintained in the presence of a secondary infection or general cardiac-specific insult. One potential mechanism to explain the elimination of autoimmunity in treated mice is that reduction of parasites caused a

reduction of parasite-associated damage and concomitant immune exposure to myosin. The absence of immune exposure to myosin and the return to a state of normalcy, free from infection-induced inflammatory conditions, caused the dampening of the self-directed immune response resulting in restoration of myosin tolerance. If this were the case, secondary infection or cardiac insult resulting in the presentation of cardiac myosin to autoreactive T cells should not elicit an autoimmune response. Reinfection with a virulent, cardiotropic parasite strain caused the reappearance of myosin-specific autoimmunity and mild myocarditis. This suggests that, while a large number of myosin-specific T cells may become unresponsive after eradication of infection, a strong enough secondary stimulus is sufficient to reactivate this population. Histologic analysis of the cardiac tissue also revealed reduced parasitosis in the hearts of mice reinfected with the hypervirulent strain compared to that seen in either the acute phase of disease or in those mice infected primarily. This suggests that the reactivation of the autoreactive cells, together with elevated parasite-specific immunity, is largely responsible for the mild, chronic inflammation. While not completely protected from *T. cruzi*, these "immunized" mice may have an enhanced ability to clear the parasite. In fact, re-infection with the original Brazil strain did not induce myosin-specific DTH (data not shown). This may be due to the strong anti-*T. cruzi* Brazil immunity (high specific DTH and antibody levels), which protected these animals (no parasitosis, parasitemia or myocarditis). The absence of myocarditis after reinfection with the original Brazil strain could mean no damage, no presentation of myosin, no myosin autoimmunity, and therefore no myosin-specific DTH. From the perspective of treating human infection in which individuals can be infected or re-infected with varying parasitic strains at any time, these results suggest that elimination of the parasite may be the best option to eliminate

autoimmunity and maintain self-tolerance. Of course, these results also suggest that an effective *T. cruzi* vaccine must prevent *T. cruzi* associated damage due to exposure to highly virulent strains, which may involve autoimmunity.

The overall importance of this last suggestion is debatable since it is not at all clear whether autoimmunity associated with *T. cruzi* infection in humans is pathogenic. The controversy surrounding autoimmunity as a major mechanism of pathogenesis in Chagas disease is currently under investigation by a number of laboratories. This study establishes a direct association between the parasite load and the magnitude of cardiac autoimmunity in experimental *T. cruzi* infection. This line of experimentation encourages future exploration of *T. cruzi* infection-induced autoimmunity and the relationship of inflammation and damage to autoimmunity in all autoimmune diseases. Areas of current study include the mechanism by which autoimmunity resolves upon drug treatment and the functional immunology of parasite-specific and myosin-specific lymphocytes in this powerful model of infection-induced myocarditis. Finally, because treatment with the trypanocidal drugs trifluralin (179) or TAK-187 also prevents cardiac damage in an experimental model of Chagas disease (180), it will be interesting to know whether autoimmunity is reduced in these animals as well.

Acknowledgments

There is no conflict of interest for any of the authors of this report due to either commercial or other affiliations.

This work was supported by NIH grant HL075822 (to DME), NIH training grant AI007476 (MDD), predoctoral fellowships from the American Heart Association (to KVH and

JSL) and a postdoctoral fellowship from the Irvington-Dana Institutes (JSL). Juan Leon assisted with the preliminary 21 and 60 days post infection experiments, contributed to the overall experimental strategic development, and performed statistical analyses on selected results. Melvin Daniels assisted with the completion of the last set of experiments, including data recording and histology analysis/scoring. Nick Giafis and LaKitta Woods each performed ELISAs as part of laboratory rotations or technician training periods, respectively. Thomas Bahk served as a scorer of cardiac histology for the acute and early chronic, d60, experiments. Kegiang Wang, our full-time animal technician, performed retroorbital bleeds for serum collection, delayed-type hypersensitivity antigen injections into the mouse ears, and assisted with terminal procedures for each experiment.

3. BIOLUMINESCENT IMAGING OF *Trypanosoma cruzi* INFECTION

Kenneth V. Hyland, Sofya H. Asfaw, Cheryl L. Olson, Melvin D. Daniels, David M. Engman*

Departments of Microbiology-Immunology and Pathology and Feinberg Cardiovascular Research Institute, Northwestern University Feinberg School of Medicine, Chicago, Illinois USA

*Corresponding author. Mailing address: Northwestern University, Department of Pathology, Ward Building 6-175, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-1288. Fax: (312) 503-1265. E-mail: d-engman@northwestern.edu.

A. Abstract

Chagas disease, caused by infection with the protozoan parasite *Trypanosoma cruzi*, is a major public health problem in Central and South America. The pathogenesis of Chagas disease is complex and the natural course of infection is not completely understood. The recent development of bioluminescence imaging technology has facilitated studies of a number of infectious and noninfectious diseases. We developed luminescent *T. cruzi* to facilitate similar studies of Chagas disease pathogenesis. Luminescent *T. cruzi* trypomastigotes and amastigotes were imaged in infections of rat myoblast cultures, which demonstrated a clear correlation of photon emission signal strength to number of parasites used. This was also observed in mice infected with different numbers of luminescent parasites, where a stringent correlation of photon emission to parasite number was observed early at the site of inoculation, followed by dissemination of parasites to different sites over the course of a 25 day infection. Whole animal imaging from ventral, dorsal and lateral perspectives provided clear evidence of parasite dissemination. The tissue distribution of *T. cruzi* was further determined by imaging heart, spleen, skeletal muscle, lungs, kidneys, liver, and intestines *ex vivo*. These results illustrate the natural dissemination of *T. cruzi* during infection and unveil a new tool for studying a number of aspects of Chagas disease, including rapid *in vitro* screening of potential therapeutic agents, roles of parasite and host factors in the outcome of infection, and analysis of differential tissue tropism in various parasite-host strain combinations.

B. Introduction

Trypanosoma cruzi, the causative agent of Chagas disease, is an intracellular, eukaryotic parasite of the family Trypanosomatidae. Endemic to vast regions of Central and South America, Chagas disease remains the leading form of infectious heart disease worldwide (1). While previous reports from the World Health Organization estimated that 16-18 million people are infected with Chagas (2, 3), a more recent analysis indicates that this number has been reduced to nearly 8 million, due to progress in the control of vectorial transmission in Latin American countries (World Health Organization, www.who.int/mediacentre/news/releases/2007/pr36/en/index.html). However, despite the optimistic nature of this statistic, the report also states that cases are now being identified outside of the typical endemic regions due to increasing incidences of blood transmission (4) and organ transplantation (5). Chagas disease can occur in an acute phase, typically characterized by high parasitism, fever and lymphadenopathy, but more commonly progresses to a chronic phase where cardiac alterations or gastrointestinal disorders are observed.

Although the tissue tropism can vary among parasite strains (181), it is generally thought that, while capable of invading virtually any cell in the body (182), *T. cruzi* preferentially targets neuronal and muscle cell-types (182) and its associated pathogenicities have typically been found to correspond to parasitosis of the myocardium (69, 70, 144, 148, 183) or digestive tract (146). These organs are often the focus of Chagas disease research, since these are the well-characterized disease manifestations. While other studies have identified trypanosomes in liver, spleen, and lung tissue, (181) and, more recently, bone and cartilage (184) these distributions generally follow the use of immunosuppressive therapy (185-187) to facilitate robust parasite

proliferation and expansion. Regardless of the mode of infection or treatment regimen, the sacrifice of animals has typically been required to obtain information on dissemination of parasites and detection of parasites in specific tissues following infection. Furthermore, quantification of whole animal and organ-specific parasite burden has been both cumbersome and inconsistent, incorporating such techniques as PCR amplification or in situ hybridization of parasite-specific genes from tissue (70, 188-190), parasite antigen-specific immunofluoresence (183, 191, 192) and the counting of either nests of parasite amastigotes in tissue sections or free-swimming trypomastigotes in the blood (193-196). While these approaches have certainly been adequate for a variety of studies of experimental Chagas disease pathogenesis, they are also accompanied by significant limitations.

During the past several years, bioluminescence imaging (BLI) techniques have overcome these limitations in the analysis of many disease processes, including various models of cancer tumorigenesis (197-201) and infections caused by bacteria, viruses, fungi and parasites, nicely summarized in a recent review (202). The incorporation of *in vivo* BLI has not only provided a means by which to evaluate the spatiotemporal progression of disease in real-time, but has brought about the opportunity to observe potentially biologically relevant interactions of host and pathogen, in the case of infectious disease, that may have otherwise gone unnoticed. In general, BLI detects light resulting from the reaction of luciferase enzymes with a specific substrate. This is made possible by utilizing either bacterial luciferase genes capable of encoding both enzyme and substrate that are typically transferred to other species of bacteria, or by using luciferase enzymes from higher organisms such as the firefly or sea pansy (202). While the use of bacterial genes precludes the use of exogenous substrate to initiate the luciferase enzymatic reaction,

employing genes from the firefly and other such organisms requires that luciferin substrate be provided to produce detectable light.

In this study, we sought to develop bioluminescent *T. cruzi* for *in vivo* BLI analysis of infection using our well-established model of experimental Chagas disease. To do this, we engineered the Brazil strain of *T. cruzi* to express firefly luciferase using standard transfection methods. Following infection of animals with different numbers of luminescent *T. cruzi* trypomastigotes, we observed clear qualitative and quantitative differences in parasite burden up to two weeks post infection, after which time a similar burden was achieved and maintained throughout the remainder of the acute infection. In addition to *in vivo* imaging of parasite infection in A/J mice, we were also able to detect luminescence in all three life cycle stages of *T. cruzi* by different methods. Finally, we illustrate the ability to detect luminescence in several harvested organs in a terminal, *ex vivo* analysis.

C. Materials and Methods

Parasites

The Brazil, heart-derived strain of *T. cruzi* (56) was used for the experiments described here. Epimastigotes, used for transfection and specific *in vitro* assays, were maintained in supplemented liver digest neutralized tryptose medium (LDNT) as described previously (203). Epimastigote transfectant cultures, consisting of differentiated metacyclic trypomastigotes, were used to infect monolayers of H9C2 rat myoblasts (American Type Culture Collection, Manassas, VA) from which trypomastigotes could be continually passaged and isolated. These trypomastigotes were used for all animal infections described.

Generation of bioluminescent *Trypanosoma cruzi*

For stable integration of the firefly luciferase gene into the tubulin locus, we used plasmid pBS:THT-x-T (the generous gift of Wesley Van Voorhis (University of Washington)). In this pBluescript (Stratagene, La Jolla, CA)-based plasmid, the HygTK gene is flanked by β-tubulin untranslated/intergenic regions (UTR/IR) and the gene of interest is flanked by α-tubulin intergenic regions (204). The *Mlu*I site in the β-α UTR/IR was converted to a unique *Sal*I site for plasmid linearization prior to transfection. The firefly luciferase gene was amplified by PCR from the pGL3 basic vector (Promega, Madison, WI) with forward primer 5'-GGATCCATGGAAGACGCCAAAAACATAAAG-3' and reverse primer 5'-TCTAGATTACACGGCGATCTTTCC-3' which included the *Bam*HI and *Xba*I restriction sites (underlined), respectively. The resulting amplicon was ligated into the pCR-blunt vector

(Invitrogen, Carlsbad, CA) and was selected for kanamycin resistance. The luciferase coding sequence was then liberated by digestion with *Bam*H1 and *Xba*1 and the purified insert was directionally cloned into pBS:THT-x-T using these sites. The resulting plasmid, pBS:THT-Luc-T was linearized with *Sal*I and 10 μg of DNA was transfected into *T. cruzi* by electroporation with a Gene Pulser (Bio-Rad, Hercules, CA) using the following conditions: 500 μF, 450 V, in a 0.2cm cuvette. Briefly, mid-log stage (~2×10^7/ml) epimastigotes of *T. cruzi* Brazil heart strain (56) were harvested by centrifugation, washed twice in PBS supplemented with 1 mg/L glucose and resuspended at a final concentration of 1×10^8 cells in 0.4 mL of electroporation buffer (phosphate buffered saline (PBS) with 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$). Following electroporation, the cells were placed on ice for 15 min and were transferred to flasks containing 5 mL of LDNT. Forty-eight hours post transfection, cells were selected for resistance to hygromycin (Boehringer–Mannheim, Mannheim, Germany) at 0.75 mg/ml. Drug-resistant parasites were analyzed for luciferase activity 6 weeks following transfection.

In vitro bioluminescent imaging

To confirm the expression of luciferase in antibiotic resistant transfectants, *T. cruzi* epimastigotes were serially diluted from 1×10^6 to 500 parasites in Dulbecco's phosphate buffered saline (PBS, GibcoBRL, Grand Island, NY) into a black 96-well plate (Costar, Acton, MA). A 50 μL cell suspension was mixed with 50 μL of Steady Glo reagent (Promega, Madison, WI), according to the manufacturer's instructions. After 5 min, the plate was read using a SpectraMax Gemini XS (Molecular Devices, Sunnyvale, CA) and analyzed with SoftmaxPro 4.8 software (Molecular Devices). Wild-type (untransfected) Brazil heart *T. cruzi* was included as a negative control.

For further analysis of luminescence in trypomastigote and amastigotes life stages, active, *in vitro* rat myoblast infections, using a variety of parasite-to-myoblast ratios (as described in Fig. 1) were also analyzed for bioluminescence. Infections were given 5 days to become established in a clear 6-well plate (BD Biosciences, San Jose, CA), luciferin was added to 150 μg/ml, and plates were imaged using the Xenogen IVIS system (see below for a description of the IVIS imaging system and analysis software) after a 5 min incubation.

Experimental animals and *T. cruzi* infections

4-6 week old male A/J mice (Jackson Laboratories, Bar Harbor, ME) were housed under specific pathogen-free conditions. Mice were infected by intraperitoneal injection of either 1×10^6, 1×10^5, or our standard quantity of 1×10^4 Brazil heart strain, luminescent *T. cruzi* trypomastigotes derived from infection of tissue culture H9C2 rat myoblasts. Wild-type Brazil heart (untransfected) *T. cruzi* and uninfected controls that had received an intraperitoneal injection of PBS were included for *in vivo* analysis. The use and care of mice were conducted in accordance with the guidelines of the Center for Comparative Medicine at Northwestern University.

***In vivo* and *ex vivo* bioluminescent imaging**

Prior to bioluminescent imaging, mice were anesthetized with 1.5% isofluorane. After anesthesia was achieved, 150 mg/kg body weight of substrate luciferin potassium salt dissolved in PBS and filtered through a 0.22 μm filter (Molecular Therapeutics, Ann Arbor, MI) was administered by a single intraperitoneal injection. Mice were placed into the camera chamber, where a controlled flow of 1.5% isofluorane in air was administered through a nose cone via a gas anesthesia system

designed to work in conjunction with the bioluminescent imaging system (IVIS 100; Xenogen, Alameda, CA). This imaging system consists of a cooled charge-coupled device camera mounted on a light-tight specimen chamber, a camera controller, and a Windows computer system. In order to allow adequate dissemination of the luciferin substrate (205), mice were maintained for 10 minutes after injection of the substrate. Mice were imaged in dorsal, ventral and left lateral positions by capturing a grayscale body image overlaid by a pseudocolor image representing the spatial distribution of the detected photons. Images were collected with 0.5 to 2 min integration times depending on signal intensity. For the analysis of parasites in specific organs, mice were administered the luciferin substrate, as described, maintained for 5 min and sacrificed for organ harvest. Data acquisition and analysis were performed by using the LivingImage software (Xenogen) where luminescence could be quantified as the sum of all detected photon counts per second within a chosen region of interest.

D. Results

Evaluation of *Trypanosoma cruzi* bioluminescence

To examine the live, *in vivo* dissemination of *T. cruzi* in a non-invasive manner, we engineered bioluminescent epimastigotes by integrating the firefly luciferase coding sequence into the tubulin locus. The luciferase coding sequence from the pGL3 basic vector was directionally subcloned into the pBS:THT-x-T tubulin integration vector (204), and integrated into the intergenic regions of parasite α- and β-tubulin after electroporation (Figure 15A). Integration of the luciferase gene was initially confirmed using pulsed-field gel electrophoresis and later with the appearance of expected band sizes from Southern blot analysis of genomic DNA digested with restriction enzymes *BglII, SacII and SphI* (204) and probed with the entire coding region of luciferase (Figure 16). Eight weeks post transfection, an antibiotic-resistant epimastigote polyclonal population displayed notable luminescence measured by a standard plate-reading apparatus, indicating successful integration and expression of the luciferase gene (Figure 15B). Further analysis of this luminescent population was conducted by allowing the epimastigote population to differentiate into metacyclic trypomastigotes. Once metacyclogenesis occurred, trypomastigotes were used to infect myoblasts at variable ratios from which both infectious trypomastigotes and intracellular, replicating amastigotes could be imaged for luminescence. The imaging of this *in vitro* infection revealed significant bioluminescence with an expected correlation of signal strength to parasite number (Figure 17).

In vivo characterization of luciferase *T. cruzi*

Figure 15. Generation and epimastigote analysis of luminescent *T. cruzi*.

(A) Construct and cloning strategy used to generate luminescent *T. cruzi*. pBS:THT-x-T (obtained from Wesley Van Voorhis, University of Washington), a plasmid with a pBluescript backbone and a hygromycin-resistance gene, was modified by insertion of the firefly luciferase gene. After linearization with *Sal*I, the plasmid was transfected into *T. cruzi* epimastigotes and the Hyg and Luc genes integrated into the tubulin locus by homologous recombination.
(B) Serial dilutions of antibiotic-resistant, *T. cruzi* epimastigote transfectants and wild-type epimastigotes were examined for luciferase activity. A black, 96-well plate containing 50 μL suspensions of parasites, mixed with 50 μL of Steady Glo reagent was read by a SpectraMax Gemini XS Microplate Spectrofluorometer and analyzed with SoftmaxPro 4.8 software.
Abbreviations: RLU, relative light units

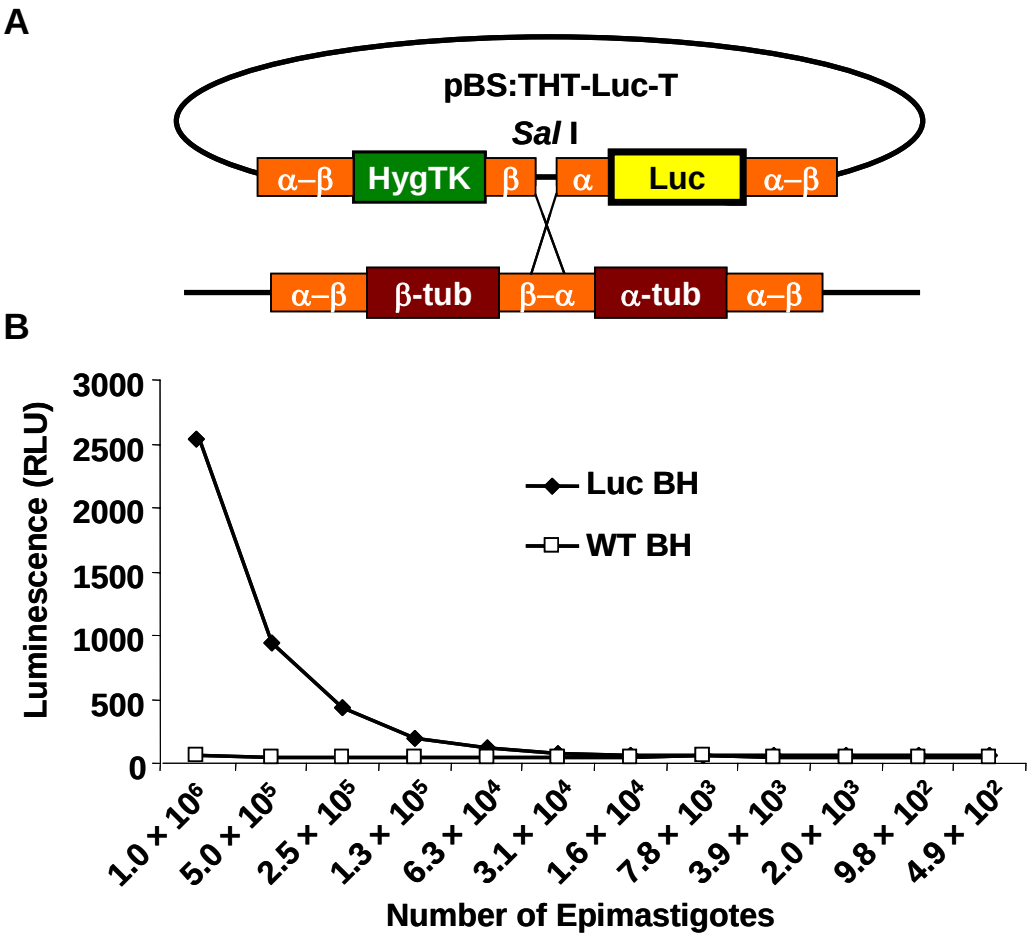

A
pBS:THT-Luc-T
Sal I
α–β
HygTK
β
α
Luc
α–β
α–β
β-tub
β–α
α-tub
α–β
B
Luminescence (RLU)
3000
2500
2000
1500
1000
500
0
Luc BH
WT BH
1.0 + 10^6
5.0 + 10^5
2.5 + 10^5
1.3 + 10^5
6.3 + 10^4
3.1 + 10^4
1.6 + 10^4
7.8 + 10^3
3.9 + 10^3
2.0 + 10^3
9.8 + 10^2
4.9 + 10^2
Number of Epimastigotes

Figure 16. Southern blot analysis of luciferase gene integration into *T. cruzi* genome. Southern blot analysis of the pTHT-Luc-T integration into the tubulin locus. Genomic DNA (5 µg) was digested with restriction enzymes Bgl II (Bg), Sac II (Sc) and Sph I (Sp) and fractionated on a 1.0% etBr-agarose gel. Blots were probed with either the entire coding region of the Luc or HygTK gene. DNA sizes are marked in kilobase pairs. Expected band sizes for each restriction enzyme digest are indicated. Strategy partially adapted from (204). Abbreviations: Luc, luciferase; HygTK, hygromycin thymidine kinase

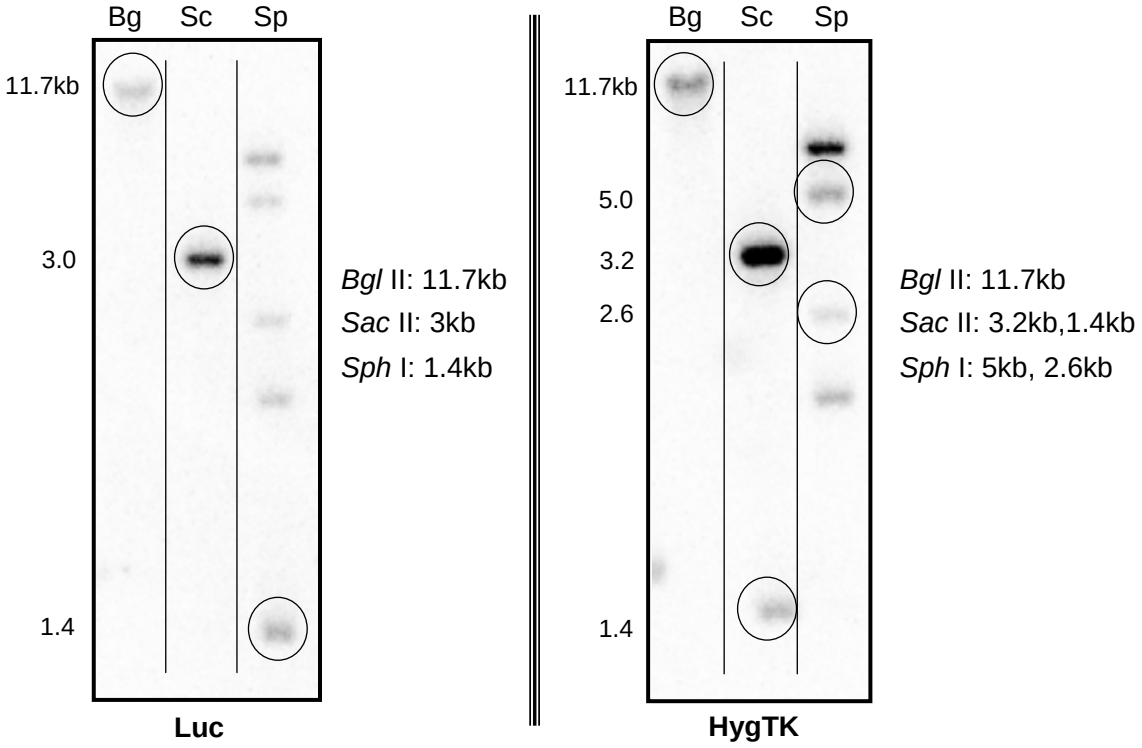
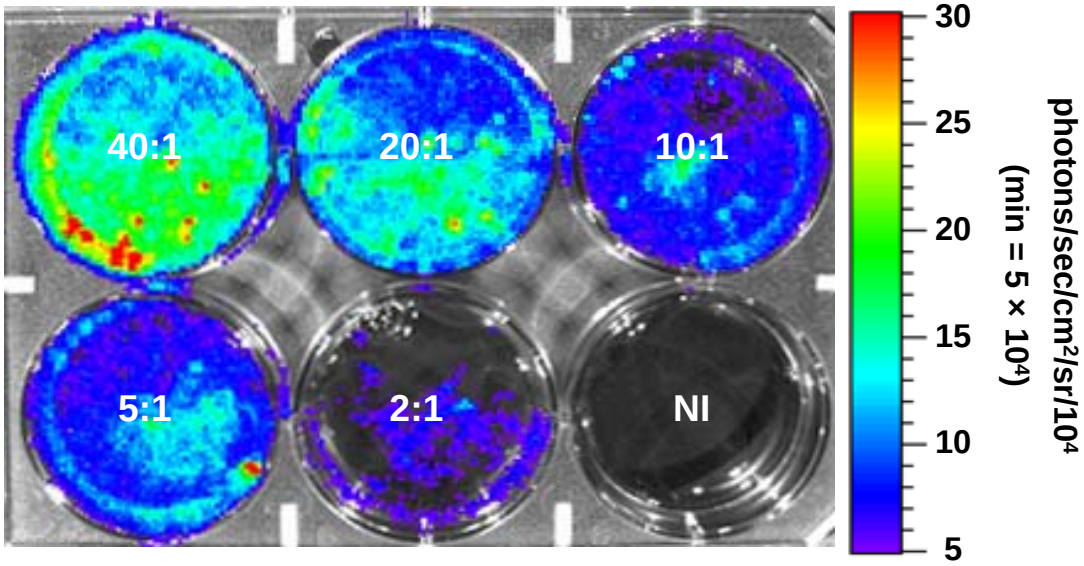

Bg
Sc
Sp
11.7kb
3.0
1.4
Luc
Bgl II: 11.7kb
Sac II: 3kb
Sph I: 1.4kb
Bg
Sc
Sp
11.7kb
5.0
3.2
2.6
1.4
HygTK
Bgl II: 11.7kb
Sac II: 3.2kb,1.4kb
Sph I: 5kb, 2.6kb

Figure 17. Bioluminescent imaging of *T. cruzi*-infected myoblasts *in vitro*.

Luminescent trypomastigotes and amastigotes were examined for luciferase activity in active, *in vitro* rat myoblast infections using parasite:myoblast ratios of 40:1, 20:1, 10:1, 5:1, 2:1 or uninfected (NI). Five days post infection, D-luciferin was added to each well and the 6-well plate was imaged with the Xenogen IVIS system after a 5-min incubation (see Materials and Methods for detailed description). In the pseudocolor image the luciferase activity or photon intensity ranges from the lowest intensity (blue) to highest intensity (red). Abbreviation: min, minimum.


40:1
20:1
10:1
5:1
2:1
NI
30
25
20
15
10
5
photons/sec/cm²/sr/10⁴
(min = 5 × 10⁴)

Once the myoblast infections were established and propagated by serial passage, it was not possible to determine with accuracy the number of parasites responsible for generating the luminescent signals shown in Figure 17. However, based on a comparison of the number of trypomastigotes used to initiate the *in vitro* infection and the numbers of epimastigotes used for the experiment of Figure 15B, it appeared that the IVIS instrument was far superior to the standard plate-reading apparatus in luminescence sensitivity. For this reason, and to determine the minimal inoculum required to follow the course of infection in our mouse model of Chagas disease, we infected mice with 10^6, 10^5, or 10^4 (our normal inoculum) luminescent trypomastigotes by intraperitoneal injection. Imaging of these mice, as soon as one hour post infection, revealed the capability of the IVIS system to detect all amounts used, with clear differences in signal strength (Figure 18). The magnitude of light emission was noticeably higher in the *in vivo* infection than in the *in vitro* infection when comparing the maximum values indicated on the scales. Interestingly, while infection with 10^4 luminescent trypomastigotes produced a signal clearly above that of wild-type parasites *in vivo* (not shown), nearly 2×10^5 epimastigotes were required to generate a luminescent signal above that of wild-type cells (Figure 15B). The dissemination of parasites was monitored in infections initiated with the different inocula over 25 days, showing the highest parasite burden at 10 days post infection when the highest number of trypomastigotes was used, but then a reduced and more dispersed burden similar among the three animals by three weeks post infection (Figure 18). When quantified, the signal intensity in all infections indicates a peak of parasite burden at 10 days post infection when using either 1×10^6 or 1×10^5 parasites, with a slight lag in peak signal (14 days post infection) when using 1×10^4 parasites (Figure 19). Additionally, at two weeks post

Figure 18. **Luminescent *T. cruzi* imaged at various times post infection in A/J mice with an IVIS imaging system.**

Trypomastigotes were isolated from *in vitro* myoblast infections and different amounts, as indicated, were injected intraperitoneally into A/J mice. Mice were imaged ventrally starting 1 hour after infection and monitored the days post infection as shown. For all images shown, the color scale ranges from blue (just above background with a minimum set to 15,000 photons/sec/cm^2/sr) to red (maximum of 1×10^6 photons/sec/cm^2/sr). Abbreviation: min, minimum.

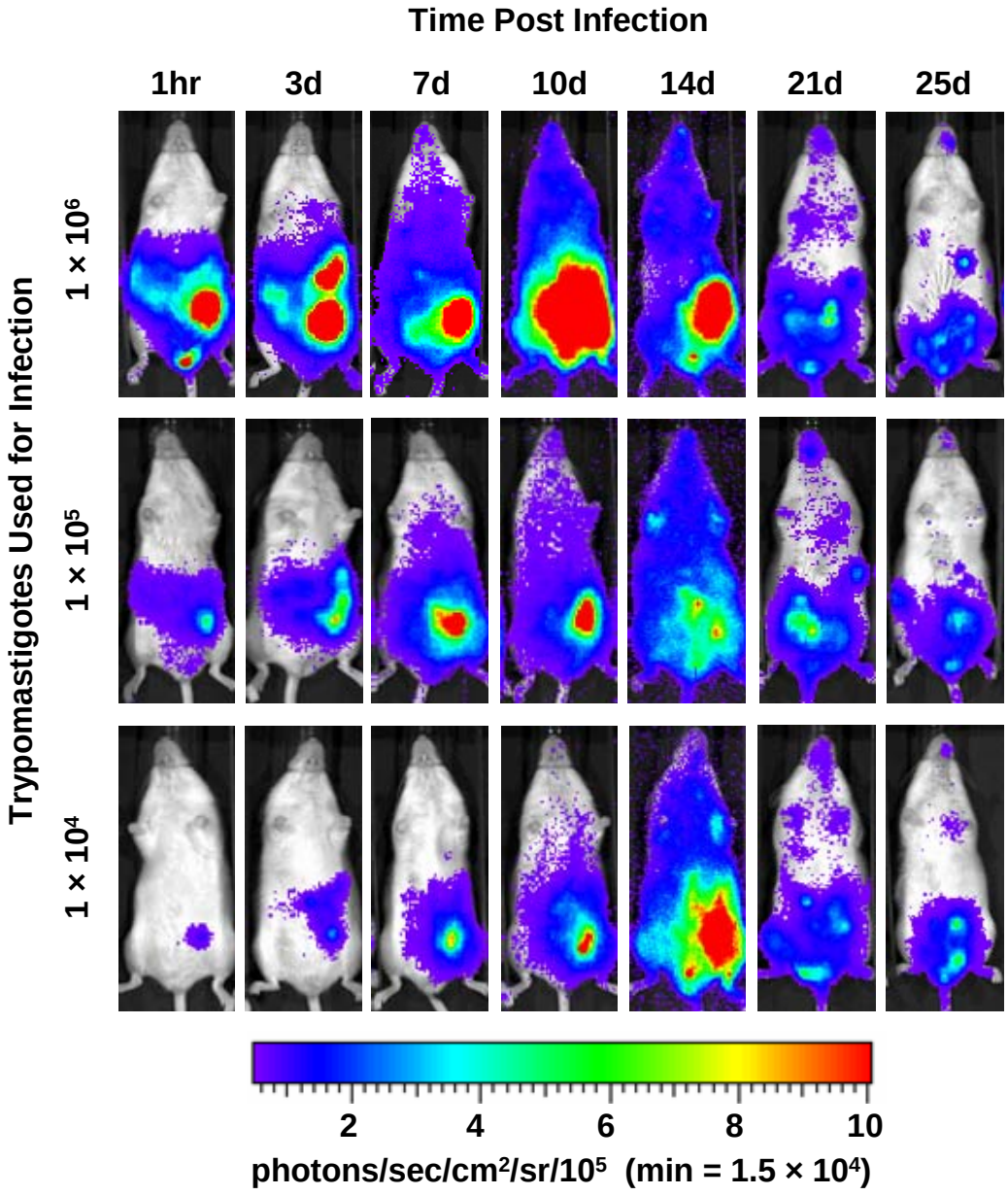
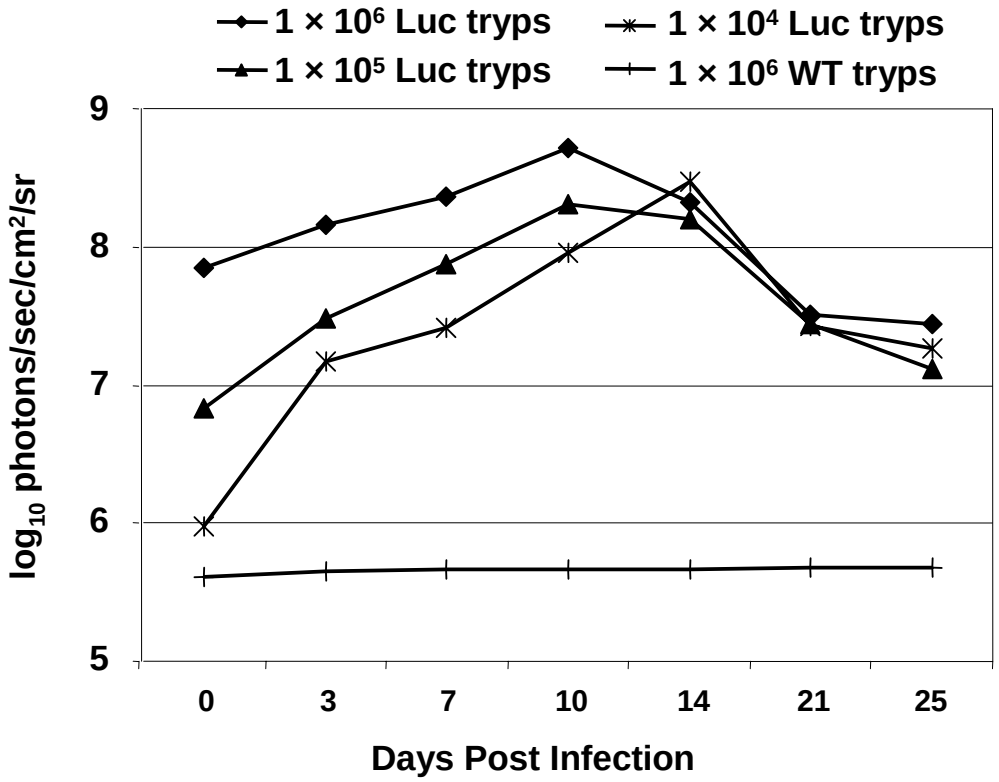

Time Post Infection
1hr
3d
7d
10d
14d
21d
25d
Trypomastigotes Used for Infection
1 × 10^6
1 × 10^5
1 × 10^4
2
4
6
8
10
photons/sec/cm^2/sr/10^5 (min = 1.5 × 10^4)

Figure 19. Whole-body parasite burden quantification from infection of A/J mice with multiple dosages of luminescent or wild-type *T. cruzi* trypomastigotes.

The course of whole-body parasite burden expressed in terms of the photonic signal resulting from infection of A/J mice with either 10^6, 10^5 or 10^4 luminescent or 10^6 wild-type *T. cruzi* trypomastigotes. The total light emission from the entire mouse body was measured and data points were generated from the analysis of at least two mice per infection condition. Day 0 post infection corresponds to measurements acquired 1 hour post infection (see Fig 18). The signal shown for wild-type infection corresponds to background noise of the IVIS instrument.


1 × 10⁶ Luc tryps
1 × 10⁴ Luc tryps
1 × 10⁵ Luc tryps
1 × 10⁶ WT tryps
log10 photons/sec/cm²/sr
9
8
7
6
5
0
3
7
10
14
21
25
Days Post Infection

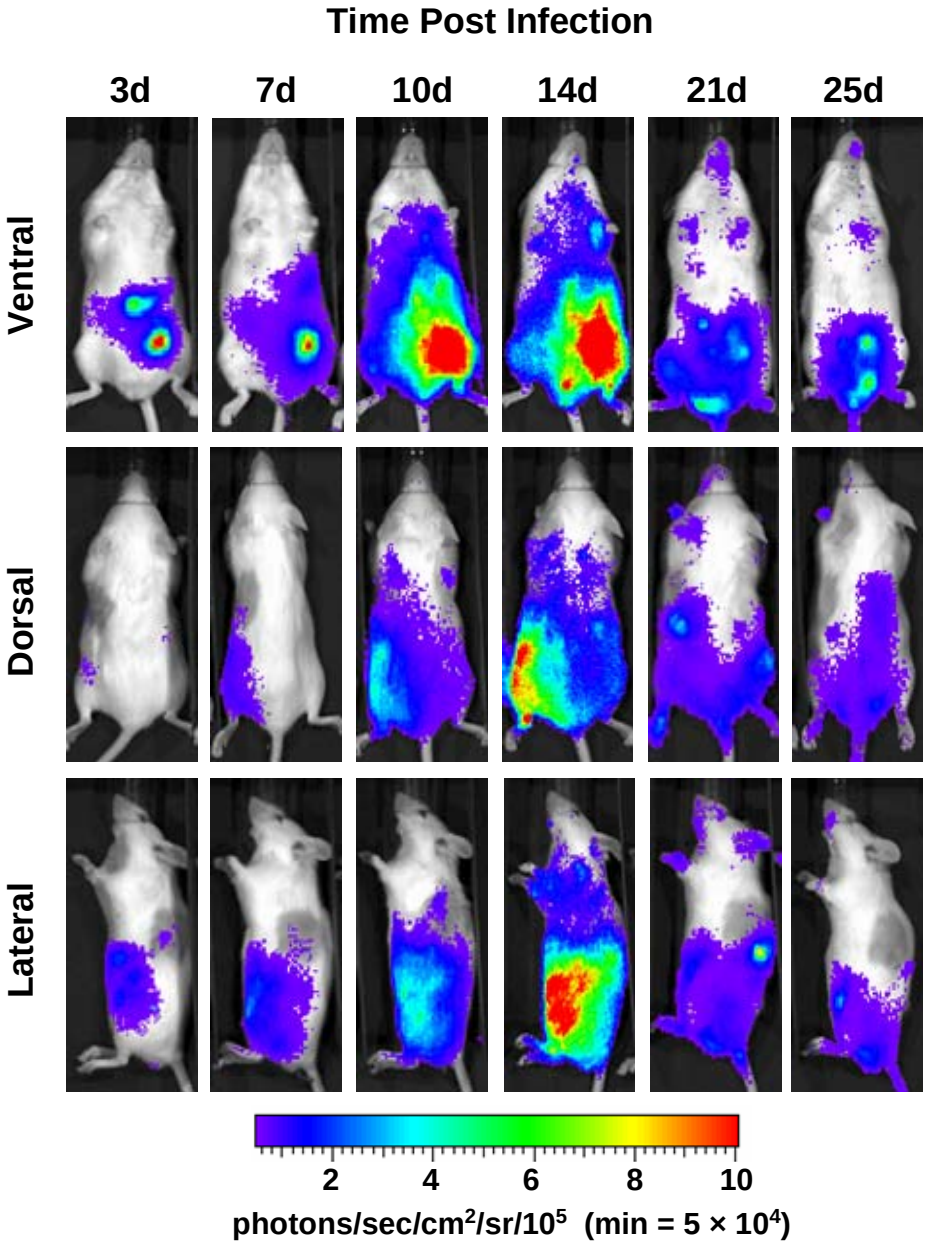
infection, the original number of parasites used for infection becomes irrelevant as photon emission, or luminescence, among the groups was indistinguishable (Figure 19) and parasitosis appears to become more organ specific at later time-points (Figure 18). It is noteworthy that the 10-fold differences of parasite quantity used for the initial infection correlates with 10-fold differences in luminescence signal intensity observed one hour post infection (Figure 19).

Course of intraperitoneal infection observed in the A/J-Brazil strain-strain model of experimental Chagas disease

Since our experimental model of acute Chagas disease is normally initiated with the intraperitoneal injection of 10^4 Brazil strain trypomastigotes, we conducted a thorough analysis of parasite dissemination from three perspectives. As expected, the majority of luminescence observed early in the infection was in the lower left quadrant, where the parasites are administered. This was consistently observed from ventral, dorsal, and left lateral viewpoints (Figure 20). As the infection progressed, the overall intensity of luminescence decreased and specific regions retained parasites, indicative of possible occupancy in specific organs. For instance, at both 21 and 25 days post infection, it appears that parasites are persistent in regions corresponding to lung or heart, as might be expected (Figure 20, ventral). The persistence of luminescence emitted from the lower portion of the mouse abdomen suggested a general maintenance of parasite proliferation in this intraperitoneal region or specific habitation of parasites in the gastrointestinal tract. To further examine the parasite burden in an organ-specific manner, several organs were harvested from animals 25 days post infection and imaged for luminescence. These animals were provided D-luciferin substrate, provided time for adequate

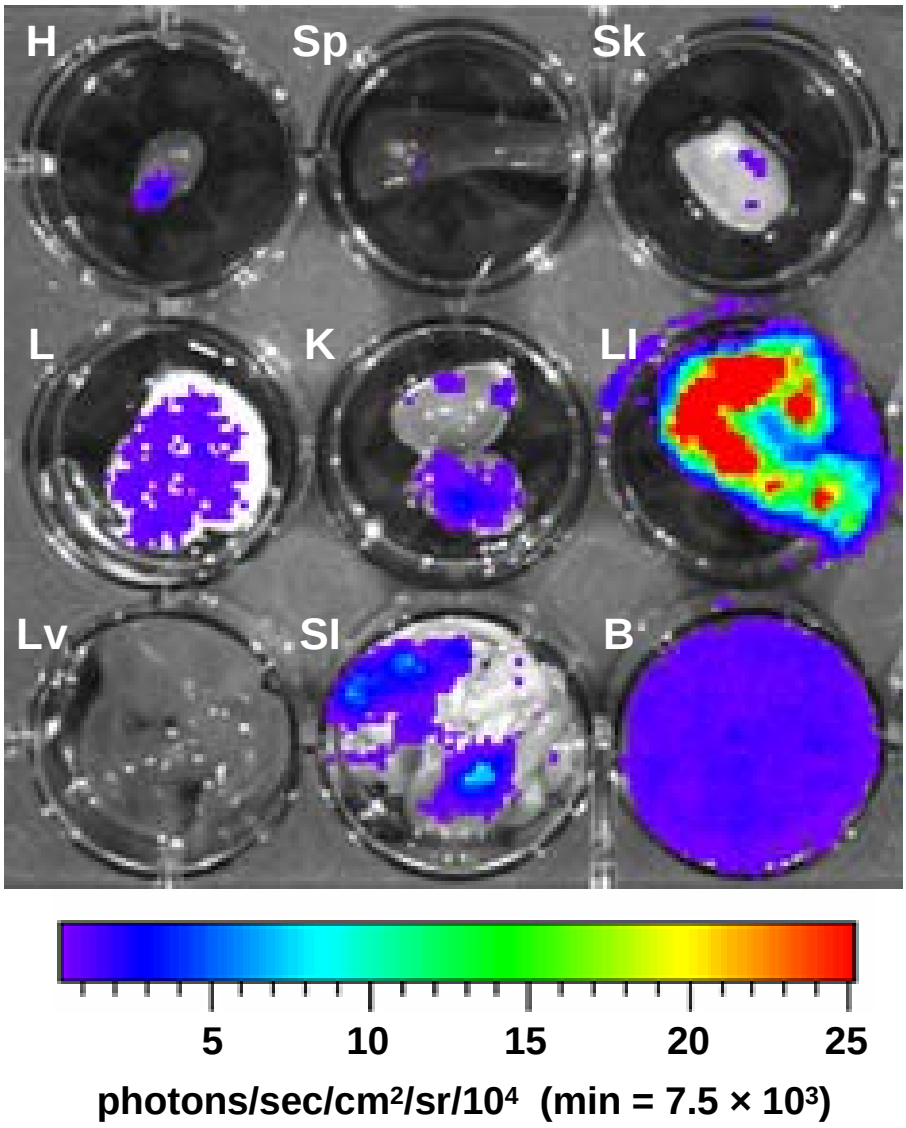
Figure 20. Course of parasite dissemination in a mouse model of experimental Chagas heart disease.

A/J mice were injected intraperitoneally with 1×10^4 luminescent *T. cruzi* trypomastigotes and imaged either ventrally, dorsally, or laterally over the course of infection prior to death typically observed by 30 days post infection. For all images shown, the color scale ranges from blue (just above background with a minimum set to 50,000 photons/sec/cm^2/sr) to red (maximum of 1×10^6 photons/sec/cm^2/sr). The minimum for this scale was adjusted to avoid signal saturation during the peak of signal intensity. The ventral, dorsal, and lateral perspectives for each timepoint were taken from the same animal and all images are representative of at least two animals. Abbreviation: min, minimum.


Time Post Infection
3d
7d
10d
14d
21d
25d
Ventral
Dorsal
Lateral
2
4
6
8
10
photons/sec/cm2/sr/105 (min = 5 × 104)

systemic dissemination, and sacrificed for organ harvest. As shown in Figure 21, the majority of *T. cruzi* was found in the gastrointestinal system, particularly the large intestine (LI), with a notable presence in the small intestine (SI), lungs (L) and kidneys (K). Smaller amounts of luminescence were also observed in the heart (H) and skeletal muscle (Sk) while either a completely absent or barely detectable luminescent signal was seen in spleen (Sp) and liver (Lv). Whole blood (B) revealed low level signal.

Figure 21. Detection of luminescent *T. cruzi* in the internal organs of infected A/J mice. Mice were infected with 10^4 trypomastigotes and injected with D-luciferin substrate (as described in Materials and Methods) prior to sacrifice and organ dissection. Twenty-five days post infection luminescence was analyzed in heart (H), spleen (Sp), skeletal muscle (Sk), lung (L), kidney (K), large intestine (LI), liver (Lv), small intestine (SI) and whole blood (B). For all images shown, the color scale ranges from blue (just above background with a minimum set to 7500 photons/sec/cm^2/sr) to red (maximum of 2.5×10^5 photons/sec/cm^2/sr). The minimum and maximum for this scale was adjusted to enhance signal detection while avoiding saturation and is consistent for all organs imaged. Abbreviation: min, minimum.


H
Sp
Sk
L
K
LI
Lv
SI
B
5
10
15
20
25
photons/sec/cm²/sr/10⁴ (min = 7.5 × 10³)

E. Discussion

To make bioluminescent imaging of *Trypanosoma cruzi* possible, a method in which the firefly luciferase enzyme could be stably expressed within the parasite was required. The pBS:THT-x-T plasmid, having been previously employed for the expression of an FL-160-GFP fusion in *T. cruzi* (204), was chosen for its ease of modification, possession of an antibiotic resistance gene for positive selection, and design for integration into an essential region of the trypanosome genome (tubulin locus) to ensure its maintained expression. This transfection strategy produced transgenic parasites that could be used for animal infections without the need for continuous drug selection, unlike those using episomal vectors. The administration of hygromycin following transfection produced a population of epimastigotes that readily display luminescent activity using a standard plate-reading luminometer (Figure 15B). Although the modest sensitivity of this instrument precludes assessment of luciferase activity at the single-cell level, there is potential of such a tool for the screening of potential parasiticidal compounds. This high-throughput, efficient method has been effective for the investigation of anti-*Leishmania* compounds (206, 207) and could similarly be applied to luminescent *T. cruzi*.

We employed the IVIS instrument, typically used for whole animal imaging, to assess bioluminescence of trypomastigotes and amastigotes from a live, *in vitro* infection of rat cardiomyocytes. This method provides a more complete picture of luminescence by providing a visualization of light and the ability to quantify emitted photons in a specific region of interest. In addition to the utility of luminescent trypanosomes for drug screening, studies of host and parasite factors involved in susceptibility and resistance to infection could be accomplished using

this technology. For instance, specific deletion or knockdown of parasite or host cell components thought to be required for invasion could be quickly analyzed by determining whether infections can be established and maintained in culture before moving to an animal model.

The ability to image *T. cruzi* with IVIS instrumentation on a whole animal level will enable investigators to conduct important studies of virulence from both host and parasite perspectives as well. We initially had to determine the threshold of detection as it relates to our standard mouse infection regimen. We previously established an experimental model of Chagas disease in which A/J mice are infected with 10^4 trypomastigotes and analyzed in the acute phase (21 days post infection) at which time severe inflammation, fibrosis and parasitosis of the heart is typically observed. To assess the sensitivity of the IVIS instrument for imaging our luciferase parasites we found that, while luminescence detection was minimal 1 hour post infection, the proliferation and dissemination over a 25 day period was sufficient to produce a maintained signal throughout the course of infection (Figure 18). Interestingly, when infecting with either 10- or 100-fold more trypomastigotes, an initial correlation of luminescence to parasite number was observed (Figure 19, one hour post infection), followed by a gradual equilibration of parasite burden by 14 days post infection. One possible explanation for this could be a combination of enhanced immunity against *T. cruzi* coupled with an impaired capacity for immune evasion typically associated with the parasite. A number of reports have described different ways in which *T. cruzi* is capable of evading host immune responses during the acute phase of infection, allowing the persistence to gradually contribute to chronic pathology (208-211). With the extreme nature of infecting with 10^5 or 10^6 trypomastigotes, an inoculum far surpassing any used

for a variety of *T. cruzi*-based experimental animal systems, the ability of the parasite to avoid the robust adaptive immune response mounted could be reduced. By 10 days post infection, we observed a peak of parasite burden in these animals followed by a decrease that approaches that seen in animals infected with our normal quantity of parasites. The timing of this event likely corresponds to the peak of the adaptive immune response, which appears to control the infection once the parasite burden reaches a certain point. Interestingly, animals infected with the lowest number of parasites show increased parasite burden until 14 days post infection, at which point the animals appear to have the same burden as those receiving higher inocula. In all cases, once the adaptive immune response presumably initiated full control of parasitism, we observed a sharp decline in burden by 21 days post infection, at which point parasites appeared to have taken up residence in specific organs, rather than being globally dispersed throughout the animal. As the animals progress to 25 days post infection, the luminescent signal plateaued, suggestive of a scenario in which host immunity has controlled the infection and parasite persistence has been achieved. In this particular experimental disease model, using the Brazil parasite strain with A/J mice, animals succumb to disease by 30 days post infection, prohibiting the examination of luminescence into the chronic phase of disease. The development of luminescent *T. cruzi* in other parasite strains, and performing infections in different strains of mice will facilitate the investigation of parasite dissemination in other, long-term chronic animal models of Chagas disease.

Further analysis of our standard experimental model, using 10^4 luminescent trypanosomes, provided a clearer picture of parasite dissemination by imaging from three perspectives (Figure 20; ventral, dorsal and lateral). The site of injection, in the lower left

quadrant, displayed the most prominent photon emission early after infection, suggesting that parasites immediately invade and initiate replication in surrounding tissue. The infection spread over the next two weeks, at which point the peak of parasite load was observed, followed by the sharp decrease in luminescent signal. By three weeks post infection, the intensity in the lower abdominal region was decreased, but maintained, and luminescence was observed in areas of the thoracic region from the ventral perspective, suggestive of parasitosis of heart or lung. In addition, the dorsal view suggested potential parasitism of either the spleen or kidney at both 21 and 25 days post infection. While the signal observed in the abdominal region diminished over time, it remained strong enough to potentially mask the parasite burden associated with nearby tissue (e.g. skeletal muscle, liver).

In order to overcome this issue, we analyzed specific organs from infected mice 25 days post infection. After allowing adequate dissemination of the injected luciferin substrate, mice were sacrificed and organs (heart, skeletal muscle, spleen, lungs, kidneys, liver, blood, large and small intestine) were imaged using IVIS (Figure 21). As anticipated, due to the typical anatomical sites associated with Chagas disease, we observed luminescent signal from heart and skeletal muscle as well as gastrointestinal organs. Surprisingly, substantial photon emission was observed in both lung and kidney tissue while nearly a complete absence of signal was observed in spleen and liver. This was surprising, given the significant roles of these organs in the reticuloendothelial system. While the blood appears to have high parasitemia based on visual appearance, the overall signal is actually quite low with respect to the scale of intensity (Figure 21; B). This low-level parasitemia is in agreement with previous findings at the acute timepoint used for analysis. While others have reported parasite distribution to lung, spleen and liver in

cases when animals have been immunosuppressed (186) and even parasitism of bone and cartilage (184), it has become increasingly clear from a number of studies that the genetics of the parasite and host play a defining role in the tissue distribution of *T. cruzi* (54, 181, 183, 212-214). Although the pathogenesis of Chagas disease is multivariate, the persistence of *T. cruzi* has been suggested as playing a fundamental role in disease progression (68-70). Despite the continued debate and variability of the mechanism of pathogenesis, the development of luminescent *T. cruzi* provides the ability to quickly screen organs and tissue samples for the presence of parasites. Further correlation of whole body and harvested organ luminescence should permit the analysis of tissue-specific parasitization in a noninvasive manner using this powerful technology. In instances where parasite persistence is thought to be maintained at extremely low levels, such as in some chronic models of disease, it is likely that methods of parasite detection proven to be far more sensitive (*i.e.* PCR of parasite genes) will remain essential for specific analyses. The observation of low-level parasitemia indicated by *ex vivo* luminescence of blood, in conjunction with the apparent absence of parasites in blood-filtering organs, implies clear limitations on the sensitivity of luminescence detection. This limitation does not come unexpectedly, since the detection of a minimal presence of trypanosomes has been challenging with a number of attempted methods, as described in the introduction. Although the development of luminescent trypanosomes certainly does not provide a solution to the obstacles frequently encountered in this field of research, it will undoubtedly provide an alternative for a number of studies that may have otherwise required an invasive, more costly approach.

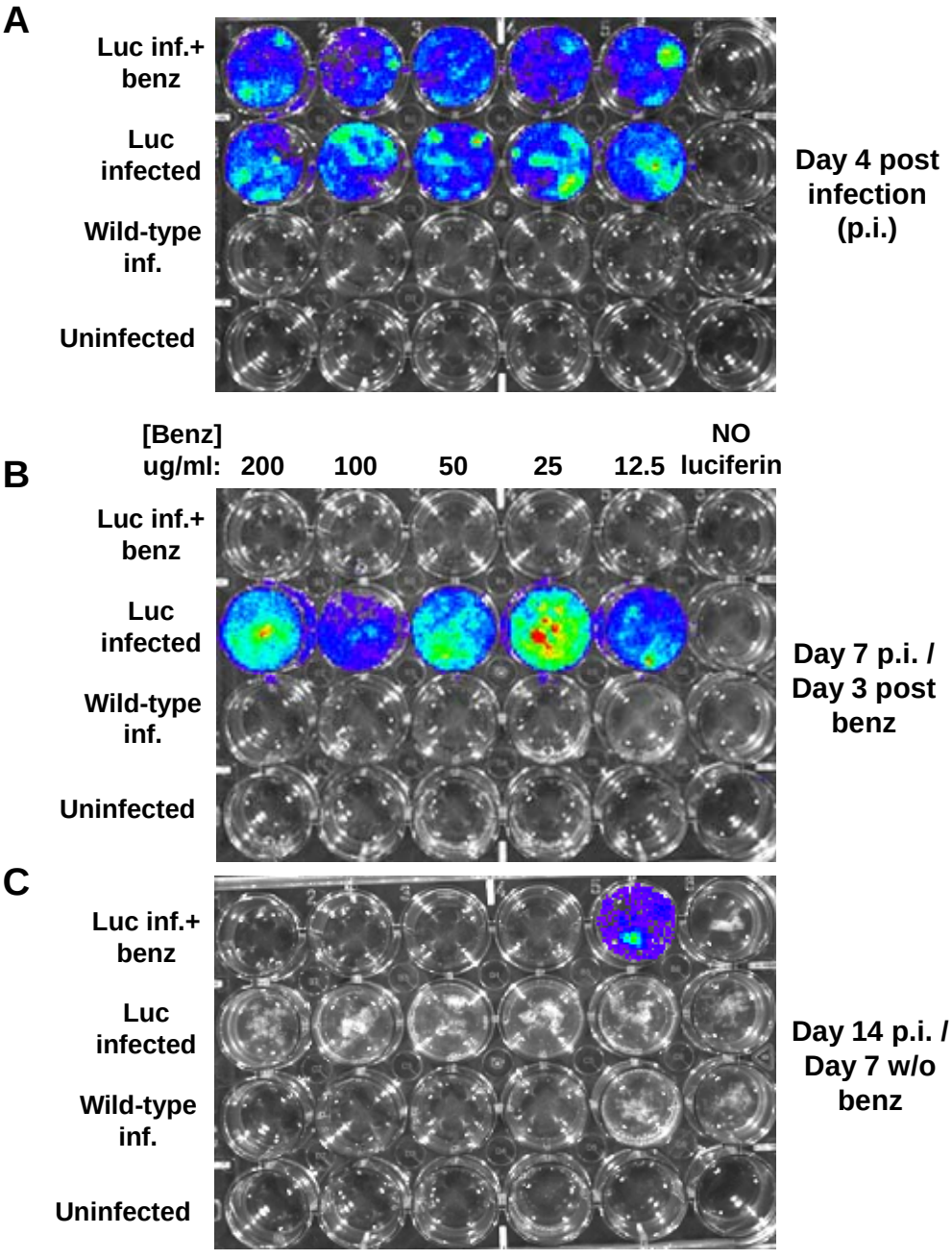
During the past several years several groups have employed IVIS technology to a variety of parasitic infections (202, 215). Recent applications to *Toxoplasma* gondii virulence have determined the importance of IFN-γ and Toll-like receptor signaling to parasite dissemination to the central nervous system following immunosuppression (216) or to overall host resistance (217), respectively. These studies were made possible using transgenic, knockout mice coupled with multiple strains of luminescent *Toxoplasma*. Others have used multiple strains to determine differences in replication capacity over time and to examine the reactivation of parasites during a chronic infection (218). Dissemination patterns resulting from different routes of *Toxoplasma* infection have also been analyzed (219). In addition to *in vitro* drug screening, bioluminescent *Leishmania amazonensis* has also been used *in vivo* and *ex vivo* to examine the response to various therapeutics on both living mice and extracted, parasitized organs (206). Results of our study confirm the applicability of IVIS technology for the study of Chagas disease pathogenesis. In addition to the experiments conducted in other parasitic disease models, such as routine *in vitro* drug screening (Figure 22), we will now able to address questions pertaining to the relevance of parasite burden to the magnitude of organ-specific autoimmunity (55, 56), conduct rapid screening of new potential parasiticidal drugs and test strategies for vaccine development against this parasite.

Acknowledgements

We are grateful to Dr. Wesley Van Voorhis for the generous gift of the pBS-THT-x-T plasmid which was easily modified for use in transfection, and to Dr. Dixon Kaufman and Courtney

Figure 22. Imaging of luminescent *T. cruzi* subjected to benznidazole *in vitro*.

Luminescent trypomastigotes were used to infect rat myoblasts at a ratio of 5:1. Wild-type trypomastigotes and uninfected myoblasts were used as controls. (A) Four days post infection, D-luciferin was added to each well and the plate was imaged with the Xenogen IVIS system after a 5-min incubation (see Materials and Methods for detailed description). (B) Various concentrations of benznidazole were added to the top row of the plate for a period of 3 days. D-luciferin was again added and the plate was imaged as previously described. (C) After thorough washing and elimination of drug, the plate was imaged 14 days post original infection, 7 days after termination of treatment, where the reappearance of live parasites is observed in the sample treated with the lowest concentration of benznidazole. (Those wells not receiving drug for the duration of the study (row 2) display no signal at 14 days post infection due to parasite-mediated destruction of myoblasts leading to inactive infection.) In the pseudocolor images the luciferase activity or photon intensity ranges from the lowest intensity (blue) to highest intensity (red).


A
Luc inf.+ benz
Luc infected
Wild-type inf.
Uninfected
Day 4 post infection (p.i.)
[Benz] ug/ml:
200
100
50
25
12.5
NO luciferin
B
Luc inf.+ benz
Luc infected
Wild-type inf.
Uninfected
Day 7 p.i. / Day 3 post benz
C
Luc inf.+ benz
Luc infected
Wild-type inf.
Uninfected
Day 14 p.i. / Day 7 w/o benz

Larson for the use of and expert technical assistance on the IVIS 100 BLI instrument and software. This work was supported by NIH grant HL075822 (to DME) and predoctoral fellowship from the American Heart Association (to KVH). Sofya Asfaw assisted with the cloning process of the p-TXT-Luc-T plasmid used for transfection into *T. cruzi*. Cheryl Olson contributed to the overall experimental strategy and assisted with the completion of all Southern blots. Melvin Daniels performed the dissections and extraction of organs for imaging analysis.

4. CONCLUSION

A. Discussion of selected unpublished studies

i. Fluorescent parasite-based adoptive transfer strategy

Perhaps the most important and controversial question surrounding autoimmunity and Chagas heart disease is whether or not the cardiac autoreactivity is pathogenic or merely benign artifact. Although a variety of experiments performed in the past have presented results of successful adoptive transfer of autoimmunity from Chagasic animal donors to naïve recipients, appropriate measures to insure that transferred T cells are void of parasitic contamination have been largely overlooked. Inducing pathogenesis upon transfer of autoreactive T cells from infected mice, in the absence of parasites, would serve as direct evidence for autoimmunity as a contributing factor to Chagas disease. In order to adequately control for potential parasitic contamination of transferred T cells, I generated a transgenic line of parasites engineered to express red fluorescent protein (RFP). Initially the dsRed monomer gene was cloned into a tubulin-based expression plasmid, containing the Hyg fusion for hygromycin resistance selection. While RFP expression was initially verified with fluorescence microscopy of live parasites, more detailed analyses illustrated that 100% of parasites were not expressing detectable levels of RFP, suggesting that either the gene had not been properly integrated into the parasite genome (suggested by Southern blot; not shown) or that the levels of RFP were below the limit of detection by the measures being used. The adoptive transfer strategy, illustrated schematically in

Figure 23, for the RFP+ trypanosomes was to employ them in a standard infection where the red fluorescence could be used as a means of exclusion of parasite-positive cells from the T cell suspension to be used for eventual transfer. To improve the quality of our transgenic parasites, a new red fluorophore (mCherry), cited for its superior brightness and photostability (220) was used to replace dsRed monomer in the fluorescent trypanosomes. Notably, 100% of mCherry parasites appeared to express detectable levels of this protein microscopically (Figure 24) but significant variability of expression was observed when using flow cytometry (Figure 25). When using untransfected *T. cruzi* to establish background fluorescence levels, a large fraction of transfectants displayed fluorescence indistinguishable from wild-type trypomastigotes. To circumvent this issue, single-cell clones were derived from the highest expressors, purified from flow cytometric sorting. Much to our surprise, the expansion of this highly fluorescent single-cell clone resulted in a complete redistribution of fluorescence intensity (Figure 26). Interestingly, single-cell clones derived from low-expressing cells also displayed this pattern of intensity distribution following expansion. This phenomenon was also recently reported by other investigators using fluorescent trypanosomes (221) where the authors speculated that additional recombination events could be occuring after drug selection or cloning procedures. Another possibility is the linkage of the transfected gene to the differential expression of tubulin that has been observed throughout the distinct life cycle stages of *T. cruzi* (222).

Using FITC-conjugated, anti-CD3 antibody, intial trials were performed to isolate purified T cells from infected animals, gating out and excluding mCherry+ cells. As expected, the differential expression of mCherry precluded the successful acquisition of parasite-free

Figure 23. Schematic of proposed adoptive transfer strategy using fluorescent *T. cruzi*. Donor mice will be infected with red-fluorescent trypanosomes for a period of 21 days. At that time, spleens and lymph nodes will be isolated and single cell suspensions prepared by conventional methods. In order to prevent parasite contamination of cell preparations, FITC-conjugated anti-CD3 antibody will be used to label all T cells. Using flow cytometry, all FITC+ cells will be purified while excluding any red cells (representative of fluorescent *T. cruzi*). The isolated T cells, representing the autoreactive population, can then be transferred to a naïve recipient that can then eventually be analyzed for autoimmunity and/or myocarditis.

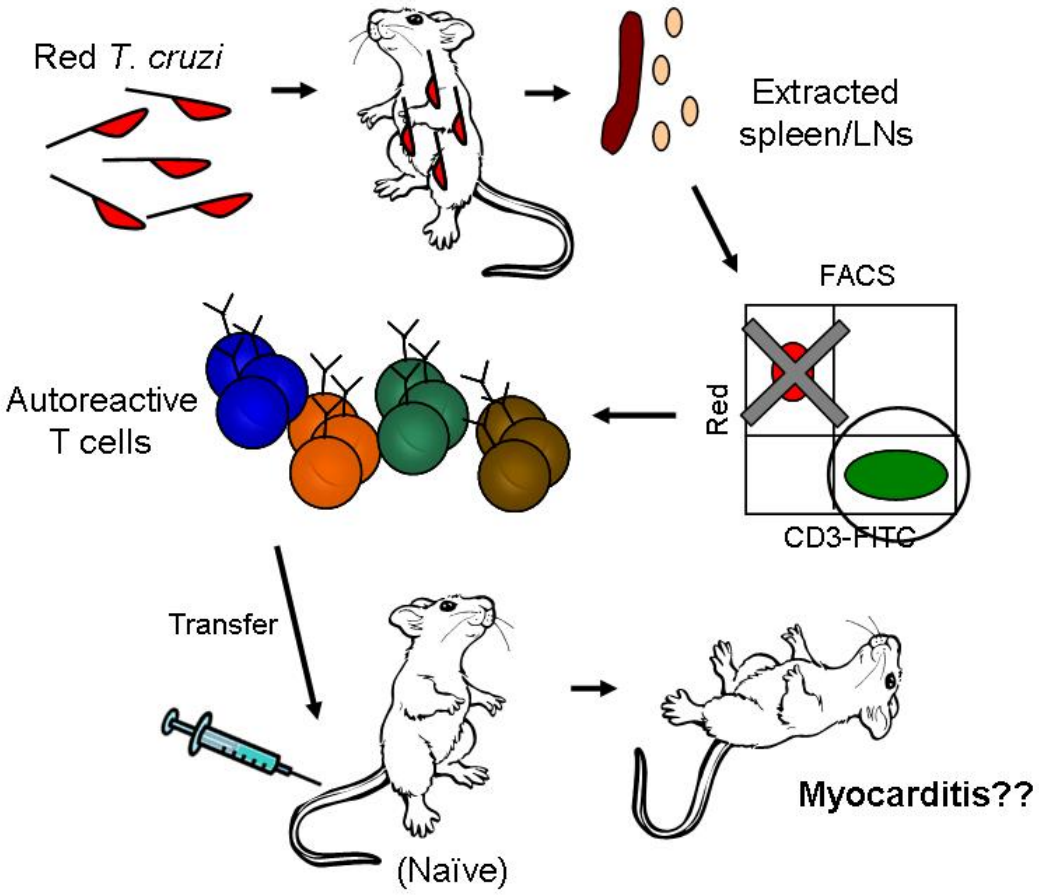

Red *T. cruzi*
Extracted spleen/LNs
FACS
Red
CD3-FITC
Autoreactive T cells
Transfer
(Naïve)
Myocarditis??

Figure 24. Fluorescence microscopy of mCherry *T. cruzi.*

Fluorescence/phase contrast microscopy of pTHT-RFP (mCherry)-T transfected Brazil *T. cruzi* epimastigotes. High magnification images (100x) of mCherry (ex-587nm, em-610nm)-transfectants illustrate 100% fluorescence among the drug-resistant population, of variable intensity. Blue fluorescence represents DAPI staining of nuclear and kinetoplast parasite DNA. mCherry is generally preferred over DsRed for its superior photostability, brightness and capabilities of detecting low-level expression (220).

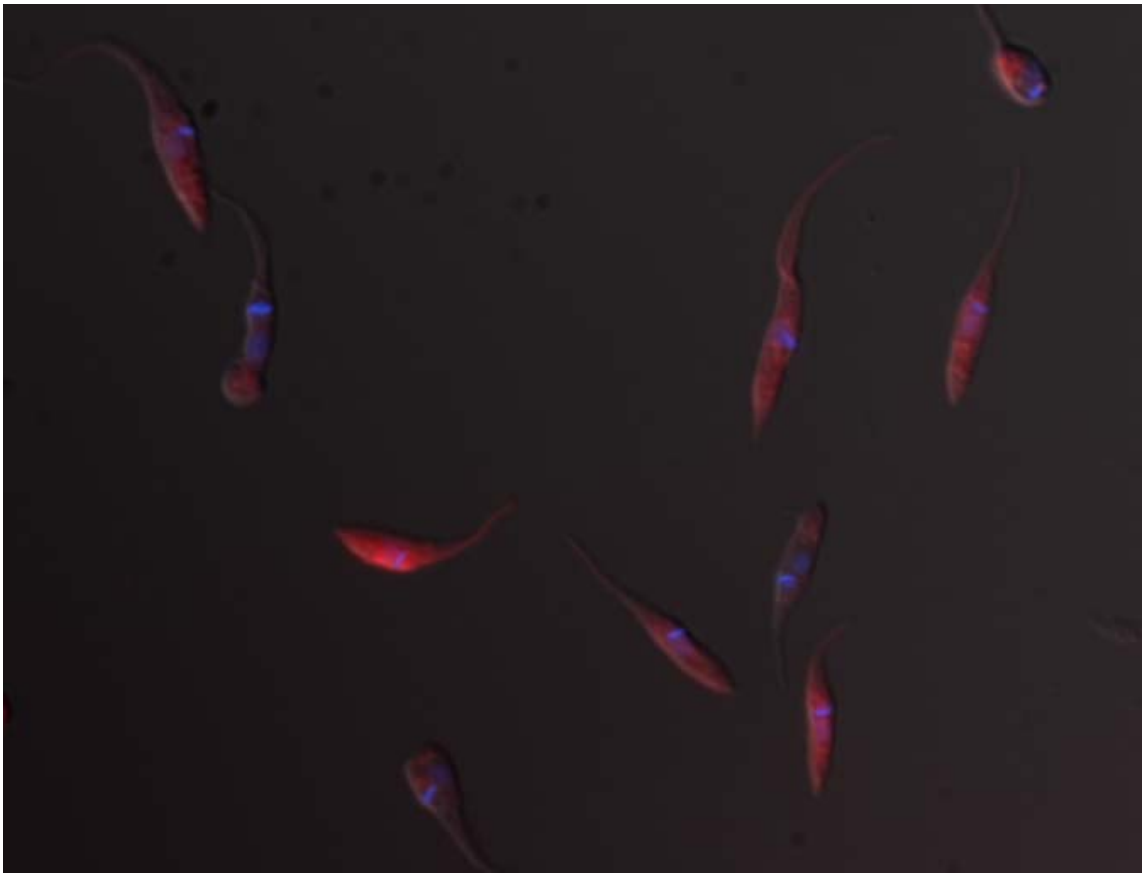
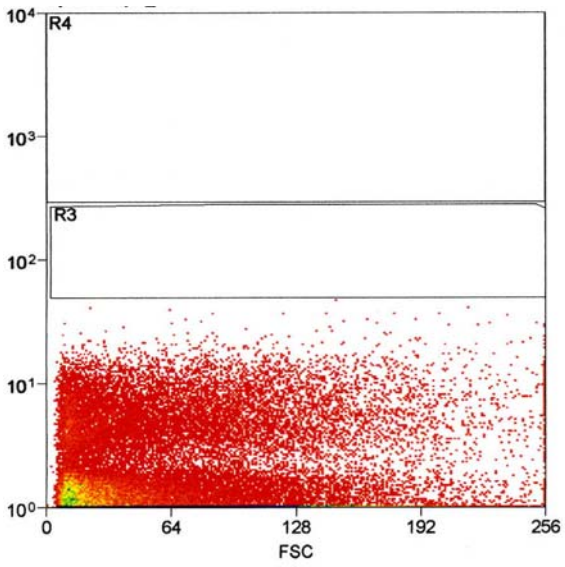
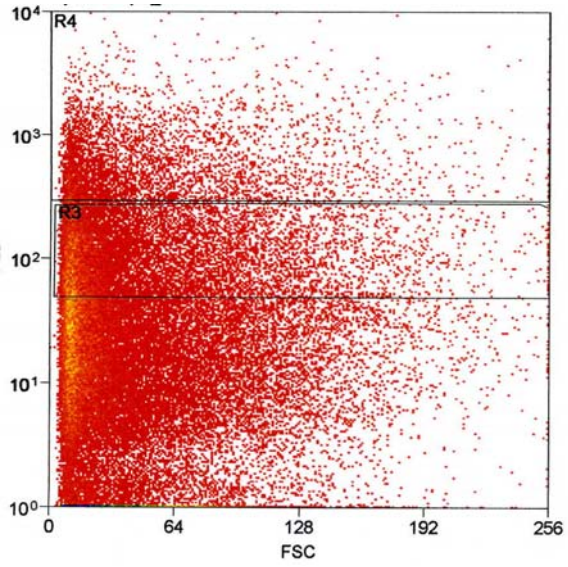


Figure 25. Flow cytometric analysis of mCherry *T. cruzi* fluorescence distribution. Fluorescence-activated cell sorting of mCherry-expressing *T. cruzi* epimastigotes. Transfectants were maintained under hygromycin drug selection in liver digest neutralized medium (LDNT) for a period of 4 weeks. Based on intensity of fluorescence, both wild-type Brazil strain and RFP+ parasites were sorted using a DakoCytomation MoFlo cell sorter. Gates R4 (high) and R3 (intermediate) were based on background fluorescence of wild-type, non-transfected epimastigotes. Parasites from R4 gate (high expressors) were obtained for subsequent single-cell cloning and expansion. The Y-axis represents fluorescence intensity, X-axis forward scatter profile.



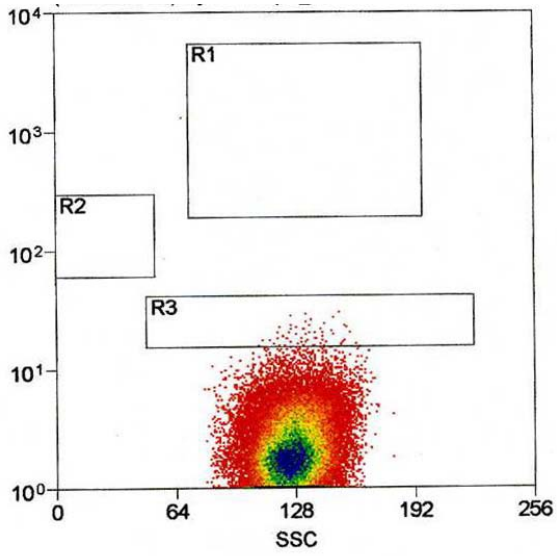
Wild-type control



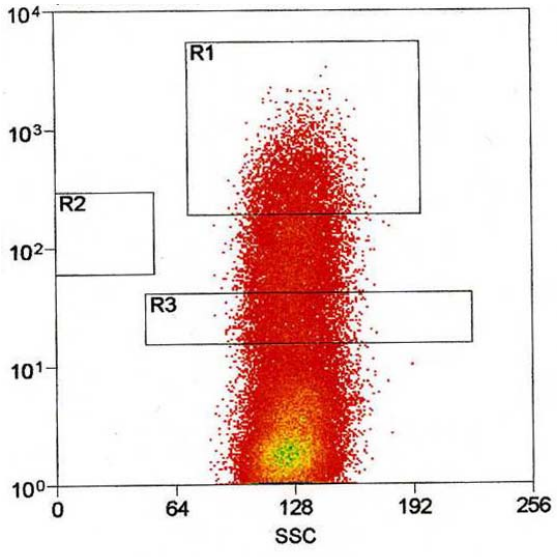
mCherry+

Figure 26. Re-distribution of fluorescence intensity from single cell clone isolated from "high-expressing" gated population.

Purified epimastigotes expressing high levels of mCherry were obtained flow cytometrically with a DakoCytomation MoFlo cell sorter. Serial dilution of this population in a 96-well plate using conditioned liver digest neutralized medium (LDNT) with hygromycin was performed for a period of 3 weeks, at which time a clonal population was expanded for further analysis. Using wild-type Brazil strain epimastigotes as a basis for background, the "high-expressing" clonal population was analyzed for fluorescence intensity via flow cytometry. Although cloned from a high-expressing population (R1 gate), the clonal population displayed a complete redistribution of fluorescence observed in the original polyclonal population (see Figure 25). The Y-axis represents fluorescence intensity, X-axis side scatter profile.



Wild-type control



mCherry+ clone

lymphocytes, since many parasites fell outside of the excluded population. Although these results were certainly unfortunate for the completion of a novel adoptive transfer strategy, these transfectants have since been useful in *in vitro* invasion studies where 100% fluorescence is not essential. From the standpoint of adoptive transfer, current studies exploring the ability to transfer autoimmunity using our purely autoimmune model of myocarditis are being performed, which will serve as a critical positive control for eventual Chagas disease transfer. The inability to use fluorescence-based exclusion will encourage alternative means of avoiding parasitic contamination. Despite its general toxicity or potential interference with normal lymphocyte activation properties, the optimization of benznidazole administration to T cells prior to transfer may be the best option to eliminate live parasites and move forward with this line of investigation.

ii. The pathogenic potential of T. cruzi protein lysate immunization

While investigating a potential mechanism by which autoimmunity and pathogenesis is induced in infected mice, it was previously shown that *T. cruzi* epimastigote lysate (completely void of viable parasites)-immunized mice develop cardiac myosin-specific DTH and autoantibodies (133). This autoreactivity was shown to be *T. cruzi* specific since immunization with lysates of *Leishmania amazonensis,* a related protozoan, did not induce myosin-specific DTH. Despite the reproducibility of this result, clearly illustrating crossreactive immunity (shown to be bidirectional with parasite-specific DTH responses observed in myosin-immunized animals and tolerization with parasite or myosin “cross-tolerizing” the other antigen), these mice have never developed significant myocarditis typically seen following either infection with live *T. cruzi* or

immunization with cardiac myosin. While the establishment of consistent autoreactivity resulting from parasite lysate immunization provides clear evidence of molecular mimicry in this experimental system, this mechanism has not appeared sufficient to drive myocardial pathogenesis.

The obvious presence of autoreactivity in the absence of disease pathogenesis prompted me to more appropriately examine the disease-inducing capability of parasite antigen by using the mammalian, infectious parasite life cycle form, trypomastigote, as a primary immunogen. Other models of infection-induced myocarditis require optimal Toll-like receptor (TLR) stimulation to attain an effective innate, and subsequent adaptive, immune response. Since TLR2 (223-227), TLR4 (228, 229) and TLR9 (227, 230) have been cited as critical modulators of *T. cruzi* induced disease, I administered the corresponding ligands, peptidoglycan, lipopolysaccharide (LPS) and unmethylated CpG DNA to trypomastigote lysate-immunized mice. The ability of these agonists to boost innate immune responses, ultimately contributing to efficient and robust adaptive immunity are critical in both the clearance of a live pathogen but can contribute to overall inflammation responsible for the cardiac damage typically observed in models of acute Chagas disease. As expected, I observed significant cellular (DTH) and humoral myosin autoimmunity in all groups, some of which was enhanced with TLR stimulation. Although moderate *in vitro* T cell proliferation was seen in response to parasite lysate stimulation, I detected negligible host-specific responses to recombinant fragments of cardiac myosin. Most importantly, I did not observe myocarditis in the immunized animals, suggesting that either (i) other proinflammatory immune components (perhaps more related to the specific

adaptive immune response) are required for pathogenesis or (ii) non-viable parasites are not a sufficient immunogen to induce pathogenesis.

I addressed the first possibility by investigating which cytokines were predominantly observed in the context of a live *T. cruzi* infection. Other experimental models of acute Chagasic cardiomyopathy indicate that high levels of proinflammatory cytokines IL-12 (22), IL-1β (122), TNF-α (231), IL-6 (232) and IL-17 are observed in the myocardium of infected animals which may further exacerbate the pathology and myocardial function. In the absence of an infectious agent (live parasite) the induction of proinflammatory conditions, perhaps essential to the development of myocardial pathology, may be absent as well. To account for this deficiency, I administered each of the indicated cytokines either individually or together to skew toward either a T-helper 1 (Th1) (IL-12/TNF-α) or Th17 (IL-17/IL-6) profile along with the trypomastigote lysate immunization. Although these mice displayed myosin-specific DTH responses and notable *in vitro* T cell activation to both parasite and host proteins, there appeared to be no enhancement of responses due to cytokine supplementation nor gross or histological signs of myocarditis.

The immune response generated following parasite protein immunization is clearly very different from that expected during a live infection. As such, the final strategy I used in testing the hypothesis that parasite antigen immunity and the subsequent evolution of host immunity are sufficient to induce myocarditis was to employ infectious agents as “adjuvants” in our immunization regimen. Recapitulating every factor – whether it be innate immune stimulant, cytokine or any mixture of inflammatory mediators, at physiologically relevant concentrations – associated with a live infection is unrealistic. By administering live pathogens that could either

be cleared or maintained in such a way as to not elicit any myocardial damage independently, natural immune responses, consisting of inflammatory components, could supplement the environment typically created during a live *T. cruzi* infection. I chose to use pathogens that were readily available and were closely related to our working disease model. Since I had already shown benznidazole to be effective at reducing parasite load and preventing myocarditis, but not directly interfering with host immune responses (Chapter 2), I used this treatment along with virulent Brazil strain trypomastigote infection in one experimental group. Other groups included infections with a previously identified, non-virulent clone of *T. cruzi*, the insect, epimastigote form of *T. cruzi* (which is known to be effectively cleared in mammals) and *Leishmania major* promastigotes (a related protozoan). The intraperitoneal administration of these infectious agents was provided concurrently with the subcutaneous parasite lysate immunization. Since experimental models of Chagas and autoimmune myocarditis are used routinely in our lab, I used both of these models to serve as positive controls for the development of heart-specific pathology. Not surprisingly, all animals immunized with *T. cruzi* lysate produced myosin-specific DTH and antibodies, as previously observed. However, as also previously observed, none of the experimental groups displayed signs of cardiac inflammation. Although all control animals infected with virulent *T. cruzi* displayed typical signs of gross and histological disease, mice immunized with Myo4 or cardiac myosin (our purely autoimmune immunization controls for myocarditis) unexplicably failed to produce any cardiomyopathy. Therefore, despite the lack of evident myocarditis resulting from this experimental procedure, the lack of myocarditis in our immunization control animals make it difficult to establish firm conclusions from this preliminary study at this point.

This final strategy was designed to test the possibility that the absence of myocarditis following immunization with parasite proteins is simply a result of missing inflammatory conditions typically present during infection. It is certainly possible for this not to be the case. As stated, no firm conclusion can be made at this point in the absence of valid controls, but after conducting numerous experiments geared toward this hypothesis, several complications of such a line of investigation can be addressed. First, it is very possible that live, virulent trypanosomes are essential for the induction of myocarditis. How? One possibility is that it is the infectious process of cardiomyocytes and subsequent destruction of tissue that causes the massive inflammatory infiltrate to the organ. The actual damage incurred through rounds of parasite replication and release could be vital to the initiation of disease that could then be exacerbated by the proceeding immune response, parasite- or host-specific or a combination thereof. Another possibility is the type of immunity resulting from infection with an intracellular organism. Recreating the immune environment resulting from *T. cruzi* infection, where CD8+ T cells outnumber the CD4+ subset 3:1in the cardiac infiltrate, with a protein immunization that elicits a predominant CD4+ type response is challenging, if not unattainable. The cytolytic activity of CD8+ T cells in the myocardium, rather than crossreactive CD4+ lymphocytes, could certainly be largely responsible for the damage observed in Chagas disease. Even though the addition of non-cardiac pathogens could invoke CD8+ responses for clearance, the absence of cardiac tissue-specific infiltrate could preclude the evolution of myocarditis regardless of the simultaneous circulation of parasite immunity resulting from immunization.

Another complication arises when considering the overall proportion of crossreactive antigen being used as an immunogen. It is clear that molecular mimicry occurs in the context of

T. cruzi infection. However, immunizing with an unfractionated mixture of bulk parasite proteins provides a limited quantity of relevant crossreactive antigen. Assuming there are only a handful of proteins, or even specific epitopes, responsible for the induction of crossreactive autoimmunity, the immune system is essentially searching for a needle in a haystack for its crossreactive antigen. Autoimmunity is truly capable of inducing myocarditis (evidenced by our purely autoimmune model), but as with any disease model, the immune system requires a sufficient depot of antigen to which it recognizes and maintains activity against. It is unlikely that this sufficient quantity will ever be attained by bulk protein immunization. Rather, if the molecular mimcry mechanism of autoimmunty induction is going to be shown to be pathogenic, the specific parasite protein, or ideally the refined peptide responsible for the crossreactivity, would need to be used as an immunogen in an adequate concentration. Despite the lack of cardiomyopathy observed with parasite lysate immunization thus far, it is important to remember that molecular mimicry does indeed occur and although not necessarily independently pathogenic, certainly capable of contributing to an established and ongoing inflammatory response initiated through other mechanisms.

iii. Impact of benznidazole therapy on T. cruzi infection-induced autoimmunity

Many investigators performing benznidazole-related studies acknowledge the autoimmune component associated with Chagas disease, yet none have examined the impact of drug treatment on the antigen-specific autoreactive response. We clearly illustrated that treatment with benznidazole is capable of eliminating autoimmunity in the chronic phase of disease but that this response can be recalled following a particular stimulus (Chapter 2). To provide a more thorough

understanding of how the immune system is modulated to reduce the overall severity of disease, a more detailed examination of how benznidazole affects subsets of responders (parasite- or host-specific) was pursued. Specifically, I sought to (i) examine how the phenotype (activation and cytokine profiles) of *T. cruzi* infection-induced autoreactive T cells is altered with benznidazole treatment during infection and (ii) establish a mechanism for the reappearance of cellular autoimmunity upon secondary infection or immunization (*i.e.* evaluating whether memory T cell population is present).

The treatment of Chagas disease with benznidazole is known to reduce tissue parasitism, eliminate acute-phase symptoms, and curtail the course of infection. This drug has been reported to enhance macrophage-associated phagocytosis, induce the selective expansion of effector and central memory CD8+ T cells, and promote resistance to reinfection (163) in some instances. Benznidazole therapy has typically been associated with an overall increase in proinflammatory cytokine production, yet some investigators claim preferential upregulation of IFN-γ from parasite-specific NK and CD8+ cells only, coupled with a more regulatory, IL-10-producing CD4+ cell phenotype (233). In most cases, IL-12 and IFN-γ have been regarded as important components for maximum drug efficacy (165).

In order to adequately evaluate the impact of benznidazole on the myosin-specific autoreactive cell population in experimental Chagas, a number of cell surface markers of activation and memory phenotypes, along with intracellular cytokine antibodies were used. Since Myo4, a recombinant fragment of cardiac myosin, has been shown by our lab to induce myocarditis comparable to immunization with whole cardiac myosin, this slightly more refined, purified antigen was used as my *in vitro* stimulus for the analysis of the autoreactive population.

In addition, *T. cruzi* protein lysate was used to examine parasite-specific immunity. Cells cultured without antigen or with anti-CD3 (a non-specific stimulant to all T cells) were included as negative and positive controls, respectively. Splenocytes were harvested at either 13 or 21 days post infection to correspond to both peaks of adaptive immunity and pathogenesis, respectively. Finally, cells were stimulated with antigen *in vitro* for either 24 or 72 hours prior to analysis in order to provide time for the expansion of antigen-specific populations.

The existing discrepancies regarding the activation of T cells in benznidazole-treated mice or humans, in addition to other issues specific to our disease model, were repeatedly encountered in this line of investigation. For instance, at 13 or 21 days post infection, autoreactive T cells from benznidazole-treated animals appeared to have a diminished activation phenotype, with downregulation of CD44, lower production of IFN-γ and elevated CD62L expression compared to untreated animals. However, CD69 appeared to be upregulated following either Myo4 or parasite lysate stimulation. After a 72 hour incubation of these cells with antigen, CD44 and CD62L displayed similar patterns of expression whereas IFN-γ production was higher and CD69 expression was reduced in drug-treated versus untreated animals (results that were opposite of those seen after 24-hour stimulation). Since the expression of CD69 is involved with early activation events and is often transient in nature, it was not entirely surprising to observe changes in this marker with long incubation times. Interestingly, in comparing anti-CD3 stimulated cells across groups, CD69 was most highly expressed in naïve animals, suggesting some overall inhibition of activation capacity in infected animals, regardless of treatment. The ability of *T. cruzi* to dampen immunity in order to evade clearance by the host (208-211) could possibly account for this observation. However, the overall incongruity of the

results made it difficult to form any definitive conclusions regarding the impact of benznidazole on antigen-specific T cells during *T. cruzi* infection.

These inconsistencies, in addition to the overall lack of clear antigen-specificity, were addressed in subsequent experiments. While the cytokines being analyzed (IFN-γ, IL-4 and IL-17) are common indicators of activation, I shifted to using more typical and less ambiguous extracellular markers of activation (CD25 and ICOS). In order to enhance antigen-specificity, I utilized magnetic cell sorting in order to isolate purified CD4+ and CD8+ T cell populations. It seemed very unlikely that an antigen-specific population (which may account for a very minor percentage of overall cells) could be extracted from an environment where a live infection is active. In order to remove T cells from the infection-induced, hyperactivated environment where the observed results were most likely due to *in vivo* activity, rather than response to antigen in culture, purified CD4+ or CD8+ T cells were cultured with antigen in the presence of irradiated splenocytes from naïve animals as a source of antigen presenting cells. This would, in theory, eliminate all possible sources of non-specific activity and potentially the discrepancy of activation marker expression previously observed.

After many attempts with this strategy, this did not entirely prove to be the case. Despite the extraction of purified subsets of T cells, the extremely low percentage of antigen-specific, autoreactive cells often failed to yield results detectable above unstimulated cells and for the most part no differences between treated and untreated groups could be identified. In terms of parasite-specific immunity (analyzed by stimulation with *T. cruzi* protein lysate), there appeared to be enhanced activation of CD4+ T cells, with upregulation of CD25 and IFN-γ, early after infection (13 days post infection) that was diminished by 21 days post infection. One possible

explanation is that the administration of benznidazole contributed to a more efficient clearance of pathogen early after infection, indicated with elevated proinflammatory cytokine production, but this response waned in the eventual absence of parasites. In untreated animals, the proinflammatory environment becomes established as the parasite infects and persists in tissue, resulting in the continued production of IFN-γ, even during the peak of disease. Nevertheless, it was the autoreactive response, not the parasite-specific response that was being primarily investigated. The lack of differences observed between treated and untreated groups and the often equivalent levels of CD25 expression and IFN-γ production between stimulated and unstimulated cells suggests that either (i) benznidazole truly exerts no direct effect on the activation of autoreactive cells in this disease model or (ii) antigen-specific responses are not truly being generated *in vitro*. The first possibility would not be surprising since we observed no impact of benznidazole on the generation of both humoral and cellular autoimmune responses in a purely autoimmune disease model (Chapter 2; Figures 8-11). However, our ability to detect autoreactive antigen-specific proliferation of T cells from *in vitro* stimulation, using a variety of methods, has also been continually challenging. With limited proliferation observed using traditional tritiated thymidine uptake assays from *in vitro*-stimulated T cells, it was not surprising to encounter difficulty in detecting markers of activation via flow cytometry. Although the presence of myosin-specific autoimmunity is clear after parasite infection, the proportion of cells responding to host antigen is likely minimal in comparison to the pathogen-specific response. Furthermore, the specific epitope(s) responsible for the autoreactivity in our disease model are unknown, forcing us to administer a large protein fragment consisting of many irrelevant epitopes. Without a more refined system of peptide specificity, administering an adequate

concentration of antigen in culture is a concern. Whether or not benznidazole plays a direct role in the ability to alter an autoimmune response has been adequately addressed with our model of experimental autoimmune myocarditis (Chapter 2). The limitations of investigating the infection-induced, cardiac autoimmune response, as described, will continue to make more in-depth analyses of this response challenging until a more detailed understanding of its specificity is established.

B. Summary and Future directions

After the establishment of a murine model of Chagas heart disease in which mice display severe cardiac inflammation and fibrosis, parasite-specific immunity and strong cardiac immunity, a number of important questions followed. In part, my thesis research was focused on answering the basic question of the relationship between *T. cruzi* levels and the magnitude of autoimmunity. Since infection with various dosages of *T. cruzi* appeared to have no impact on the magnitude of cardiac myosin autoimmunity observed, I took a different approach to examine a potential correlation between parasite burden and autoreactivity. To do so, the trypanocidal drug, benzndizole, was administered to infected mice at various times post infection (Figure 5). The impact of this drug was evaluated in terms of not only autoimmunity, but also parasite-specific immunity and overall myocardial pathogenesis (Chapter 2). This drug is currently used for the treatment of Chagasic patients and was already known to reduce or eliminate *T. cruzi* and decrease disease in the acute phase. This was also seen in our disease model of A/J mice infected with the Brazil strain of *T. cruzi* where anti-*T. cruzi* immunity, parasitosis of heart

tissue, and disease were reduced or eliminated (Figures 6 and 7). Since we had previously observed the capability of parasite protein immunization to induce myosin-specific autoimmunity, it was not entirely obvious what to expect in this line of investigation. Defining a correlation between levels of parasite and the magnitude of autoimmunity would suggest an important role for live parasites in the induction and/or maintenance of the autoreactive response. However, no correlation would suggest that either brief or prolonged exposure to *T. cruzi* is sufficient to induce similar magnitudes of myosin autoimmunity. While we had already seen that molecular mimicry was a valid mechanism for the induction of autoimmunity, this line of research was designed to evaluate the importance of bystander activation, not only for a mechanism of autoimmunity induction, but also for its role in disease pathogenesis. The consistent absence of myocarditis following immunization with parasite antigen led me to hypothesize that the magnitude of autoimmunity would be directly proportional to the amount of damage caused by the parasite, since live parasites appeared to be crucial for the inflammation (likely consisting of parasite- and host-specific cells) observed in Chagas disease.

As detailed in Chapter 2, the hypothesis was validated when we observed reduced or eliminated myosin autoimmune responses in both acute and chronic phases, along with complete eradication of or limited disease severity. Evidence of a correlation of the waning autoimmune response to the presence of parasites was illustrated by the fact that, when given time to establish infection and expand (*i.e.* treatment not initiated until two weeks post infection), parasites elicited normal autoimmune responses not significantly different than those observed in untreated animals (Figure 6). In other words, as long as the parasite levels were controlled with drug within the first week of infection, the development of cardiac autoimmunity was limited.

Therefore, a brief exposure to *T. cruzi* is insufficient to induce magnitudes of autoimmunity typically observed following longer exposure, whether it be from persistent infection or immunization with parasite antigen. This suggests that although molecular mimicry is a valid mechanism of *T. cruzi* infection-induced autoimmunity, it is likely that live parasite-induced bystander activation is responsible for the potentially pathogenic autoimmunity that ensues in the heart tissue.

Myosin autoimmunity failed to persist in the absence of *T. cruzi*, indicating that tolerance had been effectively reestablished after drug treatment and parasite clearance. We then sought to determine whether there would then be permanent protection from either secondary infection or another form of cardiac insult. The restoration of both myosin-specific autoimmunity and moderate disease showed that although the majority of myosin-specific T cells became unresponsive after the pathogen was cleared, a significant secondary stimulus could not only reactivate this population, but could also elicit recruitment and subsequent damage to cardiac tissue. The persistence of a dormant population of autoreactive memory T cells was likely responsible for such a recall response, but it is possible that after the return to a state of homestasis, free from inflammatory conditions, the immune system responded to the secondary insult as if it were its first encounter (*i.e*. the restored autoimmune response was simply a newly generated action against cardiac injury). Interestingly, only reinfection with a heart-derived, more virulent strain of *T. cruzi* or immunization with cardiac myosin resulted in the restored autoreactivity and myocarditis. The fact that reinfection with the original Brazil strain, from which the mice were adequately protected, did not result in myocarditis or autoimmunity strongly suggests and further supports the notion that, in the context of Chagas disease, live

parasites are necessary for inducing myocyte damage and releasing abundant amounts of cardiac antigen required to break self-tolerance, invoke an autoimmune response and ultimately cause inflammatory heart disease. If it were only the presence of *T. cruzi*-specific immunity required for autoimmunity and subsequent pathogenesis, we would have expected to observe the restoration of anti-myosin immunity, even in the animals capable of clearing the secondary infection.

The reduction of live parasites drastically lessens or eliminates myosin autoimmunity most likely by either attenuating the overall extent of cardiomyocyte damage and/or lowering the number of immunologically similar pathogenic epitopes (parasite antigens) to which crossreactive T cells can react. While these two possibilities correspond to bystander activation and molecular mimicry, respectively, the reported findings tend to favor bystander activation as a particularly important mechanism by which autoimmunity occurs during *T. cruzi* infection. Whether or not the autoimmune response is vital to the pathogenic process is still not entirely clear from this study. However, perhaps the most important implication derived from this study is the notion that elimination of the parasite is clearly the best strategy to eliminate autoimmunity, uphold self-tolerance and prevent disease. Despite the mechanism responsible for the induction of autoimmunity or the certainty of its benign or pathogenic nature, an effective *T. cruzi* vaccine could theoretically prevent parasite associated damage and autoimmunity. This is particularly important in the field of Chagas disease research, since several investigators have claimed that the autoimmunity hypothesis has strongly discouraged the exploration of vaccine development. Being able to potentially discontinue usage of a fairly non-specific drug like benznidazole, that has significant collateral effects on patients, for the treatment of infection

would be a substantial clinical advance for the millions affected by Chagas. Knowing that autoimmunity can be controlled, regardless of its potential contribution to inflammation, by controlling the infection should strongly promote the further engineering of prophylactic treatment for this dreadful disease.

Since the hesitation associated with vaccine development stems from the potential autoreactivity that might result from immunization with a crossreactive *T. cruzi* antigen, it would be interesting to examine the autoimmunity-inducing potential of already identified vaccine candidates in animal models of disease (234-238). Many of these studies have overlooked the very aspect of vaccine development that is hindering the progression of this strategy to a more clinically focused trial. Investigating the ability of established vaccines to induce autoreactivity in an animal disease model known to be autoimmune-susceptible would be the most appropriate way in which to confirm that elimination of the parasite, even via prophylaxis, is capable of preventing the onset of autoimmunity and subsequent pathogenesis. Even if immunization with a particular parasite antigen were shown to induce an autoreactive response, the enhanced immunity against the live pathogen may prove to supercede the onset of autoimmunity. Our studies, thus far, have not shown the presence of parasite- and host-specific immunity to be sufficient for disease induction, making the targeting of live parasites the obvious primary objective for treatment.

Another aspect of my thesis research was centered on investigating the natural course of *T. cruzi* infection. In general, the cardiac abnormalities and gastrointestinal disorders are often the focus of Chagas research, since the majority of symptomatic patients display alterations of

these organs in the chronic phase of disease. The tissue tropism of *T. cruzi* is known to vary among strains, but it has been established that neuronal and muscle cell types are often targeted. However, the occasional finding of *T. cruzi* in other organ systems, either in experimental models or human patients makes the understanding of the spatiotemporal distribution of the parasite a matter of interest. As such, I set out to adapt a bioluminescence imaging strategy to analyze the natural course of infection in our experimental Chagas model in a non-invasive manner (Chapter 3). The transfection of Brazil strain *T. cruzi* with the gene encoding the firefly luciferase enzyme, capable of producing light upon interaction with its luciferin substrate, successfully enabled the non-invasive monitoring of parasite dissemination over the course of time. In addition to the *in vivo* utility, the development of this tool was also found to be useful for *in vitro* processes as well (Figures 17 and 22). Although preliminary studies illustrated the ability to simply image *in vitro* infections and test the trypanocidal activity of drugs, more in depth studies of the invasion process could be greatly facilitated with this novel parasite line. The ability to visualize and quickly quantify parasite burden in animals or culture is an exciting advancement for numerous future studies.

Following the confirmation of luminescence in my transfectants (Figures 15 and 17), some studies were needed to determine the sensitivity of the imaging instrument for luminescent trypanosomes in mice. While these experiments, using different infection dosages in A/J mice, were intended to provide an idea of limitations of the imaging technology, the results of the timecourse analysis between groups proved to be very interesting. Although some animals were infected with 10- or even 100-fold more trypomastigotes, all groups displayed equivalent magnitudes of peak parasite burden at the same timepoint (d14 post infection), despite significant

differences early after infection (Figures 18 and 19). This observation corresponded well with previous findings where, regardless of infection dosage, animals displayed equal magnitudes of cardiac autoimmunity. In future experiments, it would be interesting to determine whether the magnitude of parasite-specific immunity changes over time to directly correlate with the differential parasite burdens observed within the first two weeks of infection. Also, since we have previously observed cardiac autoimmune responses as early as 7 days post infection (55), the analysis of this autoreactive response over time would be very informative as well. Based on earlier findings, one would expect the magnitude of cardiac autoimmunity to increase as parasite burden increases, followed by a sharp decline and plateau as parasite levels are controlled. However, in this instance, parasites are not being *eliminated* as was the case with drug treatment, but rather just being controlled. It is likely that myosin-specific immunity would be present early after infection and increase as the parasites took up residence in and began eliciting damage of heart tissue. Whatever the timecourse of autoimmunity may be, this experiment suggested that regardless of the infection dose used, levels of parasitism are gradually equilibrated, likely accounting for the equivalent magnitudes of autoimmunity observed at the peak of disease in other experiments.

The thorough analysis of dissemination in our standard experimental model illustrated the spreading from the site of infection, to what appeared to be a large part of the body, to gradual specific sites of organ habitation (Figure 20). The subsequent analysis of organs provided expected (parasites in heart, skeletal muscle and intestines) and surprising (parasites in kidneys and lungs but absent in reticuloendothelial organs) results (Figure 21). As is the case with any novel approach, the nature of these experiments produced a plethora of new questions and

applications. First and foremost, the sensitivity of this method of quantification must be thoroughly addressed in order to provide a reliable basis for comparison. As previously stated, methods of detection such as PCR that have proven to have superior sensitivity will likely continue to be needed for analyses when very small numbers of organisms are involved. However, by carefully analyzing the limit of detection of luminescence from *T. cruzi*, the quantity of parasite burden (measured in emitted photons) could be used as a correlative to such established methods. Specifically, determining what number of parasites corresponds to a particular photons/sec/cm^2 output will be very useful for future studies. Such studies will consist of an examination of the impact of the route of infection on parasite dissemination, an analysis of the course of infection in disease-susceptible and resistant mouse strains as well as with disease-inducing and benign strains of parasite (which will need to be engineered to express luciferase). The finding of intense parasitism in the intestines was not surprising when considering the relevance of this organ to Chagas disease, yet being so close to the site of intraperitoneal infection, it will be interesting to determine whether other sites of administration (subcutaneous, intravenous, oral) still produce similar patterns of dissemination. Also, those mice that are capable of clearing infection without signs of pathogenesis will be imaged to determine if parasites proliferate and expand throughout the body or are immediately cleared by the immune response.

Other areas of interest are to investigate the organs that appeared to display parasitosis in our *ex vivo* imaging (Figure 21). Although it is accepted that *T. cruzi* can infect virtually any cell type, the preference for muscle and nervous tissue has placed a focus on the corresponding organs. Since we observed parasites in lung, kidney, and intestines in our model that has been

designed for analysis of heart disease, it will be interesting to determine whether these organs are merely harboring parasites or if the infection of these tissues is causing damage and inflammation. Since we have determined that harvesting and expanding parasites from the hearts of mice results in a more virulent, cardiotropic population (56), the ability to force tropism to specific organs by repetitive rounds of harvesting and infection will be investigated. Finally, the importance of immune factors (Toll-like receptor signaling, specific cytokine environment) to *T. cruzi* dissemination and/or resistance will be examined by incorporating various conditional knockout animals into our studies; much like what has been completed for other infectious systems (216, 217).

C. Final remarks

A disease in which multiple pathogenic mechanisms are integrated and operative to different extents depending on the immunogenetics of the host, the virulence of the parasites, and a number of other physiological factors is a considerable challenge to research. Attempting to break down these mechanisms into individual entities would be considered by some to be futile. After all, a pathogenic process, such as Chagas disease, consists of intracellular parasites that not only elicit major damage by rupturing any cell they inhabit, but also cause the evolution of a massive immune response as the host desperately strives to clear the infection, as it would any other foreign pathogen. As the immune system accelerates by calling on all essential cytokines, chemokines and other inflammatory mediators, droves of macrophages, neutrophils, eosinophils, and likely an array of other subsets, are set into motion. With the sole intention of clearing the

foreign pathogen before too much damage is done, the immune system cannot avoid the destruction of some innocent bystanders and perhaps becomes confused in some instances about what is foreign and what only looks to be foreign but is, in actuality, part of its own body. Autoimmunity ensues, throwing yet another curveball into an already confounded scenario of parasite proliferation, persistence and damage and the considerable effort being made to eradicate the foreign body entirely. Amidst all of the activity, how important can the autoimmune factor really be? One would be tempted to merely brush it aside as an insignificant process that was just an unfortunate accident – a brief lapse in judgment by a hyperactive immune system. However, the fact that this very autoimmune response is capable of driving myocardial pathogenesis on its own in the right conditions begs for reconsideration of its potential importance. It is, after all, part of the pathogenic process – perhaps not the most important part, but a part nonetheless.

It is for this reason that we embrace the challenge of Chagas disease research and strive to understand just how important autoimmunity is and whether it truly should be a major concern. The complex nature of the disease undoubtedly makes it difficult to come to any concrete answers, but discoveries are built on incremental progress. Despite the apparent failures and unproven hypotheses encountered during my thesis research, I have successfully provided an encouraging basis for the continued exploration of vaccine development for Chagas disease by showing that clearance of the parasite, by whatever means, will likely remove autoimmunity and all other confounding mechanisms of pathogenesis from the equation. In addition, I have taken advantage of new and exciting technology by applying the ability to temporally monitor a live process within the same animal to *T. cruzi* infection. This tool will

open doors not only for our own lab to pursue but for many others already interested in exploring biological and physiological processes of *T. cruzi* in a non-invasive, visual manner. Chagas disease research will continue to present numerous challenges, regardless of my individual, incremental progress. Nevertheless, the scientific community owes it to the millions of people currently infected with *T. cruzi* or suffering from chronic Chagas disease to continue to make these incremental strides. Single significant discoveries are simply not possible without a multitude of incremental findings.

REFERENCES

1. Kirchhoff, L. V., L. M. Weiss, M. Wittner, and H. B. Tanowitz. 2004. Parasitic diseases of the heart. *Front Biosci* 9*:*706-723.

2. Moncayo, A. 2003. Chagas disease: current epidemiological trends after the interruption of vectorial and transfusional transmission in the Southern Cone countries. *Mem Inst Oswaldo Cruz* 98*:*577-591.

3. Moncayo, A. 1999. Progress towards interruption of transmission of Chagas disease. *Mem Inst Oswaldo Cruz* 94 Suppl 1*:*401-404.

4. Kirchhoff, L. V., P. Paredes, A. Lomeli-Guerrero, M. Paredes-Espinoza, C. S. Ron-Guerrero, M. Delgado-Mejia, and J. G. Pena-Munoz. 2006. Transfusion-associated Chagas disease (American trypanosomiasis) in Mexico: implications for transfusion medicine in the United States. *Transfusion* 46*:*298-304.

5. Nowicki, M. J., C. Chinchilla, L. Corado, L. Matsuoka, R. Selby, F. Steurer, T. Mone, R. Mendez, and S. Aswad. 2006. Prevalence of antibodies to *Trypanosoma cruzi* among solid organ donors in Southern California: a population at risk. *Transplantation* 81*:*477-479.

6. Prata, A. 1994. Chagas' Disease. *Infect Dis Clin North Am* 8*:*61-77.

7. De Souza, W. 2002. Basic cell biology of *Trypanosoma cruzi*. *Curr Pharm Des* 8*:*269-285.

8. Nickerson, P., P. Orr, M. L. Schroeder, L. Sekla, and J. B. Johnston. 1989. Transfusion-associated *Trypanosoma cruzi* infection in a non-endemic area. *Ann Internl Med* 111*:*851-853.

9. Leiby, D. A., E. J. Read, B. A. Lenes, A. J. Yund, R. J. Stumpf, L. V. Kirchhoff, and R. Y. Dodd. 1997. Seroepidemiology of *Trypanosoma cruzi*, etiologic agent of Chagas' disease, in US blood donors. *J. Infect. Dis.* 176*:*1047-1052.

10. Leiby, D. A., R. M. Herron, Jr., E. J. Read, B. A. Lenes, and R. J. Stumpf. 2002. *Trypanosoma cruzi* in Los Angeles and Miami blood donors: impact of evolving donor demographics on seroprevalence and implications for transfusion transmission. *Transfusion* 42*:*549-555.

11. Dias, J. C. 2000. [Epidemiological surveillance of Chagas disease]. *Cad Saude Publica* 16 Suppl 2*:*43-59.

12. Schmunis, G. A., and J. R. Cruz. 2005. Safety of the blood supply in Latin America. *Clin Microbiol Rev* 18*:*12-29.

13. Dodd, R. Y. 2004. Current safety of the blood supply in the United States. *Int J Hematol* 80*:*301-305.

14. Chocacir, P. R., E. Sabbage, and V. Amato Neto. 1981. Transpante de rim: Nova modalidade de transmicao de doenca de Chagas. *Revista Instituto de Medicina de Sao Paulo* 23.

15. Jorg, M. 1992. Enfermedad de Chagas con puerta de entrada en el tracto digestivo. *Centro Medico Publicacion Medica, Argentina* 5*:*71-88.

16. Tanowitz, H. B., L. V. Kirchhoff, D. Simon, S. A. Morris, L. M. Weiss, and M. Wittner. 1992. Chagas' disease. *Clin Microbiol Rev* 5*:*400-419.

17. Pontes de Carvalho, L., C. C. Santana, M. B. Soares, G. G. Oliveira, E. Cunha-Neto, and R. Ribeiro Dos Santos. 2002. Experimental chronic Chagas' disease myocarditis is an autoimmune disease preventable by induction of immunological tolerance to myocardial antigens. *J Autoimmun* 18*:*131-138.

18. Molina, H. A., and F. Kierszenbaum. 1989. Interaction of human eosinophils or neutrophils with *Trypanosoma cruzi in vitro* causes bystander cardiac cell damage. *Immunology* 66*:*289-295.

19. Reis, D. D., E. M. Jones, S. Tostes, Jr., E. R. Lopes, G. Gazzinelli, D. G. Colley, and T. L. McCurley. 1993. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor-alpha+ cells and dominance of granzyme A+, CD8+ lymphocytes. *Am J Trop Med Hyg* 48*:*637-644.

20. Higuchi Mde, L., P. S. Gutierrez, V. D. Aiello, S. Palomino, E. Bocchi, J. Kalil, G. Bellotti, and F. Pileggi. 1993. Immunohistochemical characterization of infiltrating cells in human chronic chagasic myocarditis: comparison with myocardial rejection process. *Virchows Arch A Pathol Anat Histopathol* 423*:*157-160.

21. Sztein, M. B., W. R. Cuna, and F. Kierszenbaum. 1990. *Trypanosoma cruzi* inhibits the expression of CD3, CD4, CD8, and IL-2R by mitogen-activated helper and cytotoxic human lymphocytes. *J.Immunol.* 144*:*3558-3562.

22. Cunha-Neto, E., L. V. Rizzo, F. Albuquerque, L. Abel, L. Guilherme, E. Bocchi, F. Bacal, D. Carrara, B. Ianni, C. Mady, and J. Kalil. 1998. Cytokine production profile of

heart-infiltrating T cells in Chagas' disease cardiomyopathy. *Braz J Med Biol Res* 31*:*133-137.

23. de Barros-Mazon, S., M. E. Guariento, C. A. da Silva, R. L. Coffman, and I. A. Abrahamsohn. 2004. Differential regulation of lymphoproliferative responses to *Trypanosoma cruzi* antigen in patients with the cardiac or indeterminate form of Chagas disease. *Clin Immunol* 111*:*137-145.

24. Higuchi Mde, L., L. A. Benvenuti, M. Martins Reis, and M. Metzger. 2003. Pathophysiology of the heart in Chagas' disease: current status and new developments. *Cardiovasc Res* 60*:*96-107.

25. Tarleton, R. L., L. Zhang, and M. O. Downs. 1997. "Autoimmune rejection" of neonatal heart transplants in experimental Chagas disease is a parasite-specific response to infected host tissue. *Proc Natl Acad Sci U S A* 94*:*3932-3937.

26. Mendoza, I., J. Camardo, F. Moleiro, A. Castellanos, V. Medina, J. Gomez, H. Acquatella, H. Casal, F. Tortoledo, and J. Puigbo. 1986. Sustained ventricular tachycardia in chronic chagasic myocarditis: electrophysiologic and pharmacologic characteristics. *Am J Cardiol* 57*:*423-427.

27. Rodriguez-Salas, L. A., E. Klein, H. Acquatella, F. Catalioti, V. V. Davalos, J. R. Gomez-Mancebo, H. Gonzalez, F. Bosch, and J. J. Puigbo. 1998. Echocardiographic and Clinical Predictors of Mortality in Chronic Chagas' Disease. *Echocardiography* 15*:*271-278.

28. Bocchi, E. A. 1994. [Update on indications and results of the surgical treatment of heart failure]. *Arq Bras Cardiol* 63*:*523-530.

29. Bocchi, E. A., and A. Fiorelli. 2001. The paradox of survival results after heart transplantation for cardiomyopathy caused by *Trypanosoma cruzi*. First Guidelines Group for Heart Transplantation of the Brazilian Society of Cardiology. *Ann Thorac Surg* 71*:*1833-1838.

30. Teixeira, A. R., N. Nitz, M. C. Guimaro, C. Gomes, and C. A. Santos-Buch. 2006. Chagas disease. *Postgrad Med J* 82*:*788-798.

31. Lauria-Pires, L., M. S. Braga, A. C. Vexenat, N. Nitz, A. Simoes-Barbosa, D. L. Tinoco, and A. R. Teixeira. 2000. Progressive chronic Chagas heart disease ten years after treatment with anti-*Trypanosoma cruzi* nitroderivatives. *Am. J. Trop. Med. Hyg.* 63*:*111-118.

32. Barr, S. C. 1993. Anti-heart tissue antibodies during experimental infections with pathogenic and non-pathogenic *Trypanosoma cruzi* isolates in dogs. *Int J Parasitol* 23*:*961-967.

33. Barr, S. C., V. A. Dennis, T. R. Klei, and N. L. Norcross. 1991. Antibody and lymphoblastogenic responses of dogs experimentally infected with *Trypanosoma cruzi* isolates from North American mammals. *Vet.Immunol.Immunopathol.* 29*:*267-283.

34. Amorim, D. S. 1985. Chagas' heart disease: experimental models. *Heart Vessels Suppl* 1*:*236-239.

35. Colmanetti, F. H., P. Teixeira Vde, M. L. Rodrigues, J. E. Chica, M. G. Reis, and V. M. dos Santos. 2005. Myocardiocyte ultrastructure and morphometrical analysis in hamsters experimentally infected with *Trypanosoma cruzi*. *Ultrastruct Pathol* 29*:*139-147.

36. Bilate, A. M., V. M. Salemi, F. J. Ramires, T. de Brito, A. M. Silva, E. S. Umezawa, C. Mady, J. Kalil, and E. Cunha-Neto. 2003. The Syrian hamster as a model for the dilated cardiomyopathy of Chagas' disease: a quantitative echocardiographical and histopathological analysis. *Microbes Infect* 5*:*1116-1124.

37. Ramirez, L. E., E. Lages-Silva, J. M. Soares Junior, and E. Chapadeiro. 1994. The hamster (Mesocricetus auratus) as experimental model in Chagas' disease: parasitological and histopathological studies in acute and chronic phases of *Trypanosoma cruzi* infection. *Rev Soc Bras Med Trop* 27*:*163-169.

38. Camargos, E. R., C. R. Machado, A. L. Teixeira Jr, L. L. Rocha, A. J. Ferreira, A. P. Almeida, M. Barton, and M. M. Teixeira. 2002. Role of endothelin during experimental *Trypanosoma cruzi* infection in rats. *Clin Sci (Lond)* 103 Suppl 48*:*64S-67S.

39. Piaggio, E., E. Roggero, M. Pitashny, J. Wietzerbin, O. A. Bottasso, and S. S. Revelli. 2001. Treatment with benznidazole and its immunomodulating effects on *Trypanosoma cruzi*-infected rats. *Parasitol Res* 87*:*539-547.

40. Marcipar, I. S., M. G. Risso, A. M. Silber, S. Revelli, and A. J. Marcipar. 2001. Antibody maturation in *Trypanosoma cruzi*-infected rats. *Clin Diagn Lab Immunol* 8*:*802-805.

41. Revelli, S., H. Davila, M. E. Ferro, M. Romero-Piffiguer, O. Musso, J. Valenti, J. Bernabo, E. Falcoff, J. Wietzerbin, and O. Bottasso. 1995. Acute and chronic experimental *Trypanosoma cruzi* infection in the rat. Response to systemic treatment with recombinant rat interferon-gamma. *Microbiol Immunol* 39*:*275-281.

42. Junqueira Junior, L. F., P. S. Beraldo, E. Chapadeiro, and P. C. Jesus. 1992. Cardiac autonomic dysfunction and neuroganglionitis in a rat model of chronic Chagas' disease. *Cardiovasc Res* 26*:*324-329.

43. Postan, M., J. P. McDaniel, and J. A. Dvorak. 1987. Comparative studies of the infection of Lewis rats with four *Trypanosoma cruzi* clones. *Trans R Soc Trop Med Hyg* 81*:*415-419.

44. Andersson, J., A. Orn, and D. Sunnemark. 2003. Chronic murine Chagas' disease: the impact of host and parasite genotypes. *Immunol Lett* 86*:*207-212.

45. Bustamante, J. M., H. W. Rivarola, A. R. Fernandez, J. E. Enders, R. Fretes, J. A. Palma, and P. A. Paglini-Oliva. 2002. *Trypanosoma cruzi* reinfections in mice determine the severity of cardiac damage. *Int J Parasitol* 32*:*889-896.

46. Cossio, P. M., O. Bustuoabad, E. Paterno, R. Iotti, M. B. Casanova, M. R. Podesta, N. Bolomo, R. M. Arana, and C. D. de Pasqualini. 1984. Experimental myocarditis induced in Swiss mice by homologous heart immunization resembles chronic experimental Chagas' heart disease. *Clin Immunol Immunopathol* 33*:*165-175.

47. Costa, S. C. 1999. Mouse as a model for Chagas disease: does mouse represent a good model for Chagas disease? *Mem Inst Oswaldo Cruz* 94 Suppl 1*:*269-272.

48. Dumonteil, E., J. Escobedo-Ortegon, N. Reyes-Rodriguez, A. Arjona-Torres, and M. J. Ramirez-Sierra. 2004. Immunotherapy of *Trypanosoma cruzi* infection with DNA vaccines in mice. *Infect Immun* 72*:*46-53.

49. Laguens, R. P., P. M. Cabeza Meckert, and J. G. Chambo. 1989. Immunologic studies on a murine model of Chagas disease. *Medicina (Buenos Aires)* 49*:*197-202.

50. Leon, J. S., K. Wang, and D. M. Engman. 2003. Captopril ameliorates myocarditis in acute experimental Chagas disease. *Circulation* 107*:*2264-2269.

51. Vasconcelos, J. R., M. I. Hiyane, C. R. Marinho, C. Claser, A. M. Machado, R. T. Gazzinelli, O. Bruna-Romero, J. M. Alvarez, S. B. Boscardin, and M. M. Rodrigues. 2004. Protective immunity against *Trypanosoma cruzi* infection in a highly susceptible mouse strain after vaccination with genes encoding the amastigote surface protein-2 and trans-sialidase. *Hum Gene Ther* 15*:*878-886.

52. Hontebeyrie-Joskowicz, M. 1997. Autoimmunity in Chagas disease: the mouse model. *Parasitol Today* 13*:*156-157; author reply 157.

53. Tarleton, R. L., J. Sun, L. Zhang, and M. Postan. 1994. Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. *Infect Immun.* 62*:*1820-1829.

54. Marinho, C. R., D. Z. Bucci, M. L. Dagli, K. R. Bastos, M. G. Grisotto, L. R. Sardinha, C. R. Baptista, C. P. Goncalves, M. R. Lima, and J. M. Alvarez. 2004. Pathology affects different organs in two mouse strains chronically infected by a *Trypanosoma cruzi* clone: a model for genetic studies of Chagas' disease. *Infect Immun* 72*:*2350-2357.

55. Leon, J. S., L. M. Godsel, K. Wang, and D. M. Engman. 2001. Cardiac myosin autoimmunity in acute Chagas heart disease. *Infect Immun* 69*:*5643-5649.

56. Hyland, K. V., J. S. Leon, M. D. Daniels, N. Giafis, L. M. Woods, T. J. Bahk, K. Wang, and D. M. Engman. 2007. Modulation of autoimmunity by treatment of an infectious disease. *Infect Immun* 75*:*3641-3650.

57. Bonney, K. M., and D. M. Engman. 2008. Chagas heart disease pathogenesis: One mechanism or many? *Curr Mol Med* (in press).

58. Rossi, M. A. 1990. Microvascular changes as a cause of chronic cardiomyopathy in Chagas' disease. *Am Heart J* 120*:*233-236.

59. Marin-Neto, J. A., M. V. Simoes, E. M. Ayres-Neto, J. L. Attab-Santos, L. J. Gallo, S. D. Amorim, and M. B. Carlos. 1995. Studies of the coronary circulation in Chagas' heart disease. *Sao Paulo Med J* 113*:*826-834.

60. Rossi, M. A. 1997. Aortic endothelial cell changes in the acute septicemic phase of experimental *Trypanosoma cruzi* infection in rats: scanning and transmission electron microscopic study. *Am J Trop Med Hyg* 57*:*321-327.

61. Tanowitz, H. B., D. K. Kaul, B. Chen, S. A. Morris, S. M. Factor, L. M. Weiss, and M. Wittner. 1996. Compromised microcirculation in acute murine *Trypanosoma cruzi* infection. *J Parasitol* 82*:*124-130.

62. Davila, D. F., R. O. Rossell, and J. H. Donis. 1989. Cardiac parasympathetic abnormalities: cause or consequence of Chagas heart disease. *Parasitol Today* 5*:*327-329.

63. Andrews, N. W., and M. B. Whitlow. 1989. Secretion by *Trypanosoma cruzi* of a hemolysin active at low pH. *Mol Biochem Parasitol* 33*:*249-256.

64. Kierszenbaum, F. 1996. Chronic chagasic tissue lesions in the absence of *Trypanosoma cruzi*: a proposed mechanism. *Parasitol Today* 12*:*414-415.

65. Kierszenbaum, F. 1999. Chagas' disease and the autoimmunity hypothesis. *Clin Microbiol Rev* 12*:*210-223.

66. Frank, F. M., P. B. Petray, S. I. Cazorla, M. C. Munoz, R. S. Corral, and E. L. Malchiodi. 2003. Use of a purified *Trypanosoma cruzi* antigen and CpG oligodeoxynucleotides for immunoprotection against a lethal challenge with trypomastigotes. *Vaccine* 22*:*77-86.

67. Wizel, B., M. Palmieri, C. Mendoza, B. Arana, J. Sidney, A. Sette, and R. L. Tarleton. 1998. Human infection with *Trypanosoma cruzi* induces parasite antigen- specific cytotoxic T lymphocyte responses. *J Clin Invest* 102*:*1062-1071.

68. Tarleton, R. L. 2001. Parasite persistence in the aetiology of Chagas disease. *Int J Parasitol* 31*:*550-554.

69. Anez, N., H. Carrasco, H. Parada, G. Crisante, A. Rojas, C. Fuenmayor, N. Gonzalez, G. Percoco, R. Borges, P. Guevara, and J. L. Ramirez. 1999. Myocardial parasite persistence in chronic chagasic patients. *Am. J. Trop. Med. Hyg.* 60*:*726-732.

70. Zhang, L., and R. L. Tarleton. 1999. Parasite persistence correlates with disease severity and localization in chronic Chagas' disease. *J Infect Dis* 180*:*480-486.

71. Eisen, H., and S. Kahn. 1991. Mimicry in *Trypanosoma cruzi*: fantasy and reality. *Curr Opin Immunol* 3*:*507-510.

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

73. Avila, J. L. 1992. Molecular mimicry between *Trypanosoma cruzi* and host nervous tissues. *Acta Cient Venez* 43*:*330-340.

74. Kalil, J., and E. Cunha-Neto. 1996. Autoimmunity in chagas disease cardiomyopathy: Fulfilling the criteria at last? *Parasitol Today* 12*:*396-399.

75. Leon, J. S., and D. M. Engman. 2001. Autoimmunity in Chagas heart disease. *Int J Parasitol* 31*:*554-560.

76. Girones, N., and M. Fresno. 2003. Etiology of Chagas disease myocarditis: autoimmunity, parasite persistence, or both? *Trends Parasitol* 19*:*19-22.

77. Cunha-Neto, E., V. Coelho, L. Guilherme, A. Fiorelli, N. Stolf, and J. Kalil. 1996. Autoimmunity in Chagas' disease. Identification of cardiac myosin-B13 *Trypanosoma cruzi* protein crossreactive T cell clones in heart lesions of a chronic Chagas' cardiomyopathy patient. *J Clin Invest* 98*:*1709-1712.

78. Kierszenbaum, F. 2005. Where do we stand on the autoimmunity hypothesis of Chagas disease? *Trends Parasitol* 21*:*513-516.

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

80. Kamradt, T., and N. A. Mitchison. 2001. Tolerance and autoimmunity. *N Engl J Med* 344*:*655-664.

81. Bach, J. F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 347*:*911-920.

82. Miller, S. D., C. L. Vanderlugt, W. S. Begolka, W. Pao, R. L. Yauch, K. L. Neville, Y. Katz-Levy, A. Carrizosa, and B. S. Kim. 1997. Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. *Nat. Med.* 3*:*1133-1136.

83. Miller, S. D., Y. Katz-Levy, K. L. Neville, and C. L. Vanderlugt. 2001. Virus-induced autoimmunity: epitope spreading to myelin autoepitopes in Theiler's virus infection of the central nervous system. *Adv Virus Res* 56*:*199-217.

84. Rose, N. R., and S. L. Hill. 1996. Autoimmune myocarditis. *Int J Cardiol* 54*:*171-175.

85. Beisel, K. W., J. Srinivasappa, M. R. Olsen, A. C. Stiff, K. Essani, and B. S. Prabhakar. 1990. A neutralizing monoclonal antibody against Coxsackievirus B4 cross-reacts with contractile muscle proteins. *Microb Pathog* 8*:*151-156.

86. Triantafilou, K., and M. Triantafilou. 2004. Coxsackievirus B4-Induced Cytokine Production in Pancreatic Cells Is Mediated through Toll-Like Receptor 4. *J. Virol.* 78*:*11313-11320.

87. Kyu, B., A. Matsumori, Y. Sato, I. Okada, N. M. Chapman, and S. Tracy. 1992. Cardiac persistence of cardioviral RNA detected by polymerase chain reaction in a murine model of dilated cardiomyopathy. *Circulation* 86*:*522-530.

88. Cunningham, M. W. 2000. Pathogenesis of Group A Streptococcal Infections. *Clin. Microbiol. Rev.* 13*:*470-511.

89. Cunningham, M. W. 2001. Streptococcus-induced myocarditis in mice. *Autoimmunity* 34*:*193-197.

90. Cunningham, M. W. 2003. Autoimmunity and molecular mimicry in the pathogenesis of post-streptococcal heart disease. *Front Biosci* 8*:*s533-543.

91. Bolz, D. D., and J. J. Weis. 2004. Molecular mimicry to Borrelia burgdorferi: pathway to autoimmunity? *Autoimmunity* 37*:*387-392.

92. Santos-Buch, C. A., and A. R. Teixeira. 1974. The immunology of experimental Chagas' disease. 3. Rejection of allogeneic heart cells in vitro. *J. Exp. Med.* 140*:*38-53.

93. Cossio, P. M., C. Diez, A. Szarfman, E. Kreutzer, B. Candiolo, and R. M. Arana. 1974. Chagasic cardiopathy. Demonstration of a serum gamma globulin factor which reacts with endocardium and vascular structures. *Circulation* 49*:*13-21.

94. Cossio, P. M., R. P. Laguens, C. Diez, A. Szarfman, A. Segal, and R. M. Arana. 1974. Chagasic cardiopathy. Antibodies reacting with plasma membrane of striated muscle and endothelial cells. *Circulation* 50*:*1252-1259.

95. Kierszenbaum, F. 1986. Autoimmunity in Chagas' disease. *J Parasitol* 72*:*201-211.

96. Kierszenbaum, F. 2003. Views on the autoimmunity hypothesis for Chagas disease pathogenesis. *FEMS Immunol Med Microbiol* 37*:*1-11.

97. Soares, M. B., L. Pontes-De-Carvalho, and R. Ribeiro-Dos-Santos. 2001. The pathogenesis of Chagas' disease: when autoimmune and parasite-specific immune responses meet. *An Acad Bras Cienc* 73*:*547-559.

98. Leon, J. S., and D. M. Engman. 2003. The contribution of autoimmunity to Chagas disease? In *World Class Parasites: American Trypanosomiasis*, Vol. 7. K. M. Tyler, and M. A. Miles, eds. Kluwer Academic Publishers, Boston, p. 97-106.

99. Leon, J. S., and D. M. Engman. 2003. The significance of autoimmunity in the pathogenesis of Chagas heart disease. *Front Biosci* 8*:*e315-322.

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

101. Witebsky, E., N. R. Rose, K. Terplan, J. R. Paine, and R. W. Egan. 1957. Chronic thyroiditis and autoimmunization. *J Am Med Assoc* 164*:*1439-1447.

102. Rose, N. R., and C. Bona. 1993. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). *Immunol Today* 14*:*426-430.

103. Levin, M. J., E. Mesri, R. Benarous, G. Levitus, A. Schijman, P. Levy-Yeyati, P. A. Chiale, A. M. Ruiz, A. Kahn, M. B. Rosenbaum, H. N. Torres, and E. L. Segura. 1989. Identification of major *Trypanosoma cruzi* antigenic determinants in chronic chagas' heart disease. *Am J Trop Med Hyg* 41*:*530-538.

104. Levitus, G., M. Hontebeyrie-Joskowicz, M. H. Van Regenmortel, and M. J. Levin. 1991. Humoral autoimmune response to ribosomal P proteins in chronic Chagas heart disease. *Clin Exp Immunol* 85*:*413-417.

105. Cunha-Neto, E., M. Duranti, A. Gruber, B. Zingales, I. de Messias, N. Stolf, G. Bellotti, M. E. Patarroyo, F. Pilleggi, and J. Kalil. 1995. Autoimmunity in Chagas' disease cardiomyopathy: biological relevance of a cardiac myosin-specific epitope crossreactive to an immunodominant *Trypanosoma cruzi* antigen. *Proc Natl Acad Sci USA* 92*:*3541-3545.

106. Tibbetts, R. S., T. S. McCormick, E. C. Rowland, S. D. Miller, and D. M. Engman. 1994. Cardiac antigen-specific autoantibody production is associated with cardiomyopathy in *Trypanosoma cruzi*-infected mice. *J Immunol* 152*:*1493-1499.

107. Sterin-Borda, L., C. Perez Leiros, M. Wald, G. Cremaschi, and E. Borda. 1988. Antibodies to beta 1 and beta 2 adrenoreceptors in Chagas' disease. *Clin Exp Immunol* 74*:*349-354.

108. Sterin-Borda, L., and E. Borda. 2000. Role of neurotransmitter autoantibodies in the pathogenesis of chagasic peripheral dysautonomia. *Ann N Y Acad Sci* 917*:*273-280.

109. Kerner, N., P. Liegeard, M. J. Levin, and M. Hontebeyrie-Joskowicz. 1991. *Trypanosoma cruzi*: antibodies to a MAP-like protein in chronic Chagas' disease cross-react with mammalian cytoskeleton. *Exp Parasitol* 73*:*451-459.

110. Van Voorhis, W. C., and H. Eisen. 1989. Fl-160. A surface antigen of *Trypanosoma cruzi* that mimics mammalian nervous tissue. *J Exp Med* 169*:*641-652.

111. Van Voorhis, W. C., L. Schlekewy, and H. L. Trong. 1991. Molecular mimicry by *Trypanosoma cruzi*: the F1-160 epitope that mimics mammalian nerve can be mapped to a 12-amino acid peptide. *Proc Natl Acad Sci USA* 88*:*5993-5997.

112. Girones, N., C. I. Rodriguez, B. Basso, J. M. Bellon, S. Resino, M. A. Munoz-Fernandez, S. Gea, E. Moretti, and M. Fresno. 2001. Antibodies to an epitope from the Cha human autoantigen are markers of Chagas' disease. *Clin Diagn Lab Immunol* 8*:*1039-1043.

113. Iwai, L. K., M. A. Juliano, L. Juliano, J. Kalil, and E. Cunha-Neto. 2005. T-cell molecular mimicry in Chagas disease: identification and partial structural analysis of multiple cross-reactive epitopes between *Trypanosoma cruzi* B13 and cardiac myosin heavy chain. *J Autoimmun* 24*:*111-117.

114. Borda, E. S., and L. Sterin-Borda. 1996. Antiadrenergic and muscarinic receptor antibodies in Chagas' cardiomyopathy. *Int. J. Cardiol.* 54*:*149-156.

115. Laguens, R. P., P. C. Meckert, and J. G. Chambo. 1988. Antiheart antibody-dependent cytotoxicity in the sera of mice chronically infected with *Trypanosoma cruzi*. *Infect Immun* 56*:*993-997.

116. Giordanengo, L., C. Maldonado, H. W. Rivarola, D. Iosa, N. Girones, M. Fresno, and S. Gea. 2000. Induction of antibodies reactive to cardiac myosin and development of heart alterations in cruzipain-immunized mice and their offspring. *Eur J Immunol* 30*:*3181-3189.

117. de Scheerder, I. K., M. L. de Buyzere, J. R. Delanghe, D. L. Clement, and R. J. Wieme. 1989. Anti-myosin humoral immune response following cardiac injury. *Autoimmunity* 4*:*51-58.

118. Nomura, Y., M. Yoshinaga, T. Haraguchi, S. Oku, T. Noda, K. Miyata, Y. Umebayashi, and A. Taira. 1994. Relationship between the degree of injury at operation and the change in antimyosin antibody titer in the postpericardiotomy syndrome. *Pediatr Cardiol* 15*:*116-120.

119. dos Santos, R. R., M. A. Rossi, J. L. Laus, J. S. Silva, W. Savino, and J. Mengel. 1992. Anti-CD4 abrogates rejection and reestablishes long-term tolerance to syngeneic newborn hearts grafted in mice chronically infected with *Trypanosoma cruzi*. *J Exp Med* 175*:*29-39.

120. Girones, N., C. I. Rodriguez, E. Carrasco-Marin, R. F. Hernaez, J. L. de Rego, and M. Fresno. 2001. Dominant T- and B-cell epitopes in an autoantigen linked to Chagas' disease. *J. Clin. Invest.* 107*:*985-993.

121. Santos-Buch, C. A., and A. M. Acosta. 1985. Pathology of Chagas disease. In *Immunology and Pathogenesis of Trypanosomiasis*. I. Tizard, ed. CRC Press, Boca Raton, Florida, p. 145-184.

122. Talvani, A., C. S. Ribeiro, J. C. Aliberti, V. Michailowsky, P. V. Santos, S. M. Murta, A. J. Romanha, I. C. Almeida, J. Farber, J. Lannes-Vieira, J. S. Silva, and R. T. Gazzinelli. 2000. Kinetics of cytokine gene expression in experimental chagasic cardiomyopathy: tissue parasitism and endogenous IFN-gamma as important determinants of chemokine mRNA expression during infection with *Trypanosoma cruzi*. *Microbes Infect* 2*:*851-866.

123. Cunha-Neto, E., A. M. Bilate, K. V. Hyland, S. G. Fonseca, J. Kalil, and D. M. Engman. 2006. Induction of cardiac autoimmunity in Chagas heart disease: a case for molecular mimicry. *Autoimmunity* 39*:*41-54.

124. Cunha-Neto, E., V. J. Dzau, P. D. Allen, D. Stamatiou, L. Benvenutti, M. L. Higuchi, N. S. Koyama, J. S. Silva, J. Kalil, and C. C. Liew. 2005. Cardiac gene expression profiling

provides evidence for cytokinopathy as a molecular mechanism in Chagas' disease cardiomyopathy. *Am J Pathol* 167*:*305-313.

125. Talvani, A., M. O. Rocha, L. S. Barcelos, Y. M. Gomes, A. L. Ribeiro, and M. M. Teixeira. 2004. Elevated concentrations of CCL2 and tumor necrosis factor-alpha in chagasic cardiomyopathy. *Clin Infect Dis* 38*:*943-950.

126. Gomes, J. A., L. M. Bahia-Oliveira, M. O. Rocha, O. A. Martins-Filho, G. Gazzinelli, and R. Correa-Oliveira. 2003. Evidence that development of severe cardiomyopathy in human Chagas' disease is due to a Th1-specific immune response. *Infect Immun* 71*:*1185-1193.

127. Dutra, W. O., K. J. Gollob, J. C. Pinto-Dias, G. Gazzinelli, R. Correa-Oliveira, R. L. Coffman, and J. F. Carvalho-Parra. 1997. Cytokine mRNA profile of peripheral blood mononuclear cells isolated from individuals with *Trypanosoma cruzi* chronic infection. *Scand J Immunol* 45*:*74-80.

128. Fedoseyeva, E. V., F. Zhang, P. L. Orr, D. Levin, H. J. Buncke, and G. Benichou. 1999. De novo autoimmunity to cardiac myosin after heart transplantation and its contribution to the rejection process. *J Immunol* 162*:*6836-6842.

129. Neu, N., S. W. Craig, N. R. Rose, F. Alvarez, and K. W. Beisel. 1987. Coxsackievirus induced myocarditis in mice: cardiac myosin autoantibodies do not cross-react with the virus. *Clin Exp Immunol* 69*:*566-574.

130. Oldstone, M. B. 1987. Molecular mimicry and autoimmune disease. *Cell* 50*:*819-820.

131. Cunningham, M. W. 2004. T cell mimicry in inflammatory heart disease. *Mol Immunol* 40*:*1121-1127.

132. Benoist, C., and D. Mathis. 2001. Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry? *Nat Immunol* 2*:*797-801.

133. Leon, J. S., M. D. Daniels, K. M. Toriello, K. Wang, and D. M. Engman. 2004. A cardiac myosin-specific autoimmune response is induced by immunization with *Trypanosoma cruzi* proteins. *Infect Immun* 72*:*3410-3417.

134. Leon, J. S., K. Wang, and D. M. Engman. 2003. Myosin autoimmunity is not essential for cardiac inflammation in acute Chagas' disease. *J Immunol* 171*:*4271-4277.

135. Lanzavecchia, A. 1995. How can cryptic epitopes trigger autoimmunity? *J Exp Med* 181*:*1945-1948.

136. Minoprio, P. 2001. Parasite polyclonal activators: new targets for vaccination approaches? *Int J Parasitol* 31*:*588-591.

137. Vanderlugt, C. L., W. S. Begolka, K. L. Neville, Y. Katz-Levy, L. M. Howard, T. N. Eagar, J. A. Bluestone, and S. D. Miller. 1998. The functional significance of epitope spreading and its regulation by co-stimulatory molecules. *Immunol Rev* 164*:*63-72.

138. Vanderlugt, C. J., and S. D. Miller. 1996. Epitope spreading. *Curr Opin Immunol* 8*:*831-836.

139. Chan, L. S., C. J. Vanderlugt, T. Hashimoto, T. Nishikawa, J. J. Zone, M. M. Black, F. Wojnarowska, S. R. Stevens, M. Chen, J. A. Fairley, D. T. Woodley, S. D. Miller, and K. B. Gordon. 1998. Epitope spreading: lessons from autoimmune skin diseases. *J Invest Dermatol* 110*:*103-109.

140. Craft, J., and S. Fatenejad. 1997. Self antigens and epitope spreading in systemic autoimmunity. *Arthritis Rheum* 40*:*1374-1382.

141. Croxford, J. L., J. K. Olson, and S. D. Miller. 2002. Epitope spreading and molecular mimicry as triggers of autoimmunity in the Theiler's virus-induced demyelinating disease model of multiple sclerosis. *Autoimmun Rev* 1*:*251-260.

142. James, J. A., and J. B. Harley. 1998. B-cell epitope spreading in autoimmunity. *Immunol Rev* 164*:*185-200.

143. Rizzo, L. V., E. Cunha-Neto, and A. R. Teixeira. 1989. Autoimmunity in Chagas' disease: specific inhibition of reactivity of CD4+ T cells against myosin in mice chronically infected with *Trypanosoma cruzi*. *Infect Immun* 57*:*2640-2644.

144. Jones, E. M., D. G. Colley, S. Tostes, E. R. Lopes, C. L. Vnencak-Jones, and T. L. McCurley. 1993. Amplification of a *Trypanosoma cruzi* DNA sequence from inflammatory lesions in human chagasic cardiomyopathy. *Am. J. Trop. Med. Hyg.* 48*:*348-357.

145. Brandariz, S., A. Schijman, C. Vigliano, P. Arteman, R. Viotti, C. Beldjord, and M. J. Levin. 1995. Detection of parasite DNA in Chagas' heart disease. *Lancet* 346*:*1370-1371.

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

147. Vago, A. R., A. M. Macedo, R. P. Oliveira, L. O. Andrade, E. Chiari, L. M. Galvao, D. Reis, M. E. Pereira, A. J. Simpson, S. Tostes, and S. D. Pena. 1996. Kinetoplast DNA

signatures of *Trypanosoma cruzi* strains obtained directly from infected tissues. *Am J Path* 149*:*2153-2159.

148. Bellotti, G., E. A. Bocchi, A. V. de Moraes, M. L. Higuchi, M. Barbero-Marcial, E. Sosa, A. Esteves-Filho, R. Kalil, R. Weiss, A. Jatene, and F. Pileggi. 1996. *In vivo* detection of *Trypanosoma cruzi* antigens in hearts of patients with chronic Chagas' heart disease. *Am. Heart J.* 131*:*301-307.

149. Olivares-Villagomez, D., T. L. McCurley, C. L. Vnencak-Jones, R. Correa-Oliveira, D. G. Colley, and C. E. Carter. 1998. Polymerase chain reaction amplification of three different *Trypanosoma cruzi* DNA sequences from human chagasic cardiac tissue. *Am. J. Trop. Med. Hyg.* 59*:*563-570.

150. Palomino, S. A., V. D. Aiello, and M. L. Higuchi. 2000. Systematic mapping of hearts from chronic chagasic patients: the association between the occurrence of histopathological lesions and *Trypanosoma cruzi* antigens. *Ann Trop Med Parasitol* 94*:*571-579.

151. Mortara, R. A., S. da Silva, F. R. Patricio, M. L. Higuchi, E. R. Lopes, A. A. Gabbai, P. Carnevale, A. Rocha, M. S. Ferreira, M. M. Souza, M. F. de Franco, G. Turcato, Jr., and B. H. Ferraz Neto. 1999. Imaging *Trypanosoma cruzi* within tissues from chagasic patients using confocal microscopy with monoclonal antibodies. *Parasitol Res* 85*:*800-808.

152. Castro, C., A. Prata, and V. Macedo. 2005. [The influence of the parasitemia on the evolution of the chronic Chagas' disease]. *Rev Soc Bras Med Trop* 38*:*1-6.

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

154. Urbina, J. A., and R. Docampo. 2003. Specific chemotherapy of Chagas disease: controversies and advances. *Trends Parasitol* 19*:*495-501.

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

156. Schijman, A. G., C. A. Vigliano, R. J. Viotti, J. M. Burgos, S. Brandariz, B. E. Lococo, M. I. Leze, H. A. Armenti, and M. J. Levin. 2004. *Trypanosoma cruzi* DNA in Cardiac Lesions of Argentinean Patients with End-Stage Chronic Chagas Heart Disease. *Am J Trop Med Hyg* 70*:*210-220.

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

158. Cummings, K. L., and R. L. Tarleton. 2003. Rapid quantitation of *Trypanosoma cruzi* in host tissue by real-time PCR. *Mol Biochem Parasitol* 129*:*53-59.

159. Tarleton, R. L. 2003. Chagas disease: a role for autoimmunity? *Trends Parasitol* 19*:*447-451.

160. Revelli, S., C. Le Page, E. Piaggio, J. Wietzerbin, and O. Bottasso. 1999. Benznidazole, a drug employed in the treatment of Chagas' disease, down-regulates the synthesis of nitrite and cytokines by murine stimulated macrophages. *Clin Exp Immunol* 118*:*271-277.

161. Docampo, R., and S. N. Moreno. 1984. Free radical metabolites in the mode of action of chemotherapeutic agents and phagocytic cells on *Trypanosoma cruzi*. *Rev Infect Dis* 6*:*223-238.

162. Murta, S. M., C. Ropert, R. O. Alves, R. T. Gazzinelli, and A. J. Romanha. 1999. In-vivo treatment with benznidazole enhances phagocytosis, parasite destruction and cytokine release by macrophages during infection with a drug-susceptible but not with a derived drug-resistant Trypansoma cruzi population. *Parasite Immunol* 21*:*535-544.

163. Olivieri, B. P., V. Cotta-De-Almeida, and T. Araujo-Jorge. 2002. Benznidazole treatment following acute *Trypanosoma cruzi* infection triggers CD8+ T-cell expansion and promotes resistance to reinfection. *Antimicrob. Agents Chemother.* 46*:*3790-3796.

164. Laucella, S. A., E. L. Segura, A. Riarte, and E. S. Sosa. 1999. Soluble platelet selectin (sP-selectin) and soluble vascular cell adhesion molecule-1 (sVCAM-1) decrease during therapy with benznidazole in children with indeterminate form of Chagas' disease. *Clin Exp Immunol* 118*:*423-427.

165. Michailowsky, V., S. M. Murta, L. Carvalho-Oliveira, M. E. Pereira, L. R. Ferreira, Z. Brener, A. J. Romanha, and R. T. Gazzinelli. 1998. Interleukin-12 enhances in vivo parasiticidal effect of benznidazole during acute experimental infection with a naturally drug-resistant strain of *Trypanosoma cruzi*. *Antimicrob Agents Chemother* 42*:*2549-2556.

166. Romanha, A. J., R. O. Alves, S. M. Murta, J. S. Silva, C. Ropert, and R. T. Gazzinelli. 2002. Experimental chemotherapy against *Trypanosoma cruzi* infection: essential role of endogenous interferon-gamma in mediating parasitologic cure. *J Infect Dis* 186*:*823-828.

167. de Gaspari, E. N., E. S. Umezawa, B. Zingales, A. M. Stolf, W. Colli, and I. A. Abrahamsohn. 1990. *Trypanosoma cruzi*: serum antibody reactivity to the parasite antigens in susceptible and resistant mice. *Mem Inst Oswaldo Cruz* 85*:*261-270.

168. Minoprio, P., M. C. el Cheikh, E. Murphy, M. Hontebeyrie-Joskowicz, R. Coffman, A. Coutinho, and O. G. A. 1993. Xid-associated resistance to experimental Chagas' disease is IFN-gamma dependent. *J Immunol* 151*:*4200-4208.

169. Gattass, C. R., M. T. Lima, A. F. Nobrega, M. A. Barcinski, and G. A. Dos Reis. 1988. Do self-heart-reactive T cells expand in *Trypanosoma cruzi*-immune hosts? *Infect Immun* 56*:*1402-1405.

170. Teixeira, A. R. L., G. Teixeira, V. Macedo, and A. Prata. 1978. *Trypanosoma cruzi* sensitized T-lymphocyte mediated 51Cr release from human heart cells in Chagas' disease. *Am J Trop Med Hyg* 27*:*1097-1107.

171. Abel, L. C., J. Kalil, and E. Cunha Neto. 1997. Molecular mimicry between cardiac myosin and *Trypanosoma cruzi* antigen B13: identification of a B13-driven human T cell clone that recognizes cardiac myosin. *Braz J Med Biol Res* 30*:*1305-1308.

172. Moraru, M., A. Roth, G. Keren, and J. George. 2006. Cellular autoimmunity to cardiac myosin in patients with a recent myocardial infarction. *Int J Cardiol* 107*:*61-66.

173. Mullbacher, A., and K. Flynn. 1996. Aspects of cytotoxic T cell memory. *Immunol Rev* 150*:*113-127.

174. Zevering, Y., C. Khamboonruang, K. Rungruengthanakit, L. Tungviboonchai, J. Ruengpipattanapan, I. Bathurst, P. Barr, and M. Good. 1994. Life-spans of human T-cell responses to determinants from the circumsporozoite proteins of *Plasmodium falciparum* and *Plasmodium vivax*. *Proc Natl Acad Sci U S A* 91*:*6118-6122.

175. Achtman, A. H., P. C. Bull, R. Stephens, and J. Langhorne. 2005. Longevity of the immune response and memory to blood-stage malaria infection. *Curr Top Microbiol Immunol* 297*:*71-102.

176. Chen, A. M., N. Khanna, S. A. Stohlman, and C. C. Bergmann. 2005. Virus-specific and bystander CD8 T cells recruited during virus-induced encephalomyelitis. *J Virol* 79*:*4700-4708.

177. Acosta Rodriguez, E. V., E. Zuniga, C. L. Montes, and A. Gruppi. 2003. Interleukin-4 biases differentiation of B cells from *Trypanosoma cruzi*-infected mice and restrains their fratricide: role of Fas ligand down-regulation and MHC class II-transactivator up-regulation. *J Leukoc Biol* 73*:*127-136.

178. Tarleton, R. L., and R. E. Kuhn. 1983. Changes in cell populations and immunoglobulin-producing cells in the spleens of mice infected with *Trypanosoma cruzi*: correlations with parasite-specific antibody response. *Cell Immunol* 80*:*392-404.

179. Zaidenberg, A., T. Luong, D. Lirussi, J. Bleiz, M. B. Del Buono, G. Quijano, R. Drut, L. Kozubsky, A. Marron, and H. Buschiazzo. 2006. Treatment of experimental chronic chagas disease with trifluralin. *Basic Clin Pharmacol Toxicol* 98*:*351-356.

180. Corrales, M., R. Cardozo, M. A. Segura, J. A. Urbina, and M. A. Basombrio. 2005. Comparative efficacies of TAK-187, a long-lasting ergosterol biosynthesis inhibitor, and benznidazole in preventing cardiac damage in a murine model of Chagas' disease. *Antimicrob Agents Chemother* 49*:*1556-1560.

181. Melo, R. C., and Z. Brener. 1978. Tissue tropism of different *Trypanosoma cruzi* strains. *J Parasitol* 64*:*475-482.

182. Lenzi, H. L., D. N. Oliveira, M. T. Lima, and C. R. Gattass. 1996. *Trypanosoma cruzi*: paninfectivity of CL strain during murine acute infection. *Exp Parasitol* 84*:*16-27.

183. Ben Younes-Chennoufi, A., M. Hontebeyrie-Joskowicz, V. Tricottet, H. Eisen, M. Reynes, and G. Said. 1988. Persistence of *Trypanosoma cruzi* antigens in the inflammatory lesions of chronically infected mice. *Trans R Soc Trop Med Hyg* 82*:*77-83.

184. Morocoima, A., M. Rodriguez, L. Herrera, and S. Urdaneta-Morales. 2006. *Trypanosoma cruzi*: experimental parasitism of bone and cartilage. *Parasitol Res* 99*:*663-668.

185. Calabrese, K. S. 1999. Immunosuppressive drugs as a tool to explore immunopathology in experimental Chagas disease. *Mem Inst Oswaldo Cruz* 94 Suppl 1*:*273-276.

186. Calabrese, K. S., P. G. Bauer, P. H. Lagrange, and S. C. Gon:calves da Costa. 1992. *Trypanosoma cruzi* infection in immunosuppressed mice. *Immunol Lett* 31*:*91-96.

187. Taniwaki, N. N., W. K. Andreoli, K. S. Calabrese, S. da Silva, and R. A. Mortara. 2005. Disruption of myofibrillar proteins in cardiac muscle of Calomys callosus chronically infected with *Trypanosoma cruzi* and treated with immunosuppressive agent. *Parasitol Res* 97*:*323-331.

188. James, M. J., M. J. Yabsley, O. J. Pung, and M. J. Grijalva. 2002. Amplification of *Trypanosoma cruzi*-specific DNA sequences in formalin-fixed raccoon tissues using polymerase chain reaction. *J Parasitol* 88*:*989-993.

189. Lane, J. E., R. Ribeiro-Rodrigues, D. Olivares-Villagomez, C. L. Vnencak-Jones, T. L. McCurley, and C. E. Carter. 2003. Detection of *Trypanosoma cruzi* DNA within murine cardiac tissue sections by *in situ* polymerase chain reaction. *Mem Inst Oswaldo Cruz* 98*:*373-376.

190. Lane, J. E., D. Olivares-Villagomez, C. L. Vnencak-Jones, T. L. McCurley, and C. E. Carter. 1997. Detection of *Trypanosoma cruzi* with the polymerase chain reaction and *in situ* hybridization in infected murine cardiac tissue. *Am J Trop Med Hyg* 56*:*588-595.

191. Taniwaki, N. N., C. V. da Silva, S. da Silva, and R. A. Mortara. 2007. Distribution of *Trypanosoma cruzi* stage-specific epitopes in cardiac muscle of Calomys callosus, BALB/c mice, and cultured cells infected with different infective forms. *Acta Trop* 103*:*14-25.

192. Chandler, F. W., and J. C. Watts. 1988. Immunofluorescence as an adjunct to the histopathologic diagnosis of Chagas' disease. *J Clin Microbiol* 26*:*567-569.

193. Russo, M., N. Starobinas, M. C. Marcondes, P. Minoprio, and M. Honteyberie-Joskowicz. 1996. The influence of T cell subsets on *Trypanosoma cruzi* multiplication in different organs. *Immunol Lett* 49*:*163-168.

194. Pinto, P. L., R. Takami, E. V. Nunes, C. S. Guilherme, O. C. Oliveira, Jr., J. Gama-Rodrigues, and M. Okumura. 1999. Life cycle of *Trypanosoma cruzi* (Y strain) in mice. *Rev Hosp Clin Fac Med Sao Paulo* 54*:*141-146.

195. Nunes, M. P., S. G. Coutinho, J. A. Louis, and W. J. Souza. 1990. *Trypanosoma cruzi*: quantification in tissues of experimentally infected mice by limiting dilution analysis. *Exp Parasitol* 70*:*186-192.

196. Mortatti, R. C., L. S. Fonseca, J. Coelho, A. Oliveira, and M. Moreno. 1992. Follow-up of patent and subpatent parasitemias and development of muscular lesions in mice inoculated with very small numbers of *Trypanosoma cruzi*. *Exp Parasitol* 75*:*233-239.

197. Wendt, M. K., A. N. Cooper, and M. B. Dwinell. 2007. Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells. *Oncogene* 27: 1461-1471.

198. Lyons, S. K., E. Lim, A. O. Clermont, J. Dusich, L. Zhu, K. D. Campbell, R. J. Coffee, D. S. Grass, J. Hunter, T. Purchio, and D. Jenkins. 2006. Noninvasive bioluminescence imaging of normal and spontaneously transformed prostate tissue in mice. *Cancer Res* 66*:*4701-4707.

199. Shachaf, C. M., A. M. Kopelman, C. Arvanitis, A. Karlsson, S. Beer, S. Mandl, M. H. Bachmann, A. D. Borowsky, B. Ruebner, R. D. Cardiff, Q. Yang, J. M. Bishop, C. H. Contag, and D. W. Felsher. 2004. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature* 431*:*1112-1117.

200. Edinger, M., Y. A. Cao, Y. S. Hornig, D. E. Jenkins, M. R. Verneris, M. H. Bachmann, R. S. Negrin, and C. H. Contag. 2002. Advancing animal models of neoplasia through *in vivo* bioluminescence imaging. *Eur J Cancer* 38*:*2128-2136.

201. Vooijs, M., J. Jonkers, S. Lyons, and A. Berns. 2002. Noninvasive imaging of spontaneous retinoblastoma pathway-dependent tumors in mice. *Cancer Res* 62*:*1862-1867.

202. Hutchens, M., and G. D. Luker. 2007. Applications of bioluminescence imaging to the study of infectious diseases. *Cell Microbiol* 9*:*2315-2322.

203. Kirchhoff, L. V., S. Hieny, G. M. Shiver, D. Snary, and A. Sher. 1984. Cryptic epitope explains the failure of a monoclonal antibody to bind to certain isolates of *Trypanosoma cruzi*. *J Immunol* 133*:*2731-2735.

204. Weston, D., A. C. La Flamme, and W. C. Van Voorhis. 1999. Expression of *Trypanosoma cruzi* surface antigen FL-160 is controlled by elements in the 3' untranslated, the 3' intergenic, and the coding regions. *Mol Biochem Parasitol* 102*:*53-66.

205. Contag, C. H., P. R. Contag, J. I. Mullins, S. D. Spilman, D. K. Stevenson, and D. A. Benaron. 1995. Photonic detection of bacterial pathogens in living hosts. *Mol Microbiol* 18*:*593-603.

206. Lang, T., S. Goyard, M. Lebastard, and G. Milon. 2005. Bioluminescent Leishmania expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harbouring macrophages and for quantitative real-time monitoring of parasitism features in living mice. *Cell Microbiol* 7*:*383-392.

207. Ashutosh, S. Gupta, Ramesh, S. Sundar, and N. Goyal. 2005. Use of *Leishmania donovani* field isolates expressing the luciferase reporter gene in in vitro drug screening. *Antimicrob Agents Chemother* 49*:*3776-3783.

208. Kierszenbaum, F. 1981. On evasion of *Trypanosoma cruzi* from the host immune response. Lymphoproliferative responses to trypanosomal antigens during acute and chronic experimental Chagas' disease. *Immunology* 44*:*641-648.

209. Garcia, I. E., M. R. Lima, C. R. Marinho, T. L. Kipnis, G. C. Furtado, and J. M. Alvarez. 1997. Role of membrane-bound IgM in *Trypanosoma cruzi* evasion from immune clearance. *J Parasitol* 83*:*230-233.

210. Brodskyn, C., J. Patricio, R. Oliveira, L. Lobo, A. Arnholdt, L. Mendonca-Previato, A. Barral, and M. Barral-Netto. 2002. Glycoinositolphospholipids from *Trypanosoma cruzi* interfere with macrophages and dendritic cell responses. *Infect Immun* 70*:*3736-3743.

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

212. Andrade, L. O., C. R. S. Machado, E. Chiari, S. D. J. Pena, and A. M. Macedo. 2002. *Trypanosoma cruzi*: role of host genetic background in the differential tissue distribution of parasite clonal populations. *Exp Parasitol* 100*:*269-275.

213. Franco, D. J., A. R. Vago, E. Chiari, F. C. Meira, L. M. Galvao, and C. R. Machado. 2003. *Trypanosoma cruzi*: mixture of two populations can modify virulence and tissue tropism in rat. *Exp Parasitol* 104*:*54-61.

214. McCabe, R. E., S. Meagher, and B. Mullins. 1989. *Trypanosoma cruzi*: explant organ cultures from mice with chronic Chagas' disease. *Exp Parasitol* 68*:*462-469.

215. Heussler, V., and C. Doerig. 2006. *In vivo* imaging enters parasitology. *Trends Parasitol* 22*:*192-195; discussion 195-196.

216. Dellacasa-Lindberg, I., N. Hitziger, and A. Barragan. 2007. Localized recrudescence of Toxoplasma infections in the central nervous system of immunocompromised mice assessed by *in vivo* bioluminescence imaging. *Microbes Infect* 9*:*1291-1298.

217. Hitziger, N., I. Dellacasa, B. Albiger, and A. Barragan. 2005. Dissemination of *Toxoplasma gondii* to immunoprivileged organs and role of Toll/interleukin-1 receptor signalling for host resistance assessed by *in vivo* bioluminescence imaging. *Cell Microbiol* 7*:*837-848.

218. Saeij, J. P., J. P. Boyle, M. E. Grigg, G. Arrizabalaga, and J. C. Boothroyd. 2005. Bioluminescence imaging of *Toxoplasma gondii* infection in living mice reveals dramatic differences between strains. *Infect Immun* 73*:*695-702.

219. Boyle, J. P., J. P. Saeij, and J. C. Boothroyd. 2007. *Toxoplasma gondii*: inconsistent dissemination patterns following oral infection in mice. *Exp Parasitol* 116*:*302-305.

220. Shu, X., N. C. Shaner, C. A. Yarbrough, R. Y. Tsien, and S. J. Remington. 2006. Novel chromophores and buried charges control color in mFruits. *Biochemistry* 45*:*9639-9647.

221. Pires, S. F., W. D. DaRocha, J. M. Freitas, L. A. Oliveira, G. T. Kitten, C. R. Machado, S. D. Pena, E. Chiari, A. M. Macedo, and S. M. Teixeira. 2008. Cell culture and animal infection with distinct *Trypanosoma cruzi* strains expressing red and green fluorescent proteins. *Int J Parasitol* 38*:*289-297.

222. da Silva, R. A., D. C. Bartholomeu, and S. M. Teixeira. 2006. Control mechanisms of tubulin gene expression in *Trypanosoma cruzi*. *Int J Parasitol* 36*:*87-96.

223. Ropert, C., and R. T. Gazzinelli. 2004. Regulatory role of Toll-like receptor 2 during infection with *Trypanosoma cruzi*. *J Endotoxin Res* 10*:*425-430.

224. Ouaissi, A., E. Guilvard, Y. Delneste, G. Caron, G. Magistrelli, N. Herbault, N. Thieblemont, and P. Jeannin. 2002. The *Trypanosoma cruzi* Tc52-released protein induces human dendritic cell maturation, signals via Toll-like receptor 2, and confers protection against lethal infection. *J Immunol* 168*:*6366-6374.

225. Monteiro, A. C., V. Schmitz, E. Svensjo, R. T. Gazzinelli, I. C. Almeida, A. Todorov, L. B. de Arruda, A. C. Torrecilhas, J. B. Pesquero, A. Morrot, E. Bouskela, A. Bonomo, A. P. Lima, W. Muller-Esterl, and J. Scharfstein. 2006. Cooperative activation of TLR2 and bradykinin B2 receptor is required for induction of type 1 immunity in a mouse model of subcutaneous infection by *Trypanosoma cruzi*. *J Immunol* 177*:*6325-6335.

226. Campos, M. A., and R. T. Gazzinelli. 2004. *Trypanosoma cruzi* and its components as exogenous mediators of inflammation recognized through Toll-like receptors. *Mediators Inflamm* 13*:*139-143.

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

228. Medeiros, M. M., J. R. Peixoto, A. C. Oliveira, L. Cardilo-Reis, V. L. Koatz, L. Van Kaer, J. O. Previato, L. Mendonca-Previato, A. Nobrega, and M. Bellio. 2007. Toll-like receptor 4 (TLR4)-dependent proinflammatory and immunomodulatory properties of the glycoinositolphospholipid (GIPL) from *Trypanosoma cruzi*. *J Leukoc Biol* 82*:*488-496.

229. Oliveira, A. C., J. R. Peixoto, L. B. de Arruda, M. A. Campos, R. T. Gazzinelli, D. T. Golenbock, S. Akira, J. O. Previato, L. Mendonca-Previato, A. Nobrega, and M. Bellio. 2004. Expression of functional TLR4 confers proinflammatory responsiveness to *Trypanosoma cruzi* glycoinositolphospholipids and higher resistance to infection with *T. cruzi*. *J Immunol* 173*:*5688-5696.

230. de Alencar, B. C., A. F. Araujo, M. L. Penido, R. T. Gazzinelli, and M. M. Rodrigues. 2007. Cross-priming of long lived protective CD8+ T cells against *Trypanosoma cruzi* infection: importance of a TLR9 agonist and CD4+ T cells. *Vaccine* 25*:*6018-6027.

231. Lima, E. S., Z. A. Andrade, and S. G. Andrade. 2001. TNF-alpha is expressed at sites of parasite and tissue destruction in the spleen of mice acutely infected with *Trypanosoma cruzi*. *Int J Exp Pathol* 82*:*327-336.

232. Gao, W., and M. A. Pereira. 2002. Interleukin-6 is required for parasite specific response and host resistance to *Trypanosoma cruzi*. *Int J Parasitol* 32*:*167-170.

233. Sathler-Avelar, R., D. M. Vitelli-Avelar, R. L. Massara, J. D. Borges, M. Lana, A. Teixeira-Carvalho, J. C. Dias, S. M. Eloi-Santos, and O. A. Martins-Filho. 2006.

Benznidazole treatment during early-indeterminate Chagas' disease shifted the cytokine expression by innate and adaptive immunity cells toward a type 1-modulated immune profile. *Scand J Immunol* 64*:*554-563.

234. Wizel, B., N. Garg, and R. L. Tarleton. 1998. Vaccination with trypomastigote surface antigen 1-encoding plasmid DNA confers protection against lethal *Trypanosoma cruzi* Infection. *Infect Immun* 66*:*5073-5081.

235. Morell, M., M. C. Thomas, T. Caballero, C. Alonso, and M. C. Lopez. 2006. The genetic immunization with paraflagellar rod protein-2 fused to the HSP70 confers protection against late *Trypanosoma cruzi* infection. *Vaccine* 24*:*7046-7055.

236. Garg, N., and R. L. Tarleton. 2002. Genetic immunization elicits antigen-specific protective immune responses and decreases disease severity in *Trypanosoma cruzi* infection *Infect Immun* 70*:*5547-5555.

237. Araujo, A. F. S., B. C. G. de Alencar, J. R. C. Vasconcelos, M. I. Hiyane, C. R. F. Marinho, M. L. O. Penido, S. B. Boscardin, D. F. Hoft, R. T. Gazzinelli, and M. M. Rodrigues. 2005. CD8+-T-cell-dependent control of *Trypanosoma cruzi* infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2 *Infect Immun* 73*:*6017-6025.

238. Hoft, D. F., C. S. Eickhoff, O. K. Giddings, J. R. Vasconcelos, and M. M. Rodrigues. 2007. Trans-sialidase recombinant protein mixed with CpG motif-containing oligodeoxynucleotide induces protective mucosal and systemic *Trypanosoma cruzi* immunity involving CD8+ CTL and B cell-mediated cross-priming. *J Immunol* 179*:*6889-6900.

Kenneth V. Hyland

UNIVERSITY ADDRESS

Northwestern University Feinberg School of Medicine
303 E. Chicago avenue
Ward Building 6-140
Chicago, IL 60611
(312) 503-1267 *tel*
(312) 503-1265 *fax*

HOME ADDRESS

1616 Thornfield Court
Roselle, IL 60172
(630) 306-3504 *tel*

k-hyland@northwestern.edu

EDUCATION

University of Kentucky, Lexington, KY	B.S. (Biology, *magna cum laude*)	2000-2003
University of Kentucky, Lexington, KY	B.A. (Chemistry, *magna cum laude*)	2000-2003
Northwestern University, Chicago, IL	Ph.D. (Immunology & Microbial Path.)	2008

RESEARCH EXPERIENCE

University of Kentucky, Lexington, KY 2001
Undergraduate research with Dr. Sylvia Daunert
Prostaglandin regulation in primary pulmonary hypertension patients

University of Cincinnati, Cincinnati, OH 2002-2003
Research Assistant with Dr. Nira Ben-Jonathan
Hormone modulation in human obesity and prolactin's role in breast cancer

Northwestern University, Chicago, IL 2004-2008
Graduate Student with Dr. David Engman
Mechanisms of infectious and autoimmune myocarditis

TEACHING EXPERIENCE

Western Kentucky University, Bowling Green, KY 1999
Inorganic chemistry laboratory teaching assistant

Northwestern University, Chicago, IL 2006
Graduate immunology teaching assistant

THESIS

Hyland, K.V. (2008) *The Regulation of Infection-Induced Cardiac Autoimmunity and Natural Dissemination of Trypanosoma cruzi in Experimental Chagas Heart Disease.*
Ph.D. Thesis in Immunology & Microbial Pathogenesis, Northwestern University, Integrated Graduate Program

AWARDS AND HONORS

Undergraduate Honors Program Scholarship	August 1998
Departmental Honors (Chemistry)	May 2003
Magna Cum Laude Graduate	May 2003
American Heart Association Predoctoral Fellowship	January 2006 - January 2008

BIBLIOGRAPHY: ORIGINAL JOURNAL ARTICLES

Desai, U.A., Deo, S.K., **Hyland, K.V.**, Poon, M. and Daunert, S. (2002) Determination of prostacyclin in plasma through a bioluminescent immunoassay for 6-keto-prostaglandin F-1α: implication of dosage in patients with primary pulmonary hypertension. *Anal. Chem.* 74: 3892-3898.

Yang, H.K., Sundholm-Peters N.L., Goings, G.E., Walker, A.S., **Hyland, K.V.**, Szele, F.G. (2004) Distribution of doublecortin expressing cells near the lateral ventricles in the adult mouse brain. *J. Neurosci. Res.* 76: 282-295.

Hyland, K.V., Leon, J.S., Daniels, M.D., Giafis, N., Woods, L.M., Bahk, T.J., Wang, K., Engman, D.M. (2007) Modulation of autoimmunity by treatment of an infectious disease. *Infect. Immun.* 75: 3641-50.

Hyland, K.V., Asfaw, S.H., Olson, C.L., Daniels, M.D., Engman, D.M. (2008) Bioluminescent imaging of *Trypanosoma cruzi* infection. *Int. J. Parasitol.* (*in press*)

Daniels, M.D., **Hyland, K.V.**, Wang, K., Engman, D.M. (2008) Novel cardiac myosin fragment induces experimental autoimmune myocarditis via activation of Th1 and Th17 immunity. *Autoimmunity (in press)*

Emmer, B.T., Daniels, M.D., **Hyland, K.V.**, Maric, D., Wang, K., Engman, D.M. (2008) Inhibition of the calflagins attenuates *Trypanosoma brucei* virulence. (*in preparation)*

BIBLIOGRAPHY: REVIEWS

Cunha-Neto, E., Bilate, A.M., **Hyland, K.V.**, Fonseca, S.G., Kalil, J., Engman, D.M. (2006) Induction of cardiac autoimmunity in Chagas heart disease: a case for molecular mimicry. *Autoimmunity* 39: 41-54.

Hyland, K.V., Engman, D.M. (2006) Further thoughts on where we stand on the autoimmunity hypothesis of Chagas disease. *Trends Parasitol.* 22: 101-2.

Daniels, M.D., **Hyland, K.V.**, Engman, D.M. (2007) Treatment of experimental myocarditis via modulation of the renin-angiotensin system. *Curr. Pharm. Des.* 13: 1299-305.

PRESENTATIONS: SEMINARS

"Modulation of autoimmunity by treatment of an infectious disease." Department of Pathology Calandra Seminar, Northwestern University Medical School, Chicago, IL, May 2006.

"Regulation of cardiac autoimmunity through treatment of Chagas heart disease." Department of Pathology Calandra Seminar, Northwestern University Medical School, Chicago, IL, May 2007.

"Regulation of cardiac autoimmunity through treatment of Chagas heart disease. Department of Immunology and Microbial Pathogenesis Seminar, Northwestern University Medical School, Chicago, IL, October 2007.

"The Regulation of Infection-Induced Cardiac Autoimmunity and Natural Dissemination of *Trypanosoma cruzi* in Experimental Chagas Heart Disease.." Integrated Graduate Program Thesis Seminar, Northwestern University Medical School, Chicago, IL, July 2008.

PRESENTATIONS: POSTERS

Bailey, S., McMahon, E., **Hyland, K.V.**, Miller, S.D. (2004) The role of dendritic cells in the CNS during relapsing EAE. 33rd Annual Autumn Immunology Conference. Chicago, IL.

Bahk, T.J., Daniels, M.D., **Hyland, K.V.**, Leon, J.S., Wang, K., Engman, D.M. (2004) Captopril and losartan ameliorate experimental autoimmune myocarditis. 33rd Annual Autumn Immunology Conference. Chicago, IL.

Daniels, M.D., **Hyland, K.V.**, Bahk, T.J., Wang, K., and Engman, D.M. (2004) Inhibition of the renin-angiotensin system prevents experimental autoimmune myocarditis. 33rd Autumn Immunology Conference, Chicago, IL.

Hyland, K.V., Daniels, M.D., Leon, J.S., Bahk, T.J., Giafis, N., Wang, K., Engman, D.M. (2004) Myocarditis and cardiac myosin autoimmunity are reduced by anti-*Trypanosoma cruzi* chemotherapy in experimental Chagas disease. 33rd Annual Autumn Immunology Conference. Chicago, IL.

Daniels, M.D., **Hyland, K.V.**, Bahk, T.J., Wang, K., and Engman, D.M. (2005) Potential contribution of autoimmunity to Chagas heart disease. Chicago Area Mycology and Parasitology Meeting, Chicago, IL.

Daniels, M.D., **Hyland, K.V.**, Desai, R., Wang, K., and Engman, D.M. (2005) Recombinant myosin fragment circumvents *in vitro* cardiac myosin toxicity and induces Th1-mediated experimental autoimmune myocarditis in BALB/c mice. 34th Autumn Immunology Conference, Chicago, IL.

Hyland, K.V., Olson, C.L., Daniels, M.D., Bediako, T.Y., Asfaw, S.H., Engman, D.M. (2006) The excitation of red fluorescence in *Trypanosoma cruzi*. Annual Microbiology/Immunology Retreat. Lake Geneva, WI.

Hyland, K.V., Olson, C.L., Daniels, M.D., Bediako, T.Y., Asfaw, S.H., Engman, D.M. (2006) Adoptive transfer of Chagas heart disease: Control of parasite transfer through use of fluorescent trypanosomes. 35th Annual Autumn Immunology Conference, Chicago, IL.

Bonney, K.M., **Hyland, K.V.,** Wang, K., Engman, D.M. (2006) Molecular mimicry in Chagas heart disease: identification of mimic epitopes to develop specific immunotherapy. 11th Annual Symposium. Center for Drug Discovery and Chemical Biology. Chicago, IL.

Leon J.S., **Hyland, K.V.**, Daniels M.D., Giafis N., Woods L.M., Bahk T. J., Wang K., Engman D.M. (2006) Trypanocidal drug treatment reduces cardiac autoimmunity in *Trypanosoma cruzi*-infected mice. Molecular Parasitology/Vector Biology Symposium. Athens, GA.

Emmer, B.T., Toriello, K.M., Maric, D., Daniels, M.D., **Hyland, K.V.**, Olson, C.L., Engman, D.M. (2007) Biochemical and functional investigation of *Trypanosoma brucei* flagellar calcium-binding proteins. Kinetoplastid Molecular and Cell Biology Meeting. Woods Hole, MA.

Hyland, K.V., Leon J.S., Daniels, M.D., Bahk T. J., Wang K., Engman, D.M. (2007) Regulation of cardiac autoimmunity with trypanocidal therapy. Chicago Area Mycology and Parasitology Meeting. University of Chicago, Chicago, IL.

Daniels, M.D., **Hyland, K.V.**, Wang, K., Engman, D.M. (2007) Experimental autoimmune myocarditis induced with recombinant myosin fragment. 36th Annual Autumn Immunology Conference, Chicago, IL.

Hyland, K.V., Leon J.S., Daniels, M.D., Bahk T. J., Wang K., Engman, D.M. (2007) Regulation of cardiac autoimmunity with trypanocidal therapy. 36th Annual Autumn Immunology Conference, Chicago, IL.