CD8+ T CELLS MAINTAIN PARASITE CONTROL IN NONLYMPHOID TISSUE DURING *TRYPANOSOMA CRUZI* INFECTION

by

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(Under the Direction of Rick L. Tarleton)

ABSTRACT

Trypanosoma cruzi establishes a persistent infection in mammalian hosts and causes Chagas disease in humans. CD8+ T cells are required for survival of acute infection by this parasite, but the role of these cells in chronic phase infection is not as well defined. It had been proposed that T cell dysfunction, a process observed in several chronic viral infections, could permit parasite persistence in nonlymphoid tissue. However, clearance of *T. cruzi* from skeletal muscle was not enhanced by blocking well described immunoregulatory mechanisms involving the inhibitory coreceptor PD-1 or IL-10. In contrast, this work shows that CD8+ T cells specific for *T. cruzi*-derived antigen are selectively activated in sites of parasite persistence, and depletion of CD8+ T cells during chronic phase infection results in loss of optimal parasite control. Because *T. cruzi* can be acquired via mucosal surfaces including the GI tract, the effect of oral infection on the CD8+ T cell response to *T. cruzi* was investigated. The data herein suggest that the route of infection does not alter the ability of *T. cruzi* to establish infection in muscle tissue. Also, mice generate a robust CD8+ T cell response to oral *T. cruzi* infection with a similar tissue distribution of *T. cruzi*-specific CD8+ T cells compared to foot pad infection. Finally, the vaccine efficacy of orally-administered attenuated parasites was tested. Orally immunized mice

were protected from foot pad challenge infection with wild type *T. cruzi*, supporting development of whole-organism-based vaccines that target reservoir species as a means to alleviate the burden of Chagas disease in endemic areas.

INDEX WORDS: *Trypanosoma cruzi*, Chagas disease, CD8+ T cell, chronic infection, parasitic disease, oral infection, nonlymphoid tissue

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DEDICATION

I remember being very young when the first seeds were planted that would eventually grow into a deeply rooted desire to study science. "A 60 pound boy would weigh only 10 pounds on the moon," I was told. WOW!? My teacher – who guarded *the bomb*, who can fix any car or appliance or machine or toy, who brought meals and magic to sick veterans, who made pumpkin ladies talk – has offered many more lessons than I could ever master. I have been consistently inspired by his curiosity, ability to ask good questions, inexhaustible thirst for understanding balanced by a humble recognition of human imperfection, and awe at the beautiful world in which we live. His example demonstrates that what matters most is love of family, pursuit of truth (science being but one of the rewarding paths), and enjoyment of life as a great adventure. This work is dedicated to Raymond H. Deal. Thanks Pa.

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TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 *Trypanosoma cruzi* and Chagas disease

Human infection with *Trypanosoma cruzi* was first described in 1909 by the Brazilian physician Carlos Chagas. Reporting and awareness of Chagas disease increased steadily and in the 1980s as many as 24 million people in Central and South America were infected, making Chagas disease the most important parasitic infection in the Americas according to the World Bank [1]. *T. cruzi* infection continues to plague millions, although some success in transmission control efforts and ongoing research offer hope that the burden of Chagas disease can be substantially reduced, if not eliminated.

1.1.1 Epidemiology and transmission of *T. cruzi* infection

Currently, *T. cruzi* infects approximately 10 million people with 100 million at risk, most of them living in rural areas of Latin America spanning from Argentina to Mexico. The 14,000 deaths per year along with the effects of fatigue experienced in advanced disease contribute to the 700,000 disability adjusted life years (DALYs) attributable to Chagas disease [2]. In the final decades of the 20th century, awareness and concern for Chagas disease began to rise in the US and Europe as it was estimated that over 100,000 individuals infected with *T. cruzi* were living in these regions [3]. Coupled with case reports of organ transplantation-related and autochthonous transmission in the US, the more than 500 cases of *T. cruzi* infection that were detected in 1.5 years of screening the blood supply demonstrate that Chagas disease reaches beyond endemic areas of Latin America [4].

Over a dozen hemotophagous insect species in the subfamily *Triatominae* can act as vectors for *T. cruzi* [5]. Transmission to humans is promoted by poor housing conditions that provide a habitat for triatomine bugs [5] as well as the presence of reservoir animals such as dogs and cats in the living quarters [6, 7]. Infective forms of the parasite are present in the hindgut and feces of the insect, can be inoculated into the host by contact with mucosal surfaces, through breaks in the skin, or by consumption of bugs or contaminated food or drink [8]. Large scale control efforts have targeted vector transmission with some success. However, emergence of insecticide resistance and recolonization of sprayed homes present challenges to the sustainability of control efforts that depend only on vector control [1, 8]. Other modes of transmission include mother-to-child, organ transplant and blood transfusion [8]. Oral transmission is especially relevant to this project. Although it may not be widely appreciated, this form of infection is well-documented as highlighted by several outbreak reports from regions in the Amazon [1, 9-13]. Furthermore, the prominence of this infection route among reservoirs is supported by observations of opossums [14], raccoons [15] and dogs [16] ingesting triatomine bugs.

1.1.2 Chagas disease

Clinically, Chagas disease is described as existing in three forms: acute, indeterminate, and chronic. *T. cruzi* infection is most frequently acquired in rural areas, usually during childhood or adolescence. Medical care is rarely sought during the acute phase of infection due to low access to medical facilities and absence or indiscriminate nature of symptoms, which can include fever and anemia. Parasites usually remain readily detectable in blood for two or three months following infection, and some newly-infected people exhibit a granulomatous lesion at the entry site or the characteristic unilateral edema around the eye known as Romaña sign.

Parasitemia then decreases to subpatent levels, and patients enter the indeterminate phase. This period of years to decades is symptomatically quiescent and has only recently received increased attention from researchers and clinicians who hope to better understand disease evolution, define prognostic parameters, and provide preventive therapy. Approximately 30% of *T. cruzi*-infected individuals will develop manifestations of chronic Chagas disease. Cardiomyopathy is the most important aspect of chronic Chagas disease, in which patients may experience conduction abnormalities, ventricular aneurysm, thromboembolism, and sudden death. Gastrointestinal (GI) involvement occurs in a smaller proportion of patients, usually restricted to South America, and results primarily in dysphagia or constipation. Autonomic denervation likely plays a role in cardiac and GI pathogenesis, and meningoencephalitis is seen in the very young or patients with reactivation lesions due to AIDS [17, 18].

The etiology of Chagas disease has been a matter of controversy [19, 20]. The question has been whether ongoing *T. cruzi* infection and the immune response against the parasite in heart, intestine, and esophagus are the primary mediators of disease in those locations or if parasite-induced autoimmunity could account for the pathologic changes seen in these tissues. The autoimmune hypothesis – that Chagas disease is caused by self-reactive antibodies Ab or T cells and is not dependent on parasite persistence in affected tissue – is based on observations noting a paucity of organisms in affected tissue and detection of Ab specific for self antigen (Ag) in *T. cruzi*-infected individuals, possibly arising from molecular mimicry [21]. However, a large body of evidence indicates that persistent *T. cruzi* infection in affected tissues is central in precipitating the pathologies of this disease [22, 23]. Rejection of neonatal mouse heart tissue transplanted into recipients with chronic *T. cruzi* infection was only observed when parasites were injected directly into the graft; immune responses elicited by immunization with *T. cruzi*

did not precipitate an autoreactive response to heart tissue Ag [24]. Experiments using a sensitive *in situ* PCR technique demonstrated that parasites were consistently present in tissue lesions of chronically infected mice, further establishing the link of pathology to *T. cruzi* persistence [25]. Moreover, evidence from human studies with heart [26, 27] and esophageal [28] tissue specimens support the correlation between persistent parasitism of tissue and disease development. Contrary to an autoimmune etiology, immunosuppresed patients undergoing organ transplantation or living with HIV infection are highly susceptible to resurgent parasitemia and disease manifestations including intensified myocarditis and meningoencephalitis [17, 23]. Finally, a review of drug efficacy trials and accumulating clinical experience among Latin American physicians indicate that pharmacological treatment of *T. cruzi* infection can improve clinical outcome, including disease progression in the chronic phase [29] Therefore, Chagas is best characterized as a chronic parasitic infection [22].

The only currently available drugs for treating *T. cruzi* infection are nifurtimox and benznidazole. Aggressive etiological treatment and drug discovery research have been limited by the debate over the mechanisms of pathogenesis in *T. cruzi* infection [22], the risk for unpleasant side effects, the lack of consensus on clinical efficacy of these drugs in chronic phase Chagas disease [30, 31], and the lack of a profitable pharmaceutical market in endemic areas [32]. Reflecting the prevailing clinical approach to pharmacological management of Chagas disease throughout Latin America [8], the CDC recommends drug therapy (although no generally available formulation is approved by the FDA) for persons under age 19, for most people 19 – 50 years of age in indeterminate phase of disease, and individualized decisions for older patients or those with advanced disease symptoms. Pregnancy and severe renal or hepatic insufficiency are the only contraindications [4].

There is no vaccine that prevents infection by *T. cruzi*. As with other infections, targeting conserved T cell epitopes could be beneficial [33, 34], and several studies have shown CD8+ T cells to be required for protection [35-41]. However, development of such a vaccine may be difficult given the massive expansions in *T. cruzi* gene families encoding immune targets and the highly polymorphic nature of the human MHC [34, 42]. Furthermore, protective responses would need to guard against mucosal or percutaneous routes of infection. A pragmatic strategy that has been proposed is vaccination of reservoir animals to interrupt the domestic transmission cycle [6, 7, 43].

1.1.3 *T. cruzi*: The parasite and its life cycle

T. cruzi is a protozoan pathogen belonging to the order Kinetoplastida, so named for the kinetoplast, a DNA structure in the matrix of the single mitochondrion of these organisms accounting for 20-25% of total cellular DNA [44]. Epimastigotes are the replicating form of *T. cruzi* found within the gut of the insect vector. Parasites transform into infective metacyclic trypomastigotes as they pass into the hindgut, where they are excreted with the feces. Upon entry into the mammalian host, *T. cruzi* can infect any nucleated cell in the body. Intracellular signaling in the parasite and host cell facilitates invasion by a process involving lysosome recruitment to the host cell membrane to form the parasitophorous vacuole [45]. Alternatively, *T. cruzi* can enter cells by a mechanism independent of lysosome and cell membrane fusion [46]. Within 24 hours, *T. cruzi* escapes the vacuole, converts to an amastigote, and begins to divide in the cytoplasm by binary fission. After about 5 days, the host cell is destroyed and infective trypomastigotes are released to invade other cells or complete the life cycle upon ingestion by a triatomine bug during a blood meal [18].

Recent genomic and proteomic studies mark a major step forward in our understanding of *T. cruzi* biology. Repetitive sequences account for over half of the 12,570 annotated genes predicted to encode more than 20,000 proteins. The *trans*-sialidases (TS) are the largest gene family and known targets of adaptive immunity. Sialic acid must be acquired from the host and is necessary for cell invasion by trypomastigotes [45]. However, of at least 1400 genes and pseudogenes, very few encode proteins with confirmed enzymatic activity, raising the possibility that DNA sequences of the TS gene family have been amplified and diversified in response to immune pressure in the mammalian host [47]. Gene expression is primarily regulated at the post-transcriptional level [48], and comparison of stage-specific proteomes has revealed that intriguing survival strategies are potentially employed by *T. cruzi* in the disparate environments it inhabits. For example, the gut and hemolymph of the triatomine vector contain high concentrations of histidine, probably due to the abundance of this amino acid in hemoglobin. Epimastigotes that divide in the gut of the insect were found to express enzymes to convert histidine into glutamine, a substrate of the TCA cycle. In contrast, amastigotes that reside intracellularly have a protein expression profile that implies a heavy reliance on lipid metabolism, upregulating enzymes involved in β-oxidation of fatty acids [49]

1.2 Immunity to *T. cruzi* infection: focus on CD8+ T cells

Given the complex biology of *T. cruzi*, it is not surprising that a diverse set of both innate and adaptive immune effectors are involved in control of this parasite [42, 50]. Initial recognition and control of invading pathogens, as well as activation of the adaptive immune response, is accomplished by the innate immune system, which senses pathogen associated molecular patterns (PAMP) via pattern recognition receptors (PRR) such as Toll-like receptors (TLR) [51]. Considerable research has examined the involvement of macrophages and dendritic

cells (DC) in innate immunity to *T. cruzi*, and recent work has identified a role for TLR-dependent [42] and TLR-independent [52] immune recognition and response to this pathogen. For example, deficiency in TLR4 was found to reduce resistance to acute *T. cruzi* infection in mice [53]. Subsequently, it was reported that the majority of the susceptibility to acute *T. cruzi* infection observed in MyD88 knockout (KO) mice can be attributed to TLR2 and TLR9 [54].

Multiple arms of the adaptive immune response are critical to control of *T. cruzi* infection. CD4+ T cells coordinate effector mechanisms through secretion of cytokines. An effective CD4+ T cell response to *T. cruzi* requires a type-1-biased T-helper (Th1) cell response [55], which is characterized by interferon-gamma (IFN-γ), a cytokine that is indispensable for surviving this parasitic infection [56]. Regulation of Ab class switching, activation of macrophages to kill intracellular microbes, and stimulation of CD8+ T cell immunity are among the functions of CD4+ T cells involved in pathogen control. Mice incapable of mounting Ab responses also experience increased parasitemia and enhanced mortality in acute phase *T. cruzi* infection [57]. The role for CD8+ T cells in the immune response to this parasite has been well established in CD8+ T cell-deficient models and is further discussed below [35, 58]. Thus, deficiency in various arms of adaptive immunity is detrimental to controlling *T. cruzi* infection [35, 57-59].

1.2.1 CD8+ T cell response to *T. cruzi* infection

We have focused on the role of CD8+ T cells in *T. cruzi* infection. This subset of T lymphocytes is responsible for surveying the intracellular compartment for pathogen-derived or altered self proteins presented on major histocompatability complex (MHC) molecules. When the T cell receptor (TCR) is activated by a specific peptide:MHC complex, CD8+ T cells undergo a clonal burst of proliferation and express effector molecules such as granzyme,

perforin, Fas ligand, IFN-γ, and other cytokines. Through these effector molecules, CD8+ T cells directly eliminate infected, damaged, or malignant cells or induce other cells to do so [60]. The amastigote form of *T. cruzi* replicates in the cytosol, and parasite-derived Ag are known to enter the class I MHC presentation pathway [61]. Mice cannot survive infection with *T. cruzi* in the absence of CD8+ T cells [35, 58], and this subset predominates in inflammatory lesions during Chagas disease [62]. However, parasite persistence in some tissues has been well documented [23], indicating that the immune system fails to completely clear *T. cruzi* infection.

Recently, we found that the CD8+ T cell response of mice and humans is highly focused on epitopes from proteins encoded by the *trans*-sialidase gene family of *T. cruzi* [63]. In fact, representing one of the highest frequency Ag-specific T cell responses documented in any infection, approximately 30% of CD8+ T cells in mice with acute *T. cruzi* infection recognize the *trans*-sialidase-derived peptide ANYFKTLV (TSKB20) [63]. Most of the CD8 + T cells that respond to this chronic infection have an effector (Teff)/effector memory (Tem) phenotype [64]. Additionally, CD8+ T cells in peripheral blood mononuclear cells (PBMCs) of patients with more severe disease have a more highly differentiated phenotype [65]. However, a subset of TSKB20-specific CD8+ T cells throughout chronic *T. cruzi* infection have functional and phenotypic attributes of Tcm including enhanced survival in the absence of Ag and expression of CD62L, CD127, and Bcl-2 [66]. Interestingly, very few CD8+ T cells from muscle of chronically *T. cruzi*-infected mice produce IFN-γ in response to *in vitro* stimulation, whereas those from the spleen of the same animal exhibit robust responses in this assay [67]. Factors regulating this apparent "dysfunction" of CD8+ T cells in *T. cruzi*-infected tissues have not been elucidated.

1.2.2 CD8+ T cells in nonlymphoid tissue

Because *T. cruzi* persists in tissues such as muscle [25] and adipose [68], it must be considered that much of the interaction between persistent parasites and the immune system throughout chronic infection takes place in nonlymphoid tissue. Upon activation, huge numbers of CD8+ Teff are generated. While most of this expanded population dies off, a subset is preserved as memory CD8+ T cells to protect against a second encounter with the same pathogen. Based on surface phenotype [69], memory CD8+ T cells generated after cleared infection are divided into central memory (Tcm) and Tem subsets. Tcm have the potential to rapidly supply new legions of effectors, Tem patrol peripheral tissue to combat infection at the point of attack [70]. Maintenance of Teff and Tem cells in peripheral tissue is crucial for limiting systemic spread of infections and for providing enhanced protection upon a second encounter with the same pathogen at a peripheral site [71].

Mucosal tissue, which includes the respiratory tract, the GI tract, and female genital tract, is a special subset of peripheral tissue that is often closely associated with its own organized lymphoid tissue. Collectively, mucosal tissue represents a huge surface area that is susceptible to pathogen invasion [72]. The structure and composition varies among mucosal sites, but common features include populations of resident T cells and APC, foci of organized lymphoid tissue such as Peyer's patches [73] or iBALT [74], and dependence on secretory IgA as a primary immune effector mechanism [73]. CD8+ T cells may have specific homing signatures that direct them to mucosal tissue, reflecting the unique immunological needs of this anatomic compartment [72, 75]. Also, unless an overriding danger signal is present, the cytokine environment in mucosal tissue generally favors tolerance, an imperative state due to constant contact with commensal and environmental Ag [76].

1.2.3 Regulation of CD8+ T cell responses during chronic infection

Another defining characteristic of *T. cruzi* infection is its chronicity, which likely impacts the quality of the anti-parasite T cell response. Several viruses, bacteria, protozoa, and worms are not completely cleared from the host; rather, these pathogens persist in the face of an immune response that controls but does not eliminate the infecting agent [77]. Murine infection with clone 13 lymphocytic choriomeningitis virus (LCMV), a virus that persist for months in immunocompetent mice, has been used to define much of the paradigm for T cell function during chronic infection. In this model, CD8+ T cells that are denied rest from Ag stimulation are unable to undergo cytokine-driven, homeostatic proliferation characteristic of Tcm (Ag addiction) [78] and become progressively incapable of effector functions (exhaustion) [79]. Using a mouse system that restricts class I MHC expression to hematopoietic cells, it has recently been more clearly shown that persistent antigen encounter in chronic LCMV infection is a major determining factor driving the altered function of T cells characteristic of that model [80]. However, clone 13 LCMV may represent one extreme of a continuum. The differentiation phenotype of Ag-specific CD8+ T cells varies with the local or systemic Ag load and replication rate or frequency of reactivation in different persistent viral infections [81]. In chronic infection with the protozoan pathogen *Leishmania* resistance to reinfection was lost within months of resolving the primary infection [82], agreeing with the hypothesis that "true" Ag-independent memory does not develop in chronic infections [78]. However, other evidence suggests that Tcm can develop during chronic infections [83-85] including *Leishmania* [86] and *T. cruzi* [66]. In fact, following drug-induced cure of *T. cruzi* infection, parasite-specific CD8+ T cells have a Tcm phenotype and protect against a high dose challenge [30]. Thus, prolonged exposure to Ag clearly affects the function and maintenance of CD8+ T cell populations. However, an all-

inclusive description of CD8+ T cell responses in chronic infection is not possible as the precise characteristics of CD8+ T cell responses are determined by many factors particular to each infection.

Whether responding to an infection is acute and cleared or last for years, the immune system is highly regulated to limit damage to self tissue. Two major regulators of CD8+ T cell responses are the PD-1/PD-L1 pathway and the cytokine interleukin-10 (IL-10). T cell exhaustion has been largely ascribed to programmed death-1 (PD-1) expression by Ag-specific CD8+ T cells [87]. PD-1, an inhibitory coreceptor homologous to CD28 and CTLA-4, is expressed on activated T cells, B cells and macrophages and recognizes B7-related molecules PD-1 ligand-1 and 2 (PD-L1 and PD-L2) [88]. While PD-L2 is expressed primarily on dendritic cells and macrophages, PD-L1 is distributed among many cell types including heart, skeletal muscle, kidney tubles, pancreatic islets, glia of the CNS, keratinocytes, and endothelial cells; and its expression is induced by interferons α, β, and γ [87]. PD-1 inhibits T cell proliferation and cytokine production *in vitro*. Importantly, inhibition of this pathway restores compromised T cell function in chronic LCMV infection and promotes viral clearance [89]. Interestingly in HIV-1-infected individuals, PD-1 expression on CD8+ T cells negatively correlates with viremia, CD4 counts, and impairs HIV-specific functional responses of CD8+ T cells [90-92]. This pathway is also implicated in immune regulation in other viral infections [93, 94] and parasitic infection with *Schistosoma mansoni* [95], *Leishmania* [96], and *Toxoplasma gondii* [97]. Alternatively, PD-1 involvement is notably absent in some chronic viral infections [84]. Therefore, PD-1 is a prominent regulator of CD8+ T cell responses in many chronic infections, but this pathway is not involved in all cases of persistent Ag.

A second mechanism for regulating CD8+ T cell responses to chronic infection is centered on interleukin-10 (IL-10). This cytokine is produced by many cell types including DCs and CD4+ regulatory T cells (Treg) and is known to antagonize the IL-12 IFN-γ Th1 axis and antimicrobial actions of macrophages, inhibit Ag presentation, and dampen T cell proliferation and cytokine production [98]. Viral, bacterial, and protozoan pathogens are thought to exploit actions of IL-10 as a means of persisting in their host [99-102]. Pathogen-specific CD8+ T cells display improved effector function when IL-10 is deficient or blocked during chronic viral infection [99, 103]. In acute *T. cruzi* infection, parasitemia is reduced in mice treated with anti-IL-10 Ab [104], but this cytokine may be required to prevent immune hyperactivation at early time points of infection [105, 106]. Whether IL-10 or PD-1 plays a role in facilitating parasite persistence or regulating T cell responses in chronic *T. cruzi* infection has not been explored.

1.3 Summary and questions to be investigated

T. cruzi infection is responsible for considerable mortality and morbidity mostly in rural Latin America, but people in the US and Europe, especially immigrants, are also affected. CD8+ T cells are a crucial component of the immune response to *T. cruzi* infection, but their importance during chronic phase infection is not well established. Additionally, little attention has been given to how CD8+ T cells function in various nonlymphoid tissues during *T. cruzi* infection. The hypothesis tested in Chapter 2 is that CD8+ T cells are a critical component of optimal parasite control throughout chronic infection with *T. cruzi*. Functional and parasitological readouts are used to determine if neutralizing immunoregulatory pathways enhance CD8+ T cell response. Alternatively, certain aspects of CD8+ T cell function are blocked and the detriment to host control of *T. cruzi* infection is assessed. In Chapter 3 the hypothesis is that an oral route of infection will alter the phenotype and distribution of CD8+ T

cells elicited early in *T. cruzi* infection. To address these issues the CD8+ T cell response was compared between mice infected with *T. cruzi* by oral gavage or by foot pad injection. Finally, it was tested whether oral vaccination with an attenuated strain of *T. cruzi* could provide protection against virulent challenge at a site outside of mucosal tissue (foot pad).

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CHAPTER 2

CD8+ T CELL RESPONSES AT SITES OF PARASITE PERSISTENCE IN CHRONIC *TRYPANOSOMA CRUZI* INFECTION[1]

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Abstract

CD8+ T cell responses are critical for controlling many pathogens. However in some cases, full effector T cell function is compromised, and complete clearance of the invading organism is not achieved. CD8+ T cells are an essential component of immune control of acute infection with the protozoan parasite *Trypanosoma cruzi*, which infects a range of mammalian hosts and causes Chagas disease in humans. However, few CD8+ T cells isolated from sites of parasite persistence in *T. cruzi*-infected mice are capable of IFN-γ production upon *ex vivo* restimulation, implying that a functional defect in this cell population could compromise parasite clearance. Herein we show that the well-documented regulatory pathways involving PD-1 or IL-10 have little impact on T cell responses or the ability of hosts to control *T. cruzi* infection. These results led us to further hypothesize that CD8+ T cell effector function in sites of *T. cruzi* persistence is not defective but instead is transient and crucial to optimally control this parasitic infection. A substantial proportion of CD8+ T cells in sites of parasite persistence express CD69, a marker of recent activation. Furthermore, the CD8+ T cell compartment in peripheral tissues is continually supplemented by Teff/Tem cells recruited from the circulation and the depletion of CD8+ T cells for a discrete period during chronic infection exacerbates tissue parasitism and inflammation. Thus, CD8+ T cells continue to be active players in controlling *T. cruzi* at sites of parasite persistence throughout *T. cruzi* infection.

2.1 Introduction

In response to intracellular infections, CD8+ T cells are primed by antigen presenting cells (APC) displaying specific peptide epitopes on MHC I molecules. These antigen (Ag)-specific CD8+ T cells then expand in number and acquire the ability to traffic to foci of inflammation where they may kill target cells and produce effector cytokines [1, 2]. Following the peak of an immune response, most of the effector T cells (Teff) generated undergo apoptosis, but a fraction remain as memory cells, which can be roughly classified into one subset that surveys nonlymphoid tissues (effector memory, Tem) and a second subset that recirculates through lymph nodes (LN) (central memory, Tcm) [3]. Maintenance of T cells in nonlymphoid tissue (sometimes called effector lymphoid tissue (ELT)) is crucial for controlling systemic infections and for providing enhanced protection upon a second encounter with the same pathogen at a peripheral site [4].

An additional level of complexity to the above paradigm of T cell responses to infection is that Ag clearance is not the only possible outcome. Ag is persistently present in chronic infectious diseases (as well as autoimmune disease and tumors). Some of the most deadly (HIV) and ubiquitous (CMV, EBV) viral infections fall into this category, and there is a crucial need to better understand the relationship between persistent infections and the T cell responses they elicit [5].

It has been suggested that "true" Ag-independent memory does not develop in chronic infections [6], and that CD8+ T cells become dysfunctional in a progressive yet often reversible process termed exhaustion [7, 8]. However, there are counterexamples that demonstrate that these generalizations do not necessarily apply to all cases in which the infecting agent persists

[9-11]. Thus, a comprehensive description of CD8+ T cell responses during chronic infection is difficult to represent in any single, existing model.

The protozoan pathogen *Trypanosoma cruzi*, which causes Chagas disease in humans, establishes a chronic infection in a range of mammalian hosts. The primary clinical complications of this infection, cardiomyopathy and gastrointestinal pathology, occur in nonlymphoid tissue and are contingent upon parasite persistence in the affected tissues [12, 13]. CD8+ T cells are the predominant cell type in tissue lesions seen in *T. cruzi* infection [14] and are critical for control of this parasitic infection [15-17]. However, CD8+ T cells from skeletal muscle of mice chronically infected with *T. cruzi* have been labeled "dysfunctional" due to their inability as a population to secrete IFN-γ and exhibit cytolytic activity [18]. Whether compromised CD8+ T cell function permits persistence of *T. cruzi* in peripheral tissues has not been directly tested. In this study we have taken advantage of the recent identification of a class I MHC-restricted immunodominant epitope [19] to analyze CD8+ T cell-mediated immunity to *T. cruzi* in nonlymphoid tissues. The results provide evidence that "dysfunction" does not aptly describe T cell responses in this persistent parasitic infection. Rather, functional CD8+ T cells are present throughout the chronic phase and actively limit parasitism by *T. cruzi*.

2.2 Materials and Methods

2.2.1 Mice, parasites, and infections

C57BL/6 (Ly5.2$^+$) (B6) and B6.SJL (Ly5.1$^+$) mice were purchased from either The Jackson Laboratory or National Cancer Institute at Frederick (Frederick, MD). IL-10 KO mice were a kind gift from Dr. Julie Moore, University of Georgia. PD-L1 KO mice were a kind gift of Dr. Arlene Sharp, Brigham and Women's Hospital, Boston, MA. Mice were maintained at the University of Georgia animal facility in microisolator cages under specific pathogen-free

conditions. For *T. cruzi* infections, tissue culture trypomastigotes (TCT) of the Brazil strain or CL strain of *T. cruzi* were obtained from passage through Vero cells. Mice were infected either intraperitoneal injection (ip) or by subcutaneous infection into the foot pad (fp) with 1000 TCT and sacrificed by CO_2 inhalation. All animal protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

2.2.2 PBMC collection

Mice were anesthetized with halothane and blood was obtained from the retroorbital space with a glass Pasteur pipette. Alternatively, 20 to 30 ml of blood was taken from the nicked tail of mice in a physical restraint. Whole blood was then used for staining.

2.2.3 Isolation of lymphocytes from nonlymphoid tissues and adoptive transfers

Before tissue removal, mice were perfused with 20 ml of PBS containing 0.8% sodium citrate as an anticoagulant. Tissue-derived lymphocytes were obtained by teasing tissues apart and vigorously pushing through a 40-μm nylon mesh screen. In some cases, cells were further purified by collection from the interface of 44% percoll in RPMI underlain with 67% percoll in PBS. For transfers, spleens from naïve or chronically *T. cruzi*-infected mice were homogenized with frosted glass slides (Fisher Scientific, Pittsburg, PA, USA) in a hypotonic ammonium chloride RBC lysis buffer and washed in RPMI with 10% FBS (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA). $CD8^+$ T cells were negatively selected through magnetic sorting with a $CD8a^+$ T cell isolation kit (Miltenyi, Bergisch Gladbach, Germany). CD8+ cells were transferred i.v. into infection-matched (or simply chronically infected mice for naïve transfers) congenic mice, and recipients were sacrificed at various days post transfer to analyze donor cell populations in recipient tissues.

2.2.4 T cell phenotyping

RBCs in single-cell suspensions of spleen cells (SC) were lysed in a hypotonic ammonium chloride solution and washed in staining buffer (2% BSA and 0.02% azide in PBS (PAB)). In some cases, mouse peripheral blood was obtained by retro-orbital venipuncture, collected in sodium citrate solution, and washed in PAB. SC and whole blood were incubated with tetramer-PE and the labeled Abs. Cells were stained for 45 min at 4°C in the dark, washed twice in PAB, and fixed in 2% formaldehyde. The ELC-Ig chimera was used for detecting CCR7 expression and was a gift from Dr. K. Klonowski (University of Georgia, Athens, GA). For CCR7 detection, cells were incubated at 37°C for 1 h, stained with ELC-Ig for 45 min, and washed. Cells were then stained with goat anti-human IgG Alexa Fluor 488 (Invitrogen) for 30 min, washed, and stained with surface markers as indicated above. For whole blood, RBCs were lysed after surface staining in a hypotonic ammonium chloride solution and washed twice in PAB. At least 500,000 lymphocytes were acquired using a CyAn flow cytometer (DakoCytomation) and analyzed with FlowJo software (Tree Star). MHC I tetramer TSKB20 (ANYKFTLV/K^b) was synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA). Antibodies for flow cytometric analysis were purchased from BD Biosciences, eBioscience, and Caltag Laboratories/Invitrogen.

2.2.5 Intracellular cytokine staining

Lymphocytes isolated from spleen or peripheral tissue were stimulated with plate-bound anti-CD3 or *T. cruzi* peptides (1 μM) for 5 h at 37°C in the presence of 1 μg/ml brefeldin A (BD PharMingen, San Diego, CA, USA), an inhibitor of the secretory pathway. *T. cruzi* peptides comprised a pool of TSKB20 (ANYKFTLV) and TSKB18 (ANYDTLV) (Signa-Genosys, St. Louis, MO, USA) [19]. Cells were surface stained, washed, fixed and permeablized, and

intracellular cytokine staining was performed to detect IFN-γ expression using a Cytofix/Cytoperm kit (BD PharMingen) in accordance with the manufacturer's instructions.

2.2.6 Quantitative real-time PCR

Parasite equivalents in tissue were determined as previously described [20]. Briefly, tissue was collected from mice and finely minced. Samples were incubated at 55 °C for 4 hours in SDS-proteinase K lysis buffer. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 100% ethanol, and resuspended in nuclease free water. Samples were analyzed on an iCycler (Biorad, Hercules, CA, USA)

2.2.7 Histology

Heart and Skeletal muscle was obtained from *T. cruzi*-infected mice and controls, fixed in 10% buffered formalin and embedded in paraffin. Five-micron-thick sections were obtained and stained with hematoxylin-eosin. Inflammation was evaluated qualitatively according to the presence or absence of myocyte necrosis and infiltrates according to distribution (focal, confluent or diffuse) and extent of inflammatory cells present [21]. Images of muscle tissue were taken with an OLYMPUS DP70 digital camera on an OLYMPUS BX60 microscope.

2.2.8 Statistical analysis

We calculated statistical significance with a two-tailed Student's *t*-test.

2.3 Results

2.3.1 Phenotype and function of CD8+ T cells in nonlymphoid tissue in *T. cruzi* infection

We first asked if reduced *ex vivo* functional responsiveness of CD8+ T cells from skeletal muscle of mice chronically infected with *T. cruzi* [18] was due to trafficking to that specific tissue. To address this question, we determined the memory phenotype and responsiveness to polyclonal stimulation of CD8+ T cells from additional nonlymphoid tissues of *T. cruzi*-infected

mice. In particular, we were interested in cells from adipose tissue, which has recently been described as a target for persistent *T. cruzi* infection [22]. We confirmed persistent *T. cruzi* infection in adipose tissue in three of three mice at over 100 days post infection (dpi) by real-time PCR (0.63 ± 0.51, mean parasite equivalents/50 ng tissue DNA ± SEM). Alternatively, *T. cruzi* is scarcely or not detectable in the lung during the chronic phase of infection (data not shown), allowing us to study CD8+ T cells in nonlymphoid tissue in which *T. cruzi* does or does not persist. CD44 is a marker of previous Ag encounter, and Ag-experienced cells can broadly be designated as either Tcm or Tem based on CD62L expression [23]. Consistent with previous findings [18, 24], splenocytes from chronically *T. cruzi*-infected mice are enriched for CD8+ Tem cells (Fig 2.1*A*). CD8+ T cells in peripheral tissues are nearly uniformly $CD44^{hi}$ and $CD62L^{lo}$, although CD44 expression is slightly less intense on CD8+ T cells in the lung (Fig 2.1*A*). In all tissues examined, mice maintain a population of CD8+ T cells specific for the immunodominant epitope TSKB20 [19] for > 200 dpi (Fig 2.1*B*). Thus, CD8+ T cells display specificity for *T. cruzi* Ag and a similar memory phenotype regardless of anatomical location. When stimulated with anti-CD3, a low frequency of CD8+ T cells from adipose tissue produces IFN-γ in comparison to CD8+ T cells from the spleen of the same mouse (Fig 2.2*A*). In contrast, a relatively high frequency of CD8+ T cells from lung and spleen makes IFN-γ upon polyclonal stimulation and in response to *trans*-sialidase gene family-encoded peptides (TS peptide, Fig 2.2*B*). Therefore, CD8+ T cells trafficking to sites of parasite persistence during chronic *T. cruzi* infection (i.e. muscle [18] and fat) exhibit diminished cytokine production upon *ex vivo* stimulation, but those circulating through non-infected tissue such as the lung remain highly responsive.

2.3.2 T cell exhaustion and IL-10-mediated immune regulation are not dominant determinants of parasite persistence

Persistent Ag stimulation can lead to CD8+ T cell dysfunction (exhaustion) [7], an often reversible process associated with expression of the inhibitory receptor programmed death-1 (PD-1) on the surface of CD8+ T cells [8, 25-27]. To test if the PD-1 pathway has a role in regulating CD8+ T cell responses during chronic *T. cruzi* infection, we infected mice lacking PD-L1 (PD-L1 KO), the ligand for PD-1 that is widely distributed in nonlymphoid tissue including heart, skeletal muscle, and endothelial cells [28]. To determine if there was a greater amount of proliferation or survival of immune cells in PD-L1 KO mice infected with *T. cruzi*, total spleen cells and the number and frequency of CD8+ T cells and TSKB20-specific CD8+ T cells were calculated in acute and chronic phase of infection (Fig 2.3*A*-*E*). In response to acute *T. cruzi* infection, both groups of mice generate comparable proportions of TSKB20-specific CD8+ T cell populations. These populations contract similarly, though there is a higher frequency and absolute number of TSKB20-specific CD8+ T cells per spleen in PD-L1 KO mice in the chronic phase (Fig 2.3*D* and 2.3*E*). Like wild type (WT) mice, PD-L1 KO mice control but do not completely clear *T. cruzi* infection (Fig 2.3*F*). Unexpectedly, the PD-L1 KO mice have a greater average parasite load in skeletal muscle than WT mice at 26 and 176 dpi, but this difference was not statistically significant. PD-L1-deficient mice also do not develop a different degree of inflammation in muscle or heart tissue relative to WT mice (Fig 2.3*G*). Finally, the absence of PD-L1 expression does not alter the inability of CD8+ T cells isolated from muscle to produce IFN-γ *ex vivo* following *ex vivo* stimulation with plate-bound anti-CD3 or *T. cruzi* TS peptide (Fig 2.3*H*). Therefore, the PD-1 inhibitory pathway is not a major factor in shaping the CD8+ T cell response to chronic *T. cruzi* infection.

Interleukin-10 (IL-10) has recently been shown to be a major regulator of T cell responses to chronic infections [29-31]. We examined whether this cytokine contributes to the control of T cell responses at sites of parasite persistence in *T. cruzi* infection. Our initial approach was to infect IL-10 KO mice and assess *T. cruzi*-specific T cell frequency and phenotype as mice progressed into chronic phase. This was not possible because mice succumbed to infection by 45 dpi, a result in agreement with previous studies [32]. We did not observe enhanced parasite clearance (Fig 2.4*A*), but inflammation in skeletal muscle was notably increased (Fig 2.4*B*) in acutely-infected IL-10 KO mice compared to WT. To address our original question of whether IL-10 dampens the anti-*T. cruzi* immune response and prevents clearance of parasites in chronic phase infection, we administered an IL-10 receptor (IL-10R)-blocking antibody (Ab) to mice with established *T. cruzi* infection. Persistent parasites were detected in 3 of 5 anti-IL-10-treated mice and 4 of 5 rat IgG-treated controls, and the average parasite load among the positive samples in the two groups was not statistically different (Fig 2.4*C*). Taken together, these data suggest that neither PD-1 nor IL-10 alone is a major factor inhibiting the clearance of *T. cruzi* during chronic infection.

2.3.3 Recently activated CD8+ T cells are preferentially found in sites of *T. cruzi* persistence

Because major regulatory pathways are apparently dispensable during chronic *T. cruzi* infection, we questioned the amount of CD8+ T cell stimulation caused by persistent parasites. To investigate the extent of Ag encounter in chronic *T. cruzi* infection, we stained for the early activation marker CD69 on CD8+ T cells from lymphoid and nonlymphoid tissues of infected mice. Interestingly, CD69 is selectively expressed on CD8+ T cells at sites of parasite persistence in both acute and chronic *T. cruzi* infection (Fig 2.5*A*). At 35 dpi, nearly 50% or more of TSKB20-specific CD8+ T cells isolated from heart, skeletal muscle and adipose tissue

express CD69. More than 200 dpi, a substantial fraction (~15-35%) of both TSKB20-specific and other CD8+ T cells in heart, skeletal muscle, and adipose tissue continue to exhibit a recently activated phenotype (Fig 2.5*A*). We next examined surface expression of KLRG1, a molecule induced after multiple rounds of Ag encounter [33]. A high percentage (70-85%) of TSKB20-specific CD8+ T cells in all tissues examined express KLRG1 (Fig 2.5*B*), implying that most *T. cruzi* –specific CD8+ T cells present in chronic phase infection have encountered Ag several times. Collectively, these data demonstrate that Ag–specific CD8+ T cells transit to sites of persistent *T. cruzi* infection and probably become activated upon Ag encounter in peripheral tissue, but not to the point of exhaustion as reported for a number of persistent viral infections [34].

2.3.4 CD8+ T cell effector function is required for optimal control of chronic *T. cruzi* infection

The simple presence of recently activated CD8+ T cells in *T. cruzi*-infected tissue does not address the question of how these cells are functioning and whether or not their function is involved in control of parasite levels in these tissues. A principle effector function of activated CD8+ T cells is IFN-γ production [35], and this cytokine is a crucial element in the immune response to many intracellular infections [36] including *T. cruzi* [15]. To test whether this cytokine was an imperative component of parasite control in the chronic phase of infection, mice were treated for 30 days with an anti-IFN-γ Ab beginning at >100 dpi and then examined for changes in parasite load. Mice treated with anti-IFN-γ had higher parasite burden in skeletal muscle tissue (Fig 2.6). Although this experiment does not address the cellular source of IFN-γ, this result shows that chronic phase control of *T. cruzi* depends on actions of this cytokine.

To directly test the importance of CD8+ T cells in maintaining control *T. cruzi* in the chronic phase, anti-CD8 Ab was administered for 30 days to mice beginning at >100 dpi. Treatment markedly reduced circulating CD8+ T cells in all mice (Fig 2.7*A*), although a small population of residual CD8+ T cells was present in some animals. Compared to IgG-treated controls, parasite load in skeletal muscle was generally higher in the CD8-depleted group, with some mice in this group having parasite loads 10-100-fold higher than any mice in the control group (Fig 2.7*B*). Anti-CD8 treated mice also exhibited increased inflammation in muscle tissues in concert with the increased parasite tissue burden (Fig 2.7*C*). These data support the hypothesis that CD8+ T cells play a vital role in maintaining parasite control in persistently infected tissues.

2.3.5 Maintenance of CD8+ T cell response to *T. cruzi* in peripheral tissue

We next asked how hosts maintain CD8+ T cells in sites of parasite persistence over the long course of *T. cruzi* infection. To test the hypothesis that T cell responses in the peripheral tissue must be seeded by recruitment of Teff from a common pool of Tem, CD8+ T cells were isolated from the spleens of chronically *T. cruzi*-infected SJL mice (CD45.1+) and transferred into infection-matched B6 (CD45.2) recipients. Donor cells were promptly incorporated into the CD8+ T cell response in recipient tissue as early as 2 days post transfer and are detectable in the tissue at least one month after transfer (data not shown). As expected, an increased proportion of the transferred cells (as well as the endogenous T cells) recovered from the sites of parasite persistence or the LN that drain these sites express CD69 as compared to those obtained from the spleen or lung (Fig 2.8*A*). These data show that even in the setting of low-level chronic inflammation, CD8+ T cell trafficking is a dynamic process, and CD8+ T cells responding to *T. cruzi* move readily from the circulation into tissues harboring parasite-infected cells. To

determine if frequent recruitment of effector cells to infected tissue was a key mechanism of controlling *T. cruzi* during chronic infection, trafficking was disrupted by the treatment of infected mice with Ab against VLA-4, a molecule involved in leukocyte extravasation [37]. Mice receiving anti-VLA-4 Ab exhibited an increased parasite load in skeletal muscle indicating that infected tissues depend on continuous leukocyte trafficking to suppress outgrowth of parasites (Fig 2.8*B*).

In addition to continued recruitment of Ag-experienced CD8+ T cells into responses to persistent pathogens, *de novo* priming of naïve T cells during an ongoing immune response may be necessary to sustain Teff levels [38, 39]. To determine whether naïve T cells are also being primed during chronic *T. cruzi* infection, we purified CD8+ T cells from naïve SJL mice (CD45.1) and transferred them into chronically infected B6 mice (CD45.2). Clear evidence of naive priming was found in only 1 of 12 *T. cruzi*-infected mice receiving naïve cell transfers. In other mice, the transferred cells detected in blood maintained a naïve phenotype ($CD11a^{lo}CD62L^{hi}CD127^{hi}$). Importantly, when priming of naïve donor cells was detected, these cells expanded, upregulated CD11a (marker of T cell activation), and trafficked to nonlymphoid tissue (Fig 2.8*C*).

2.4 Discussion

It is well-established that CD8+ T cells are an essential component of the immune response to acute *T. cruzi* infection [15-17]. However, the significance of this T cell population in chronic infection is not as well understood. The vast majority of patients with Chagas disease are diagnosed years after initial infection with *T. cruzi* [40], developing symptoms primarily due to damage of nervous, heart, and other muscle tissues [41]. Cellular infiltrates are associated with tissue lesions in Chagas disease, but the critical connection in this scenario is that *T. cruzi*

persists in affected tissues [42]. Therefore, we sought to better define the interaction between CD8+ T cells infiltrating peripheral tissue and the parasites chronically present in those same tissues. We found that CD8+ T cells continue to be required for optimal control of *T. cruzi* infection, even during chronic infection (approximately 120 – 180 dpi in most experiments) when the parasite burden is relatively low. The data that most directly supports this conclusion comes from our CD8 depletion experiments (Figure 2.6). All of the mice that fail to control tissue parasitism belonged to the anti-CD8-treated group, and striking inflammatory lesions are readily observable in muscle tissue of CD8-depleted mice. The incomplete penetrance of these observations likely derives from other arms of the immune system compensating for the defect in CD8+ T cell-mediated control of *T. cruzi* infection.

As with CD8+ T cells from skeletal muscle, we show that CD8+ T cells infiltrating adipose tissue in chronic *T. cruzi* infection display a Teff/Tem phenotype and produce IFN-γ in low frequency upon polyclonal *ex vivo* stimulation (Figure 2.1). These experiments highlight the role of persistent Ag, rather than the tissue environment at a particular site, in influencing T cell function. Although it is increasingly being appreciated as an endocrine organ with considerable connection to the immune system [43], infection of adipose tissue is not well-studied. It would be interesting to know if adapting to an intracellular niche in adipocytes is a unique strategy of parasitism employed by *T. cruzi* [22]. It is intriguing that the amastigote stage of *T. cruzi* may rely heavily on a lipid-based energy metabolism, providing a possible link between the tropism of *T. cruzi* in the chronically infected host and parasite physiology [44].

The selective upregulation of CD69 in adipose and muscle tissue is strong support that CD8+ T cells remain responsive to Ag stimulation and carry out effector function in persistently-infected tissues. Interestingly, increased frequencies of CD69+CD8+ T cells are also present

within LN of acutely and chronically *T. cruzi*-infected mice compared to baseline levels in naïve mice. These values are consistently above those in the spleen of the same mouse and below those in persistently infected peripheral tissues. Presently, it is not known if *T. cruzi*-specific CD8+ T cells are activated in LN and then concentrated in peripheral tissue in response to some chemotactic signal or if CD8+ T cell are activated in infected nonlymphoid tissue and then passively or actively move to LN. Recent work has highlighted a role for CCR7 in facilitating migration of CD8+ T cells in peripheral tissues to the draining LN [45, 46] and sphingosine-1-phosphate receptor-1 in retaining T cells in peripheral sites of inflammation [47]. Further experiments are needed to determine if these findings apply to T cell trafficking during *T. cruzi* infection.

How is the pattern of CD69 expression reconciled with *ex vivo* cytokine production of CD8+ T cells formerly labeled as "dysfunctional [18]?" First, it should be pointed out that some of the CD8+ T cells in adipose or muscle tissue (range ~1-15%) do produce IFN-γ; therefore, "functional" cells are detected by this assay in sites of persistent infection. Others have noted that when CD8+ T cells are maximally stimulated, removed from APC contact, and immediately re-exposed to Ag; they are incapable of a cytokine response to the second stimulation [48]. CD8+ T cells responding to Borna disease virus in the brain can similarly be underestimated by functional assays performed directly *ex vivo* [49]. Thus, the lower frequency cytokine response exhibited by populations of nonlymphoid CD8+ T cells *ex vivo* when compared to splenic CD8+ T cells could actually indicate a greater degree of anti-parasitic activity *in vivo*. The enrichment of CD69+ CD8 T cells at sites of persistence lends credence to this claim. CD69 is detectable on the surface of T cells as early as 2-3 hours after activation, peaks by 24 hours, and diminishes with a half-life of approximately 24 hours, although sustained stimulus may prolong its

expression [50]. In agreement with our observations, work in other systems has shown that CD69 is upregulated on CD8+ T cells trafficking to sites of active infection [51] and that CD69+ cells are producing IFN-γ [35]. In our experiments, the lung is not a major effector site, but rather a control to rule out nonspecific activation of all nonlymphoid populations of CD8+ T cells in *T. cruzi* infection. Accordingly, we see little CD69 expression on CD8+ T cells in lung or spleen, indicating that this activation marker is preferentially expressed by T cells in tissues where they are responding to pathogen. Thus, T cell function and activation status reflect Ag distribution within the *T. cruzi*-infected host. These data underscore the general importance of studying T cell immunity at the host-parasite interface and not relying only on assays with cells from spleen and peripheral blood.

In contrast to other chronic infections [52], well-described immunoregulatory pathways do not appear to be pivotal factors in determining the extent of parasite persistence or in diminishing the potency of CD8+ Teff cells in *T. cruzi* infection. Mounting data support that PD-1 is a chief inhibitor of T cell function in some models of chronic viral infection [8, 25-27, 31, 53-56]. Recently, CD8+ T cells responding to brain infection by *Toxoplasma gondii* were found to have diminished effector functions after trafficking to this nonlymphoid site and becoming PD-1+ [57]. Conversely, there are examples of chronic infections that do not exhibit PD-1 involvement [11]. Mice infected with *T. cruzi* group with the latter. Humans live for decades with *T. cruzi* infection and progress differently (if at all) to more severe stages of Chagas disease. CD27-CD28- terminally differentiated CD8+ T cells account for a larger proportion of the memory CD8+ T cell population in individuals with the most advanced disease [58]. Whether PD-1 is more frequently expressed by CD8+ T cells from patients with more severe disease compared to individuals with milder or no clinical symptoms is currently being

investigated. We suspect that duration of infection could yield a distinct phenotype in humans compared to that seen in mouse studies. It is important to note that the level of Ag in a mouse infected with *T. cruzi* is sufficient to induce $CD62L^{lo}$ phenotype on the majority of *T. cruzi*-specific cells [9, 24]. Most Ag-specific CD8+ T cells also express KLRG1 (Fig 2.5*B*), a phenotype reminiscent of inflationary CD8+ T cells described in MCMV infection [11]. *klrg1* is one of the most relatively abundant transcripts in Teff [59], and is thought by some to denote a population of short-lived T cells that do not contribute to memory responses [60, 61]. However, KLRG1 expression does not preclude the ability of CD8+ T cells to survive for extended periods of time in the memory phase [62]. Thus, during chronic *T. cruzi* infection, CD8+ T cells experience sufficient Ag stimulation to confer a Tem/Teff phenotype on the majority of parasite-specific CD8+ T cells, but this does not lead to T cell exhaustion.

Even with less intense Ag stimulation, CD8+ T cell function may still require modulation by immunoregulatory mechanisms. IL-10 is recognized as an antagonist to Th1-biased immune responses elicited against intracellular pathogens [63]. This cytokine is thought to be exploited by viral, bacterial, and protozoan pathogens as a means of persisting in their hosts [29-31, 64]. IL-10 has been shown to delay mortality or lessen some of the pathology associated with acute *T. cruzi* infection in mice [32, 65], but its role during the chronic phase is less clear. We observed early mortality in IL-10 KO mice. These data signify that cytokine may be necessary to prevent lethal pathology due to unbridled T cell activity. However, when IL-10 signaling was interrupted during chronic *T. cruzi* infection, treated mice could not clear parasites from skeletal muscle (Fig 2.4*C*) and did not develop increased inflammation in that tissue compared to control mice, meaning that IL-10 is much more critical in the acute phase as a means to protect the host from immunopathology when systemic parasite load is at a maximum. In a related study, we

observed that mice with a T cell-specific defect in TGFβ signaling (DNRII) [21] exhibited enhanced mortality, T cell hyperproliferation, and greater histopathology in comparison to WT controls. However, CD8+ T cells from skeletal muscle of DNRII mice infected with *T. cruzi* responded similarly to WT in *ex vivo* restimulation assays. Although the CD8+ T cell compartment was increased in DNRII mice with *T. cruzi* infection, the frequency of TSKB20+CD8+ T cells was decreased in comparison to that in B6 mice, meaning that *T. cruzi*-specific cells may not depend on an inhibitory signal from TGFβ to prevent excessive proliferation. In a separate study, depleting CD4+ regulatory T cells did not improve the ability of mice to control a lethal *T. cruzi* challenge or alter the development of anti-*T. cruzi* T cell responses [66].

In each of our experiments, we have tested single regulatory pathways individually. The absence of both IL-10 and TGFβ leads to severe inflammation and parasite clearance from muscle tissue in *T. spiralis* infection [67]. Others have observed that blocking the PD-1 pathway and IL-10 in combination yields even greater T cell function and viral clearance compared with interrupting either pathway alone [68]. While it is possible that simultaneously blocking multiple regulatory mechanisms may lead to eventual clearance of parasites, substantial Ag reduction, and/or exacerbation of tissue pathology; any one regulatory pathway appears to be dispensable for avoiding rampant T cell-mediated pathological responses during chronic *T. cruzi* infection. Overall, it is remarkable that *T. cruzi* infection seems to stimulate the immune system at a precise intensity that yields a controlling immune response that appears to be sufficiently mild such that fundamental regulatory pathways are not required for limiting T cell responses.

Though an overly exuberant CD8+ T cell response may not be provoked by persistent parasites in peripheral tissue, *T. cruzi* must be under thorough surveillance in the chronically-

infected host. Our adoptive transfer studies indicate that T cells are consistently trafficking to tissue from a common pool of circulating T cells (Fig 2.7*A*). Work in CCR5-deficient mice showed that the inability of T cells to traffic to peripheral tissue results in increased tissue parasitism and mortality during acute phase *T. cruzi* infection [69]; but, this model precludes experiments beyond early time points. Our results suggest that trafficking of CD8+ Teff to sites of parasite persistence remains a critical aspect of *T. cruzi* control in the chronic phase of infection (Fig 2.8*A* and 2.8*B*). Priming of naïve cells in chronic phase polyoma virus infection has been shown to maintain Ag-specific responses over long periods of time in mice [39]. Conditions in chronic *T. cruzi* can also support naïve priming. Though perhaps an infrequent event or minor factor in maintaining the anti-*T. cruzi* cellular immune response in affected tissues, it is important to note that even in the chronic phase, an appropriate environment for priming naïve *T. cruzi*-specific CD8+ T cells is preserved. The idea that persistent infections give rise to Ag-addicted CD8+ T cells [6] does not apply to *T. cruzi* as we have shown that a stable population of CD8+ T cells with Tcm characteristics is present throughout infection [9]. *T. cruzi*-specific cells in tissue likely derive primarily from Tem/Teff cells in general circulation, which are in turn refreshed by Tcm cells generated early in infection as well as infrequent naïve priming.

In conclusion, we present new data demonstrating that Ag-specific CD8+ T cells are selectively activated at sites of parasite persistence and show that depletion of this population has a detrimental impact on host control of parasites and disease. We believe that sustaining effective CD8+ T cell responses in *T. cruzi*-infected tissue is an active and dynamic process, providing competent CD8+ Teff cells to sites of infection as they are needed to control parasites.

Augmentation of this population may be an attractive therapeutic option for combating chronic *T. cruzi* infection.

Figure 2.1. CD8+ cells populate nonlymphoid tissues of *T. cruzi*-infected mice and display a Tem phenotype and specificity for *T. cruzi*-derived Ag. *A*, Tem CD8+ T cells are present in nonlymphoid tissues of *T. cruzi*-infected mice. Surface expression of CD44 and CD62L was analyzed by flow cytometry on cells isolated from isolated from, fat, lung, skeletal muscle, heart, and spleen of mice with chronic *T. cruzi* infection and spleen cells of naïve mice. Flow plots in the left column indicate the percentage of CD44hi cells among CD8+ T cells. Histograms in the right column show the percentage of $CD62L^{lo}$ cells within the CD44+CD8+ population. Cells were obtained from pooled tissue of three mice, and data are representative of at least two additional similar experiments. *B*, TSKB20-specific cells traffic to peripheral tissues in chronic *T. cruzi* infection. Flow plots are gated on CD8+ T cells isolated from indicated pooled tissues from 2 chronically-infected mice (>200 dpi), and numbers indicate the percentage of CD8+ T cells binding TSKB20-loaded class I MHC tetramer. Five similar experiments provided similar results. LN, lymph node.

Figure 2.1

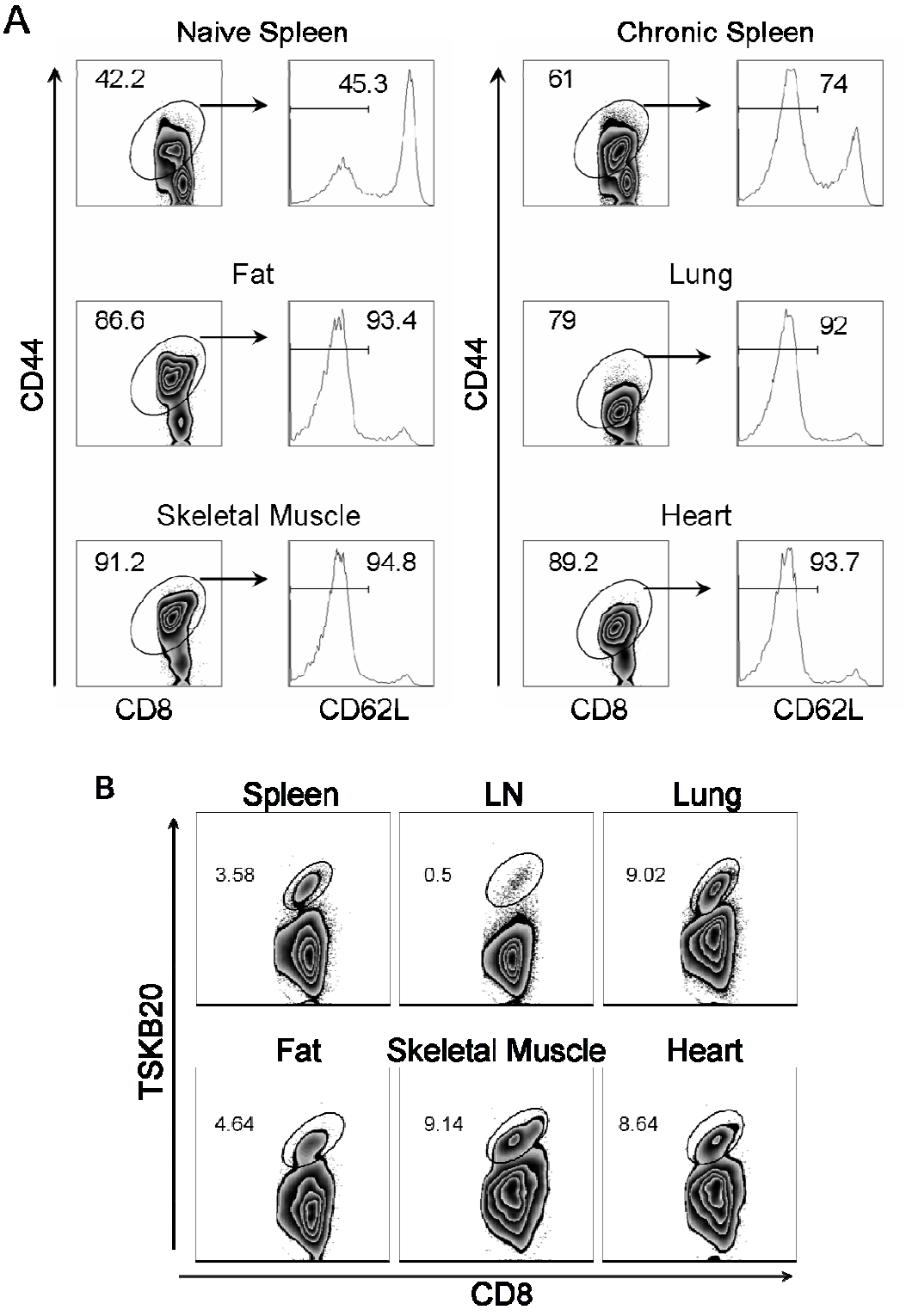

A
Naive Spleen
42.2
45.3
Chronic Spleen
61
74
Fat
86.6
93.4
Lung
79
92
Skeletal Muscle
91.2
94.8
Heart
89.2
93.7
CD44
CD44
CD8
CD62L
CD8
CD62L
B
Spleen
LN
Lung
3.58
0.5
9.02
Fat
Skeletal Muscle
Heart
4.64
9.14
8.64
TSKB20
CD8

Figure 2.2. Distinct functional responses of CD8+ T cells from nonlymphoid tissue of *T. cruzi*-infected mice. *A*, CD8+ T cells from fat are poor producers of IFN-γ. Cells isolated from fat and spleen of chronically-infected mice were stimulated *ex vivo* with plate-bound anti-CD3 for 6 hours, then stained for surface expression of CD8 and intracellular expression of IFN-γ, and analyzed by flow cytometry. Numbers are percentages of CD8+ cells producing IFN-γ. *B*, CD8+ T cells in lung are responsive to *ex vivo* stimulation. Cells isolated from lung and spleen of chronically infected mice were processed and analyzed as in *A* except cells were also stimulated with a mixture of defined CD8+ T cell peptide epitopes (TSKB18 and TSKB20) derived from *T. cruzi trans*-sialidase genes [19]. Similar results were obtained in two other experiments.

Figure 2.2

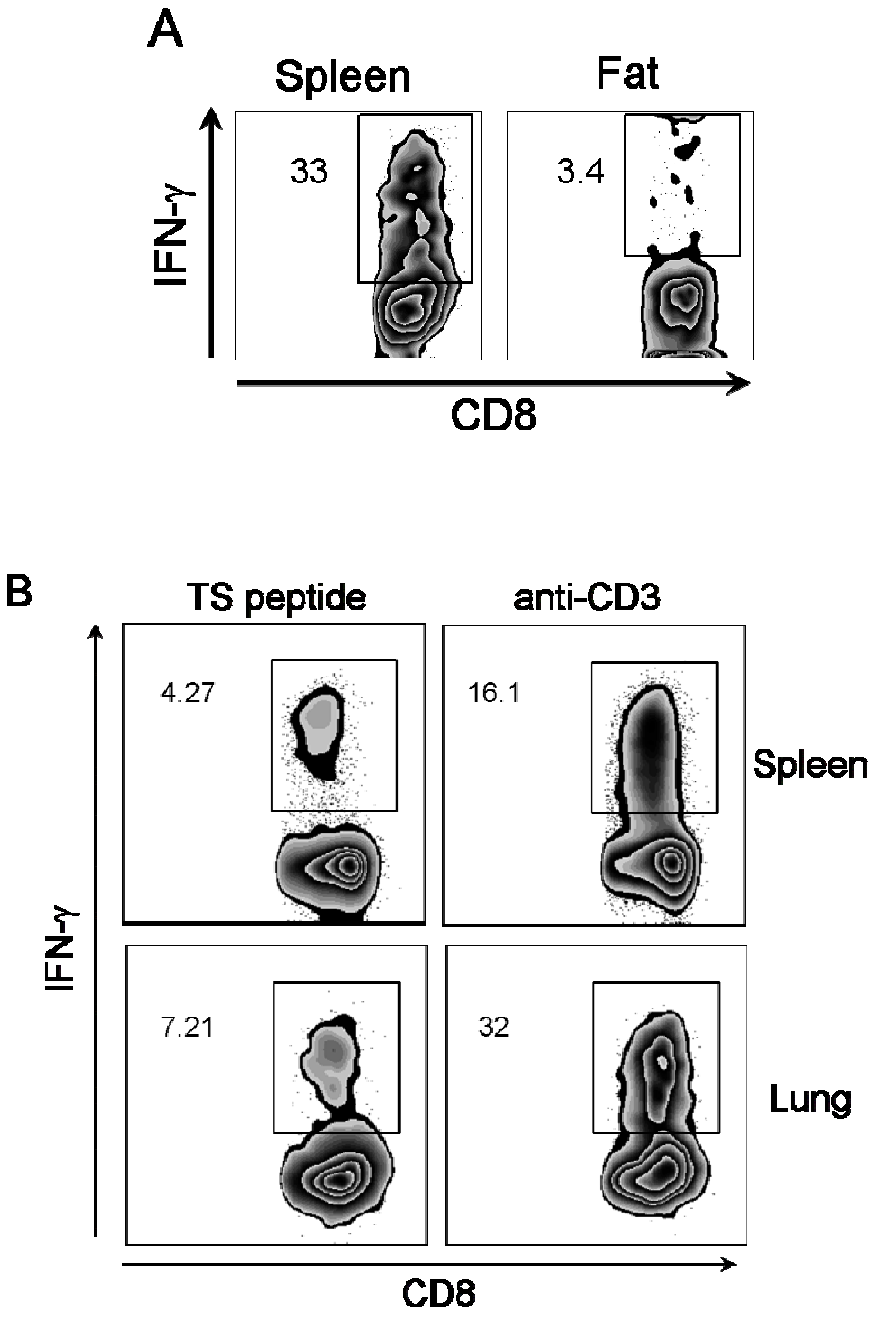

A
Spleen
Fat
33
3.4
IFN-γ
CD8
B
TS peptide
anti-CD3
4.27
16.1
Spleen
7.21
32
Lung
IFN-γ
CD8

Figure 2.3. PD-1 dependent T cell exhaustion does not account for low *ex vivo* responsiveness or for parasite persistence in chronic *T. cruzi* infection. *A-E* PD-L1 KO mice do not exhibit lymphocyte hyperproliferation during *T. cruzi* infection. Total spleen cell counts are shown in *A*. Frequency of CD8+ T cells and TSKB20+CD8+ T cells was analyzed by flow cytometry and used to calculate total numbers of these subsets per spleen. Averages ± SEM are graphed. * indicates $p<0.05$ by two-tailed student's t test compared to WT. *F*, Mice lacking PD-L1 do not exhibit enhanced clearance of *T. cruzi* infection. Parasite load in skeletal muscle of PD-L1 KO and WT mice during acute (26 dpi, *left*) and chronic (167 dpi, *right*) *T. cruzi* infection was assessed by real-time PCR. Number of animals in each group (n) is indicated in parentheses. Bar shows mean. *G*, PD-L1 KO mice experience levels of inflammation similar to WT mice during acute and chronic *T. cruzi* infection. Skeletal muscle was fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. *H*, PD-L1 deficiency does not restore ability of CD8+ T cells from muscle to produce IFNγ. Cells were isolated from muscle and spleen of chronically-infected PD-L1 KO and WT mice, processed and analyzed as in 2*B*. In *A-F*, n = 10 for all groups except n = 6 for acute WT. Histology slides in *G* are representative images from a different experiment with three mice per group. Cells restimulated in *H* are pooled from two similarly treated mice.

Figure 2.3

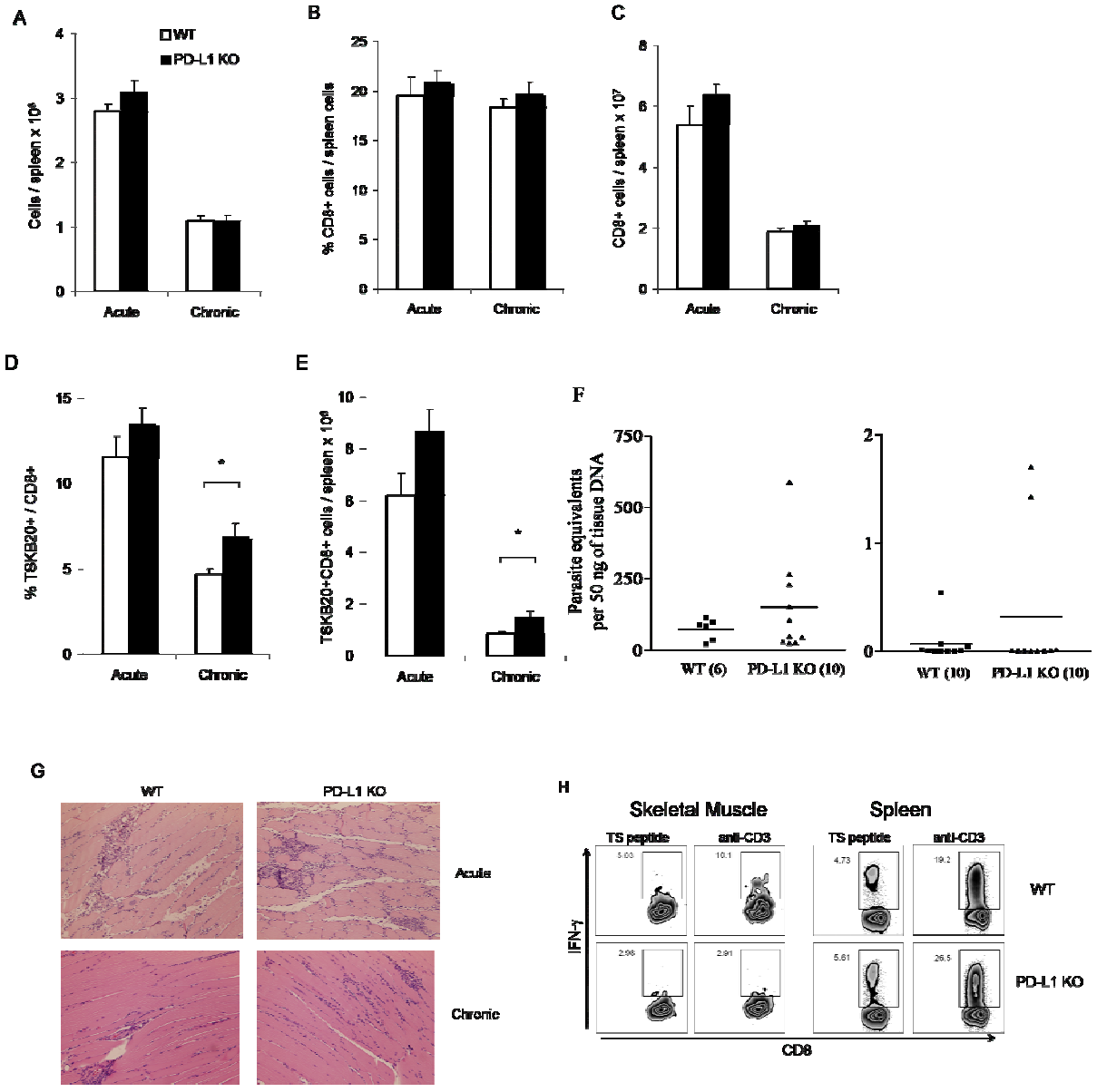

A
WT
PD-L1 KO
Cells / spleen x 10⁶
Acute
Chronic
B
% CD8+ cells / spleen cells
Acute
Chronic
C
CD8+ cells / spleen x 10⁷
Acute
Chronic
D
% TSKB20+ / CD8+
Acute
Chronic
E
TSKB20+CD8+ cells / spleen x 10⁶
Acute
Chronic
F
Parasite equivalents per 50 ng of tissue DNA
WT (6)
PD-L1 KO (10)
WT (10)
PD-L1 KO (10)
G
WT
PD-L1 KO
Acute
Chronic
H
Skeletal Muscle
Spleen
TS peptide
anti-CD3
TS peptide
anti-CD3
WT
PD-L1 KO
IFN-γ
CD8

Figure 2.4. Immunoregulation by IL-10 in not a major factor controlling CD8+ T cell immunity in chronic *T. cruzi* infection. *A*, IL-10 KO (n = 8) and WT (n = 8) mice exhibit similar parasite burden. Parasite load in skeletal muscle of IL-10 KO and WT mice during acute (30 dpi) *T. cruzi* infection was assessed by real-time PCR. Bar shows mean. *B*, IL-10 KO mice cannot control the inflammatory response to *T. cruzi*. H&E sections of skeletal muscle from acutely-infected IL-KO and WT mice. *C*, Interrupting IL-10 signaling does not allow clearance of *T. cruzi*. Parasite loads in skeletal muscle of chronically *T. cruzi*-infected mice receiving anti-IL-10R (n = 5) Ab or rat IgG (n = 5) are plotted. Bar shows mean. Data in *C* were repeated in a similar experiment.

Figure 2.4

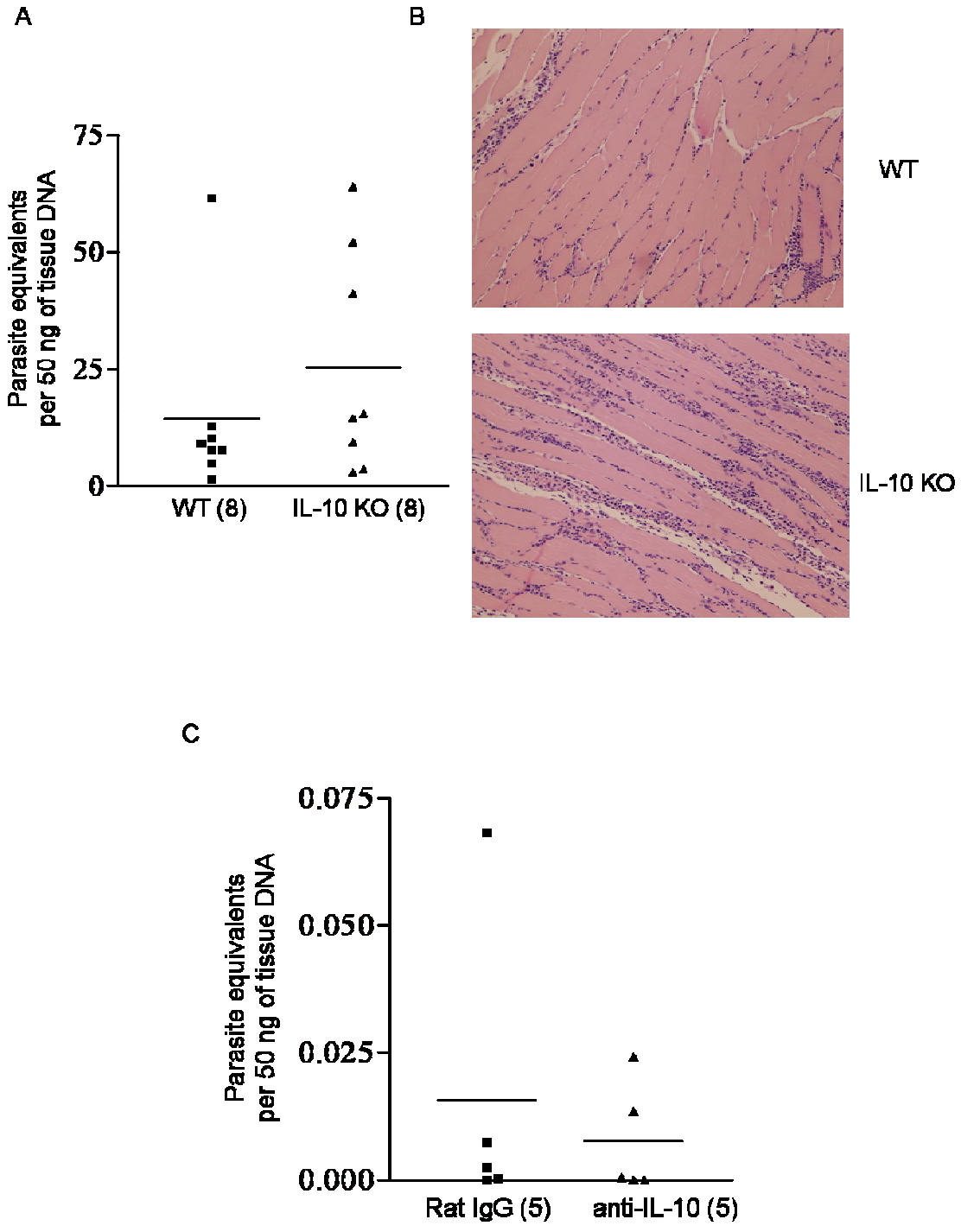

A
B
75
50
25
0
Parasite equivalents per 50 ng of tissue DNA
WT (8)
IL-10 KO (8)
WT
IL-10 KO
C
0.075
0.050
0.025
0.000
Parasite equivalents per 50 ng of tissue DNA
Rat IgG (5)
anti-IL-10 (5)

Figure 2.5. CD8+ T cells encounter antigen and express markers of activation in chronic *T. cruzi* infection. *A*, Recently-activated CD8+ T cells are found preferentially at sites of parasite persistence. Flow plots show surface expression of CD69 for cells isolated from indicated tissue during acute (37 dpi) and chronic (230 dpi) *T. cruzi* infection. Plots gated on CD8+ T cells. Numbers in upper right indicate the percentage of CD69+TSKB20+ cells; numbers in lower right indicate the percentage of TSKB20- cells expressing CD69. Data are representative of five experiments. *B*, KLRG1 is induced on CD8+ T cells during *T. cruzi* infection. Flow plots show surface expression of KLRG1 and TSKB20 specificity of CD8+ T cells isolated from indicated tissue of chronic (>100 dpi) or naïve mice. Plots are gated on CD8+ T cells and are representative of three similar experiments. Numbers indicate the percentage of cells in each quadrant.

Figure 2.5

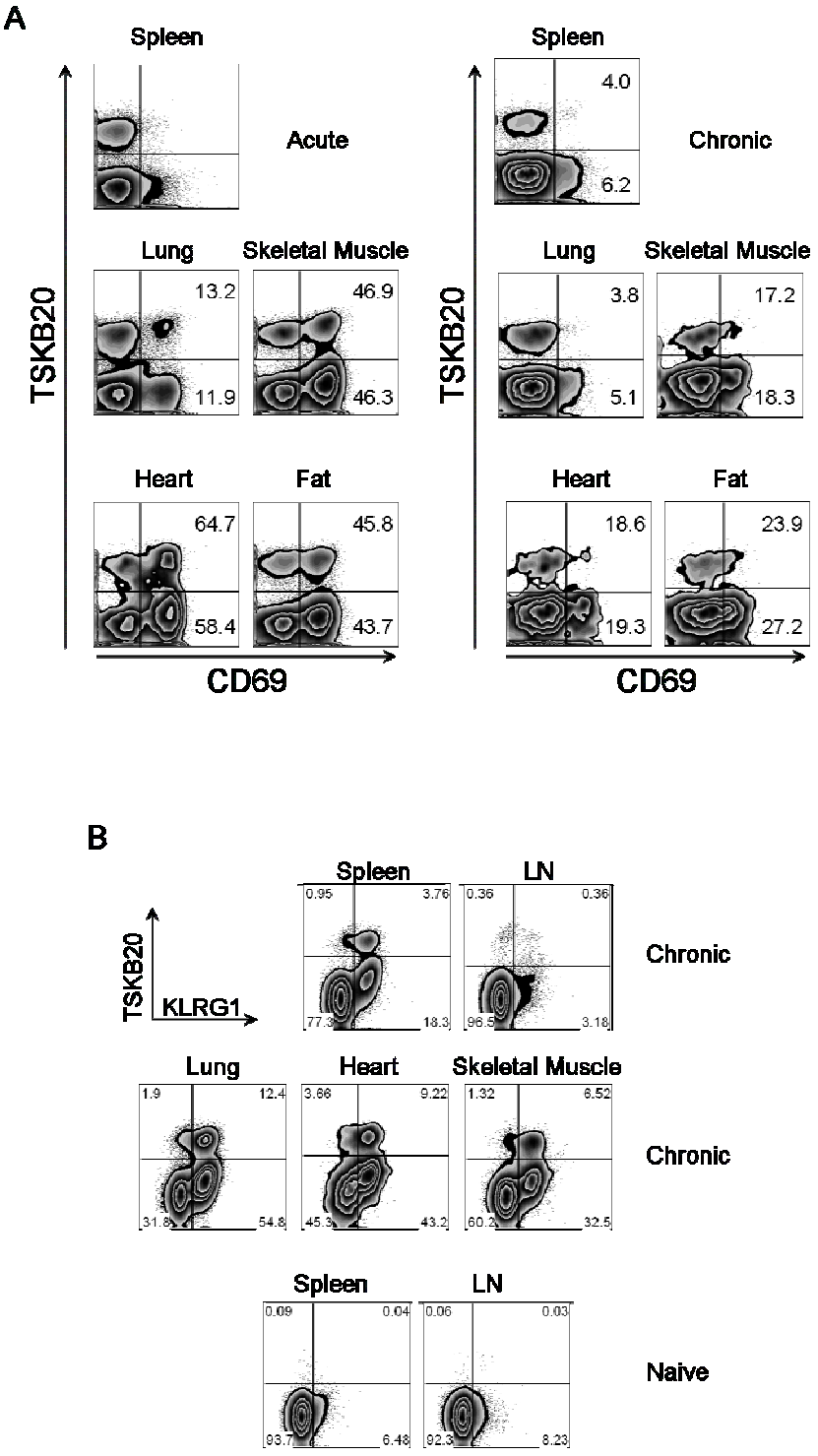

A
Spleen
Acute
Chronic
Lung
Skeletal Muscle
Heart
Fat
TSKB20
CD69
4.0
6.2
13.2
11.9
46.9
46.3
64.7
58.4
45.8
43.7
3.8
5.1
17.2
18.3
18.6
19.3
23.9
27.2
B
Spleen
LN
TSKB20
KLRG1
Chronic
Lung
Heart
Skeletal Muscle
Naive
0.95
3.76
77.3
18.3
0.36
0.36
96.5
3.18
1.9
12.4
31.8
54.8
3.66
9.22
45.3
43.2
1.32
6.52
60.2
32.5
0.09
0.04
93.7
6.48
0.06
0.03
92.3
8.23

Figure 2.6. IFNγ is an important component of immunity in chronic *T. cruzi* infection.

Mice (>100 dpi) were treated for 1 month with anti-IFNγ (n = 5) or rat IgG (n = 4) control, and parasite load was assessed in skeletal muscle tissue by real-time PCR. * designates $p<0.05$ compared to control. A similar trend ($p>0.05$) was observed in another independent experiment.

Figure 2.6

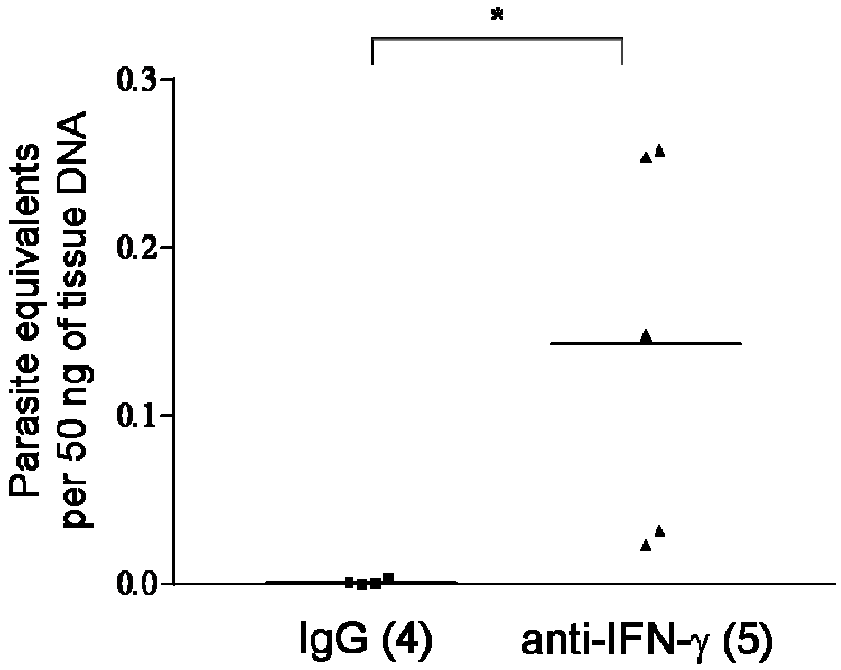

*
0.3
0.2
0.1
0.0
Parasite equivalents per 50 ng of tissue DNA
IgG (4)
anti-IFN-γ (5)

Figure 2.7. CD8+ T cells are required for optimal control of chronic *T. cruzi* infection. *A*, anti-CD8 treatment is effective. Mice (>130 dpi) received anti-CD8 Ab (n = 5) or rat IgG control (IgG, n = 5)) for 30 days. Bar graphs show the percentage of CD8+ cells in the total lymphocyte gate, * $p<0.05$ vs. control.. *B*, CD8-depleted mice exhibit a deficit in controlling chronic *T. cruzi* infection. Parasite load was determined in skeletal muscle by real-time PCR in both groups at the end of the treatment protocol. Data are combined from 2 different experiments for a total number of 8 mice per group. Bar shows mean. A third experiment with similar results was performed. *C*, CD8 depletion results in greater inflammation at sites of parasite persistence. Heart and skeletal muscle were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E.

Figure 2.7

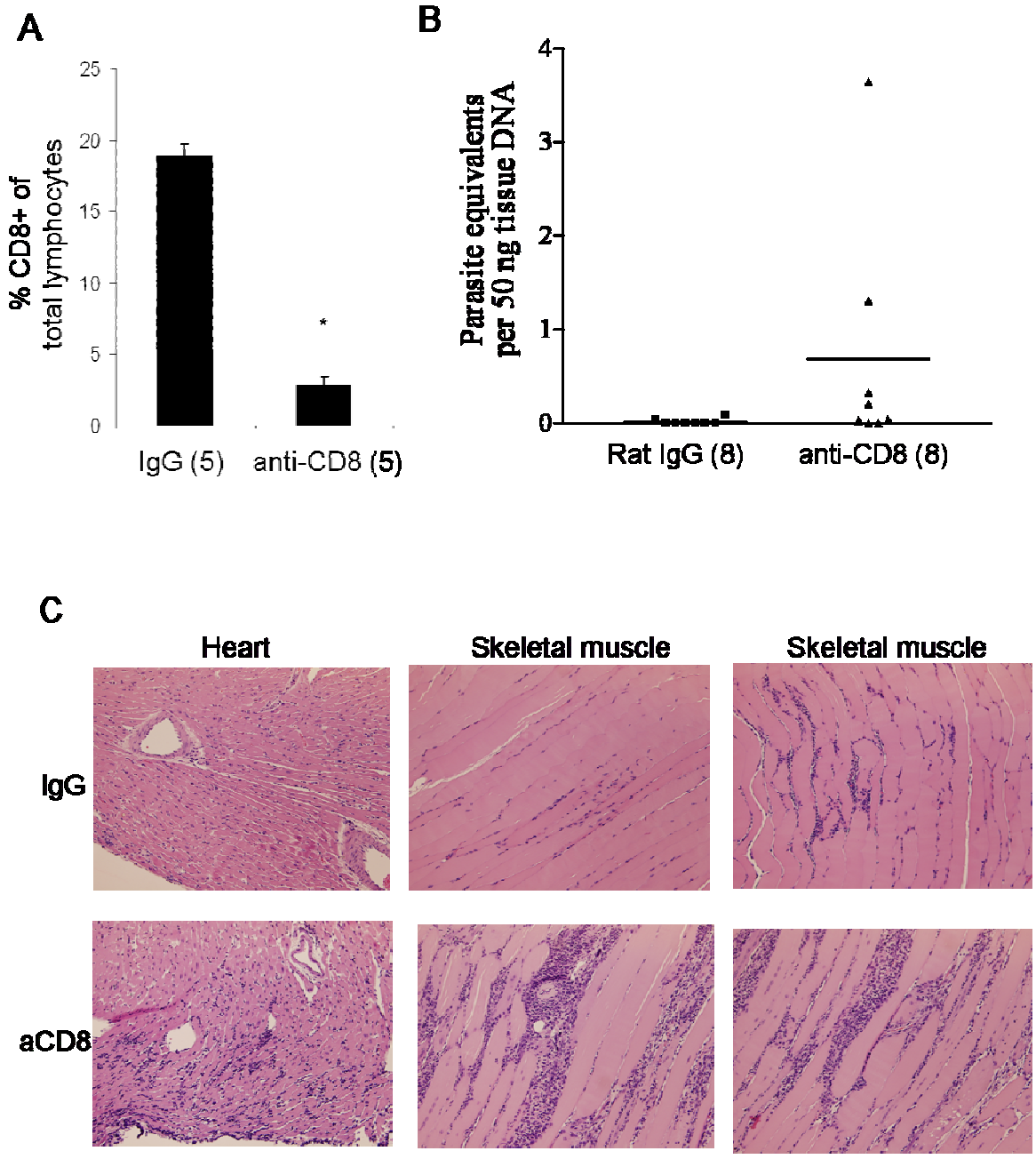
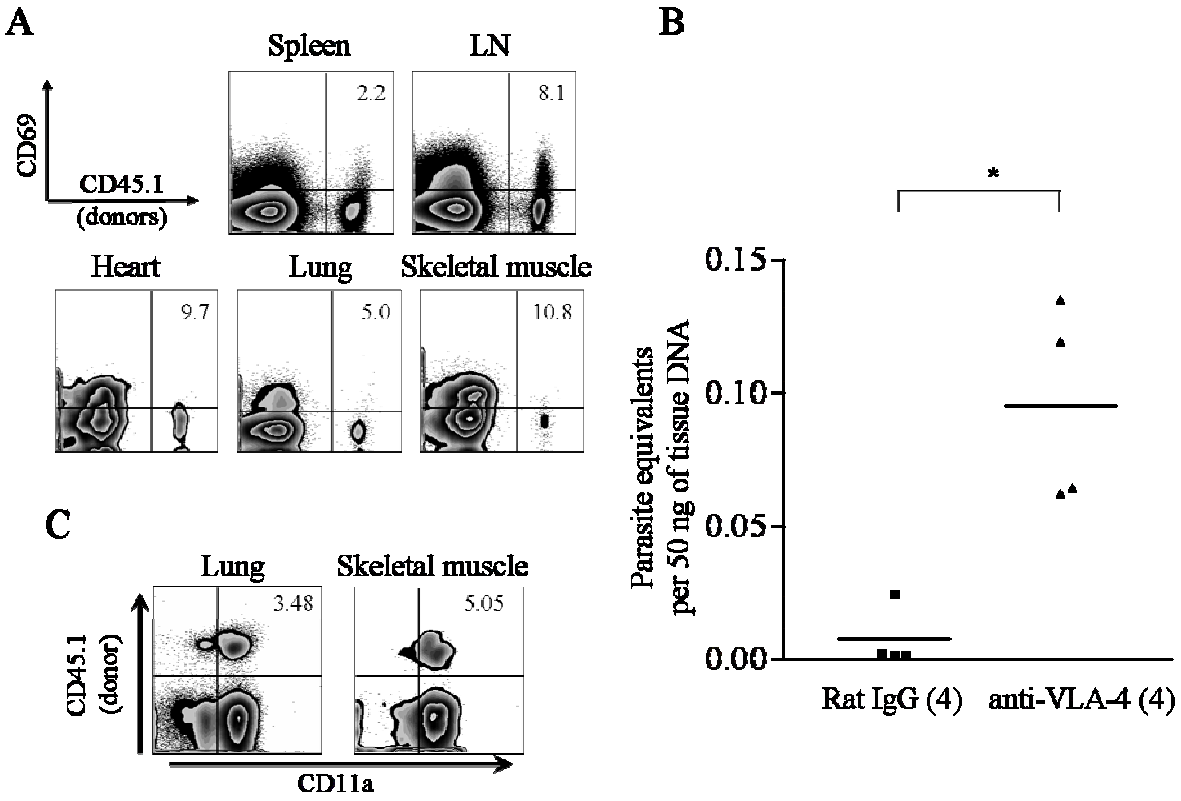

A
% CD8+ of total lymphocytes
25
20
15
10
5
0
IgG (5)
anti-CD8 (5)
B
Parasite equivalents per 50 ng tissue DNA
4
3
2
1
0
Rat IgG (8)
anti-CD8 (8)
C
Heart
Skeletal muscle
Skeletal muscle
IgG
aCD8

Figure 2.8. Trafficking and maintenance of CD8+ T cell response to *T. cruzi* in peripheral tissue is a dynamic process. *A*, CD8+ T cells are recruited from a central pool to nonlymphoid tissues in chronic *T. cruzi* infection. CD8+ T cells were magnetically purified from infection-matched CD45.1+ congenic mice (>100 dpi) and transferred into CD45.2+ congenic mice. Cells were isolated from spleen, lymph node, and peripheral tissue 14 days later. Plots gated on CD8+ cells. Numbers indicate the percentage of transferred cells expressing CD69. Donor cells could consistently be detected in tissue of eighteen mice between 2 and 20 days post transfer. B, Interruption of leukocyte trafficking is detrimental to parasite control. Mice (>100 dpi) were treated with a blocking anti-VLA-4 Ab (4) or rat IgG (4) as a control for 30 days. Parasite load was determined in skeletal muscle by real-time PCR. * indicates $p<0.05$ compared to control. A similar trend ($p>0.05$) was observed in 2 of 3 additional experiments. *C*, Naïve CD8+ T cells can be primed and enter nonlymphoid tissue during chronic *T. cruzi* infection. CD8+ T cells from mice (CD45.1) not infected with *T. cruzi* were purified and transferred into chronically *T. cruzi*-infected mice. More than 1 month post-transfer, cells were isolated from peripheral tissue of recipient mice. Flow plots are gated on CD8+ cells and show surface expression of CD11a (x-axis) against the congenic marker CD45.1 (y-axis). Donor cells were only seen in tissue when peripheral blood CD8+ T cells of that mouse displayed evidence of priming (one out of twelve, data not shown). Numbers indicate the percentage of CD8+ T cells in recipient mice derived from naïve donors.

Figure 2.8


A
Spleen
LN
2.2
8.1
CD69
CD45.1
(donors)
Heart
Lung
Skeletal muscle
9.7
5.0
10.8
B
*
0.15
0.10
0.05
0.00
Parasite equivalents
per 50 ng of tissue DNA
Rat IgG (4)
anti-VLA-4 (4)
C
Lung
Skeletal muscle
3.48
5.05
CD45.1
(donor)
CD11a

2.5 References

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CHAPTER 3

CD8+ T CELLS RESPOND TO ORAL *TRYPANOSOMA CRUZI* INFECTION AS A SYSTEMIC INFECTION[1]

[1] Collins, M.H. and Tarleton, R.L., *et al*. To be submitted to *The Journal of Immunology*

Abstract

Trypanosoma cruzi infects millions of people in Latin America and often leads to the development of Chagas disease. *T. cruzi* infection can be acquired at the bite site of the triatomine vector, but *per os* infection is also a well documented mode of transmission, as evidenced by recent microepidemics of acute Chagas disease attributed to consumption of parasite-contaminated foods and liquids. It would also be convenient to deliver vaccines for *T. cruzi* by the oral route, particularly live parasite vaccines intended for the immunization of reservoir hosts. For these reasons, we were interested in better understanding immunity to *T. cruzi* following oral infection or oral vaccination, knowing that the route of infection and site of antigen encounter can have substantial effects on the ensuing immune response. Here, we show that the route of infection does not alter the ability of *T. cruzi* to establish infection in muscle tissue, nor does it impair the generation of a robust CD8+ T cell response. Importantly, oral vaccination with attenuated parasites provides protection against WT *T. cruzi* challenge. These results strongly support the development of whole-organism-based vaccines targeting reservoir species as a means to alleviate the burden of Chagas disease in affected regions.

3.1 Introduction

Millions of people throughout Latin America are affected by Chagas disease. This condition is caused by persistent infection with the hemoflagellate protozoan parasite *Trypanosoma cruzi*, which sustains infection in mammalian hosts by replication of amastigotes in a cytoplasmic niche [1]. Accounting for nearly 700,000 DALYs [2], Chagas disease is a prominent public health challenge, and current approaches to treatment and prevention are far from optimal [3]. Metacyclic trypomastigotes in the feces of various triatomine vectors are infective to a wide range of mammals, which primarily acquire *T. cruzi* through breaks in the skin, exposure to mucosal surfaces, or ingestion [1]. Domestic spraying campaigns have had some degree of success in controlling transmission, most notably in Chile, Uruguay, and parts of Brazil [4]. In this context, more attention has been given to outbreaks of *T. cruzi* infection acquired from food or drink tainted with *T. cruzi*-laden triatomine excreta [5-7], raising the question of whether oral transmission may be an underappreciated route of *T. cruzi* infection. The prominence of oral infection among reservoirs is supported by observations of opossums [8], raccoons [9] and dogs [10] ingesting triatomine bugs. It is not known how frequently humans acquire *T. cruzi* infection from contaminated food or ingestion of fomites. From an epidemiological perspective, it would be beneficial to distinguish people who acquired *T. cruzi* by oral route from other means of transmission, perhaps by a phenotypic signature of the T cell response. This aim necessitates a better understanding of the host immune response to *T. cruzi* following exposure via the gastrointestinal (GI) tract.

Oral infection may lead to distinct parasitological and/or immunological outcomes compared to other routes of infection. GI mucosal tissue forms an interface between the

organism and its environment and constitutes an immense surface area constantly in contact with potential pathogens and commensals. Accordingly, the immune system associated with mucosae has evolved a unique capacity to determine when an aggressive response is appropriate, balancing regulation and activation [11]. Ag encounter at the GI mucosa or in gut-associated lymphoid tissue often results in tolerance, particularly for T cell responses, a process largely mediated by the cytokines TGFβ and IL-10 [12, 13]. We questioned whether parasite-specific CD8+ T cell responses may develop differently during oral infection and explored the possibility that *T. cruzi* may exhibit a different tropism when infecting by the GI tract than that previously observed in systemic routes of infection [14]. T cell populations found in the mucosal tissue along the GI tract have several characteristics that separate them from T cells in peripheral circulation. For example, homing to the GI mucosa is controlled by expression of distinct adhesion molecules. T cells primed by dendritic cells (DC) from Peyer's patches (PP) or mesenteric lymph nodes (mesLN) express specific integrins on their surface that confer the ability to home to gut tissue [15, 16]. We also asked if the mucosal route of infection would bias responding CD8+ T cells to accumulate in the intestines, possibly at the expense of a parasite-specific CD8+ response in other peripheral tissue.

If a robust immune response is generated with oral *T. cruzi* infection, one would hypothesize that vaccination by this route could be effective. Although not currently available, it is a major goal to develop a vaccine that protects against *T. cruzi* infection, especially one that elicits T cell-based immunity [17, 18]. CD8+ T cells, which respond to foreign Ag processed from the intracellular compartment and presented on molecules from the class I major histocompatability complex (MHC), are essential for controlling infection by *T. cruzi* [19]. In mice, a population of immunodominant CD8+ T cells recognizes epitopes derived from the *T.*

cruzi trans-sialidase gene family [20], and CD8+ T cells are strongly implicated in experimental vaccine-induced protection against this parasite [21-25]. An attainable, practical approach to reducing the burden of Chagas disease would be to implement a transmission-reducing vaccine targeting reservoirs of *T. cruzi*. In endemic areas, dogs are an integral component of domestic transmission of *T. cruzi* [26-28] and mathematical models have suggested that removing infected dogs from homes in some regions could almost completely prevent vector transmission to humans [28]. To that end, our lab is working to generate genetically-attenuated strains of *T. cruzi* as vaccine candidates. As this vaccine would likely be administered to reservoir dogs by feeding, we carried out protection studies in mice following oral immunization with an attenuated strain of *T. cruzi*.

Here, we address several specific questions: 1) Does entry via the oral route alter the capacity of *T. cruzi* to establish infection systemically in muscle tissue? 2) Does the immune system recognize and respond to *T. cruzi* as a localized gut infection? 3) Can the phenotype of *T. cruzi* –specific T cells be used to distinguish route of exposure to *T. cruzi* in an already-infected host? 4) Can oral vaccination with attenuated parasites induce protective immunity to systemic challenge with *T. cruzi*?

3.2 Materials and Methods

3.2.1 Mice, parasites, infections, and vaccinations

C57BL/6 (Ly5.2^{+}) (B6) mice were purchased from either The Jackson Laboratory or National Cancer Institute at Frederick (Frederick, MD). Mice were maintained at the University of Georgia animal facility in microisolator cages under specific pathogen-free conditions. Mice were infected with metacyclic forms of Brazil or CL strain *T. cruzi* by oral gavage (po), intraperitoneal injection (ip) or by subcutaneous infection into the foot pad (fp). Mice could be

infected by *ad libitum* ingestion of food that had been contaminated with *T. cruzi* metacyclic trypomastigotes (data not shown); however, a gavage needle was used in the majority of experiments to maximize infection efficiency and allow consistent dosing between animals. As previously described [29], epimastigote cultures were maintained in LIT media, and metacyclic trypomastigotes were generated by stressing cultures with TAU media. Alternatively, tissue culture trypomastigotes were obtained from passage through Vero cells and mice were infected by ip or fp injection with 1,000 tissue culture trypomastigotes (TCT). Attenuated parasites ($ECH1^{+/-}$ $ECH2^{-/-}$) were generated by targeting the tandem genes Enoyl-CoA hydratase 1 and 2 in a high through put knock out system recently developed by our lab [30]. For vaccine protection studies, attenuated $ECH1^{+/-}$ $ECH2^{-/-}$ metacyclic trypomastigotes were administered by gavage. Mice received 3 doses separated by approximately 2 weeks. 5×10^5 metacyclic trypomastigotes were given for doses 1 and 3, and 1.35×10^5 metacyclic trypomastigotes were given in dose 2. Control mice received an equal volume (100 µl) of PBS at each dose. Mice were sacrificed by CO_2 inhalation followed by cervical dislocation. All animal protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

3.2.2 Assessing protection with *in vivo* imaging

For protection studies, *T. cruzi* parasites of the CL strain were transfected with the *T. cruzi*-specific expression plasmid pTREX [31] containing the sequence coding for the far-red tdTomato protein [32]. Drug selected parasites displayed stable fluorescence through the different stages and 2.5×10^5 tissue culture fluorescent trypomastigotes were subcutaneously inoculated in 4 µl into superficial subcutaneous tissue of the footpads. Mice feet were imaged every other day using the Maestro 2 *In Vivo* Imaging System (CRi, MA) with the green set of filters (acquisition settings: 560 to 750 in 10 nm steps; exposure time 88.18 ms and 2 x 2

binning). Total fluorescent signal was quantitated and normalized by exposure time and the area of the camera field corresponding to the source of the fluorescence, and values are reported as photons / cm^2 / second.

3.2.3 Isolation of lymphocytes from nonlymphoid tissues and adoptive transfers

Before tissue removal, mice were perfused with 20 ml of PBS with containing 0.8% sodium citrate as an anticoagulant. Perfusion was done by opening the abdominal and thoracic cavities, nicking the portal vein, and forcing PBS into the heart ventricles with a ½ inch 35 gauge needle and 10 ml syringe. Tissue-derived lymphocytes were obtained by teasing tissues apart and vigorously pushing through a 40-μm nylon mesh screen. Lymphocytes were obtained from lamina propria and intestinal epithelium as previously described [33]. In brief, small intestines were isolated, cleaned, and cut into small segments. Pieces were stirred at 37°C for 20 min in CMF containing 10% FCS and 1mM dithioerythritol to free intraepithelial lymphocytes (IEL). Gut pieces were further digested by stirring for 1 hour at 37°C in RPMI 1640 medium containing 5% FCS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 150 Units/ml type II collagenase (Sigma). Tissue homogenate was then passed over a 40 μm nylon mesh screen and both IEL and lamina propria (LP) cell populations were further purified by collection from the interface of 44% percoll in RPMI underlain with 67% percoll in PBS.

3.2.4 T cell phenotyping

Spleens were homogenized and RBCs were lysed in a hypotonic ammonium chloride solution. Washes and staining was done in PAB (2% BSA and 0.02% azide in PBS). Peripheral blood was obtained by retro-orbital venipuncture or by nicking the tail and collecting blood with a capillary, collected in sodium citrate solution, and washed in PAB. Cells were obtained from peripheral tissue as described below. Cells were incubated with class I MHC tetramer-PE

complexes loaded with TSKB20 peptide [20]. and the labeled Abs. Cells were stained for 30 min at 4°C in the dark, washed in PAB, and fixed in 2% formaldehyde. For whole blood, RBCs were lysed after surface staining in a hypotonic ammonium chloride solution and washed twice in PAB. Data was acquired using a CyAn flow cytometer (DakoCytomation) and analyzed with FlowJo software (Tree Star). MHC I tetramer TSKB20 (ANYKFTLV/K^b) was synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA). Antibodies for flow cytometric analysis were purchased from BD Biosciences, eBioscience, and Caltag Laboratories/Invitrogen.

3.2.5 PCR

Quantitative real-time PCR was performed as previously described [34]. Briefly, tissue was collected from mice and finely minced. Samples were incubated at 55 °C for 4 hours in SDS-proteinase K lysis buffer. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 100% ethanol, and resuspended in nuclease free water. Samples were analyzed on an iCycler (Biorad). For real-time and standard PCR the following primers were to amplify a 182-bp product from genomic *T. cruzi* DNA:

TCZ-F* 5′-GCTCTTGCCCACAMGGGTGC-3′, where M=A or C and

TCZ-R 5′-CCAAGCAGCGGATAGTTCAGG-3′ [34]

3.2.6 Histology

Heart and Skeletal muscle was obtained from *T. cruzi*-infected mice and controls, fixed in 10% buffered formalin and embedded in paraffin. Five-micron-thick sections were obtained and stained with hematoxylin-eosin. Inflammation was evaluated qualitatively according to the presence or absence of myocyte necrosis and severity of leukocyte infiltration. Images of muscle tissue were taken with an OLYMPUS DP70 digital camera on an OLYMPUS BX60 microscope.

3.2.7 Statistical analysis

We calculated statistical significance with a two-tailed Student's *t*-test in all cases and a one-way, non-parametric ANOVA was used in Fig 3.6*C*.

3.3 Results

3.3.1 *T. cruzi* infects skeletal muscle following oral exposure

We first asked if parasites infecting via the oral route establish a systemic infection similar to that generated using other routes of infection. Metacyclic trypomastigotes were administered to mice by oral gavage (po) or injected intraperitoneally (ip) or subcutaneously in the foot pad (fp) for control infections. PCR analysis revealed parasite DNA in the skeletal muscle at 21-25 days post infection (dpi) in both po and fp infected mice (Figure 3.1*A*). Similar patchy mononuclear cell infiltrates were detectable in skeletal muscle at 35 dpi in mice infected by po and fp routes (Figure 3.1*B*). These results show that within ~3 weeks after oral infection, *T. cruzi* has disseminated and colonized skeletal muscle. The emergence of a population of CD8+ T cells capable of binding class I MHC tetramers loaded with the *T. cruzi*-derived immunodominant peptide TSKB20 [20] was also observed in mice infected po. At 14 dpi, a robust TSKB20-specific response could be detected in PBMC of po and fp infected mice (Fig 3.1*C*). By 140 dpi, the TSKB20-specific CD8+ T cell population measured in the spleen of po infected mice contracted and stabilized at frequencies typical of chronic *T. cruzi* infection in mice (Fig 3.1*D*) [20].

We hypothesized that parasite specific CD8+ T cells would accumulate in gut tissue following po but not ip infection with *T. cruzi* if parasite Ag accumulated at the infection site. To examine this possibility, lymphocytes isolated from lymphoid and nonlymphoid tissue were analyzed. The frequency and distribution of TSKB20+CD8+ T cells did not vary depending on

infection route as mice infected ip developed parasite-specific CD8+ T cells able to traffic to gut tissue, and mice infected po had proportions of TSKB20-specific cells in spleen, lung, and skeletal muscle comparable to those in mice infected ip (Fig 3.2*A*). We have recently shown that *T. cruzi*-specific CD8+ T cells express CD69 selectively in sites of parasite persistence (Collins et al., submitted). CD8+ T cells isolated from skeletal muscle of po and ip infected expressed similar levels of this marker of recent activation, whereas little expression is detected in the spleens of these animals (Fig 3.2*B*). Taken together, these results indicate that regardless of infection route, *T. cruzi* achieves a systemic infection and thus has access to many tissue compartments.

3.3.2 T cells primed during oral *T. cruzi* infection are not imprinted for gut homing

It has been suggested that T cells are educated to adopt a particular homing pattern when Ag is acquired in specific anatomical sites. Most notably, dendritic cells (DC) from intestines or mesLN have been shown to confer a gut-homing program on T cells by induction of gut homing receptors such as CCR9 and α4β7 integrin [15, 16]**.** While expression of β7 integrin is key for homing to the LP and IEL compartments of the gut [35], CD103 (α_E integrin) expression overlaps with that of β7 on Ag-specific CD8+ T cells in the gut [36] and has been implicated in the migration and localization of T cells in the intestine [37]. To determine whether initial T cell priming associated with oral exposure to *T. cruzi* shows signs of imprinting, the expression of CD103 was investigated. CD103 was not expressed on TSKB20+CD8+ T cells in peripheral blood at 14 dpi (Fig 3.3*A*). Although approximately 35-60% and 95% of CD8+ T cells from LP and IEL, respectively, express CD103, very few of the TSKB20-specific CD8+ T cells were CD103+ in these locations (Fig 3.3*B*). CD103 was also undetectable on TSKB20+CD8+ T cells in LN. Analyzing the expression of CD103 among total CD8+ T cells revealed that the

expression of this integrin was not enhanced in either peripheral (pLN) or gut-draining LN (mesLN) depending on the route of infection (Fig 3.4*A*). Thus, no evidence of imprinting is observed after po infection with *T. cruzi*. Considering that CD69 is a marker of recent activation and that it associates with shingosine-1-phosphate receptor 1 (S1P1) to retain lymphocytes in lymph nodes [38], we compared CD69 expression on CD8+ T cells in pLN and mesLN as an alternative method for investigating anatomically-restricted antigen (Ag) presentation. The expression of CD69 on TSKB20-specfic CD8+ T cells in pLN or mesLN was not favored by one infection route over the other (Fig 3.4*B*). Taken together, all these data suggest that *T. cruzi* enters its host and disseminates early in infection; it is not anatomically confined or immunologically recognized as an infection of any one tissue compartment.

3.3.4 Can orally-administered attenuated strains of *T. cruzi* provide protection against a challenge infection?

Given the robust CD8+ T cell response observed in spleen, blood, gut, and other nonlymphoid tissues following po infection by *T. cruzi*, we hypothesized that oral vaccination could provide protection against a heterologous route of *T. cruzi* challenge. To test this hypothesis, we exposed mice to an attenuated strain of *T. cruzi* by the oral route and later challenged them with virulent parasites. Mice received three doses of CL strain *T. cruzi* attenuated by deletion of one allele of enoyl-CoA-hydratase-1 (ECH1) and both alleles of enoyl-CoA-hydratase-2 (ECH2) (Xu, et al., unpublished). Both ECH genes encode an enzyme involved in fatty acid oxidation, a process thought to be important in amastigote energy metabolism [39]. Mice received $ECH1^{+/-}$ $ECH2^{-/-}$ parasites by oral gavage (oral vax). Two weeks after the final vaccination, PBMCs in the majority of the vaccinated mice used for the protection study exhibited upregulation of CD44, with over 50% of CD8+ T cells becoming

$CD44^{hi}$ in some cases (Fig 3.5A). This group was designated as "high responders" (HR). Additionally, TSKB20+CD8+ populations with low CD127 expression were apparent in the blood of HR mice (Fig 3.5B), indicating a significant induction of *T. cruzi*-specific T cell responses in these mice. "Low responder" (LR) mice were indistinguishable from PBS controls based on the CD44 expression profile of CD8+ T cells (Fig 3.5A) and did not have measurable levels of *T. cruzi*-specific CD8+ T cells. The 7 HR and 3 LR mice were challenged with 2.5 x 10^5 CL trypomastigotes in each foot pad and parasite load at the site of infection was monitored from 2 to 10 days post challenge (dpc) by *in vivo* imaging of WT parasites transfected with a gene encoding TdTomato fluorescent protein [32].

Orally vaccinated mice displayed a significant reduction in fluorescence signal compared to control unvaccinated mice at all times measured (Fig 3.6*A* and *B*). While fluorescence signal progressively amplified in PBS mice until 8 dpc, a consistently low signal was displayed from 2-6 dpc in oral vax mice, and measurements were statistically indistinguishable from background (naïve) signal by 8 dpc. As expected, HR mice controlled fp infection with *T. cruzi* more efficiently than LR mice, but LR mice still displayed a significantly lower fluorescence signal than PBS mice at this time (Fig 3.6*C*). At 25 dpc, oral vax mice exhibited lower parasite loads in skeletal muscle compared to controls, although both groups controlled parasites well after these early time points (Fig 3.6*D*). Together, these results indicate that oral vaccination with attenuated parasites stimulated effective adaptive immunity that successfully protected mice from challenge with WT *T. cruzi*.

3.4 Discussion

Mucosal surfaces are the target of infection for many human pathogens [40], including those that cause HIV, tuberculosis, respiratory tract infections and diarrheal illnesses and account

for an enormous portion of global disease burden [2]. The diverse biology of potential pathogens requires an array of immune effector mechanisms to protect the host from infection and pathology [40]. Oral *T. cruzi* infection is a prominent public health issue in *T. cruzi*-endemic areas [5-7]. Some mucosal pathogens remain localized to the airways, gut lumen, mucosal surface epithelium, or lamina propria; but like HIV or *Salmonella typhi* [40], *T. cruzi* transits these superficial compartments and establishes a systemic infection. Immune responses to infection or immunization may be shaped differently depending on the route of Ag exposure. For example, the distribution of rotavirus and the responding cytotoxic T lymphocytes (CTL) varies according to route of infection [41, 42]. CD8+-dependent protection against mucosal HIV challenge could be generated by mucosal vaccination, whereas subcutaneous vaccination failed to elicit protective CTL at mucosal sites [43]. Even simultaneously within the same animal, CD8+ T cells specific for the same tumor Ag can acquire different homing phenotypes if the Ag is introduced in multiple distinct environments [44]. These data from other systems may predict that the immune system would respond differently to oral *T. cruzi* infection compared to fp or ip infection. In this study, we investigated whether the CD8+ T cell response to *T. cruzi* is affected by an oral route of parasite entry.

The nature of the immune response to parasitic infection can be heavily influenced by the earliest events of host-parasite encounter, and there have recently been exciting advances in this area of parasitology [45-48]. The precise cells first infected and/or acting as APCs that signal the adaptive immune system during oral *T. cruzi* infection are not known, but one can envisage at least two possible scenarios as to how the immune system recognizes *T. cruzi* as it progresses from mucosal exposure to a systemic infection. Dissemination could occur early after infection, with trypomastigotes entering the blood stream soon after invasion or following the first round of

replication in cells at the site of infection. If this were the case, differences in phenotype, frequency, and distribution of parasite-specific CD8+ T cells would not be expected. Alternatively, a few rounds of initial parasite replication may be largely relegated to local GI cells, and Ag presentation would then be confined to mucosal associated lymphoid tissue, possibly leading to imprinting of primed T cells. Others have documented local proliferation of parasites following mucosal *T. cruzi* infection with early involvement of draining LN, but inflammatory lesions and parasites can be observed at distal sites by 14 dpi [49, 50]. Our data (Fig 3.6 and [51]) support local parasite amplification following fp infection, but parasite DNA is also detectable in dLN within hours of infection [51]. This observation suggests that either some parasites migrate out of the infection site prior to replication or that parasites are shuttled to the dLN in lymph or associated with host cells such as DC.

Interestingly, we show that the CD8+ T response to *T. cruzi* infection develops similarly, regardless of infection route. Ag-specific CD8+ T cells were found in all lymphoid and nonlymphoid tissues assayed following either fp or po infection (Fig 3.2-3.4). The frequency of TSKB20-specific CD8+ T cells was much lower among intraepithelial lymphocytes (IEL) compared to other compartments (Fig 3.2), mimicking what is observed for Ag-specific T cells in HIV infection, another pathogen that persists in mucosal and other systemic tissue [52]. This is to be expected as only a limited proportion of IEL CD8+ T cells are capable of recognizing epitopes presented by the classical MHC I Ag processing pathway due to their diverse expression patterns of Ag receptor and coreceptor molecules [53]. The localization of TSKB20-specific cells to the LP following fp infection was intriguing given that this compartment is known for displaying stringent "gating" that is permissive to only some T cell populations [54]. One possible explanation is that intestinal tissue can be infected from the bloodstream just as easily as

from the GI lumen. *T. cruzi* exhibited tropism for infecting skeletal muscle after oral administration (Fig 3.1*A*), and CD69+TSKB20+CD8+ T cells were selectively enriched at that site as seen in fp infection (Fig 3.2*B*). Expression of CD69, a molecule expressed within hours of stimulation via the TCR [55], is indicative that CD8+ T cells are recognizing and actively responding to persistent parasites. Finally, comparable proportions of *T. cruzi*-specific CD8+ T cells were activated in lymph nodes draining skeletal muscle or the gut regardless of infection route (Fig 3.2*D*). Overall, these results support a model in which the CD8+ T cell response is initiated by APC that have acquired Ag in sites dispersed throughout the host. From very early time points after infection, Ag is not confined to the site of parasite entry, which is consistent with our inability to detect phenotypic differences in CD8+ T cells following *T. cruzi* infection by distinct routes. One goal of this work was to ask whether the route of *T. cruzi* infection could be distinguished by an immunological metric. However, our results indicate that it may be difficult to identify immune parameters that would allow the mode of transmission to be determined in individuals with *T. cruzi* infection.

A second reason for studying the immune response following oral infection is that oral vaccination of reservoir hosts is an attractive means of limiting vector transmission of *T. cruzi*. An effective vaccine that serves individuals at risk for Chagas disease is still not available, but substantial effort has identified Ag and immunization schemes that protect mice against *T. cruzi* challenge [17]. However, *T. cruzi* is a complex pathogen, with at least 12,000 genes encoding more than 20,000 proteins and a large amount of repetitive sequences that could be a depot for recombination [56]. Given the massive expansions in gene families encoding immune targets [20], selecting an optimal set of Ag that would be effective across human populations with diverse MHC genotypes may be a difficult task [18, 57]. Live attenuated parasites have been

proposed as an appealing alternative to subunit vaccination [18]. While a preventive vaccine for humans may be the ideal intervention, a transmission-reducing vaccine for domestic reservoirs is a pragmatic compromise. Thus, interruption of *T. cruzi* transmission to humans could be achieved by decreasing the ability of dogs to transmit *T. cruzi* to insects, a primary factor promoting household infections in endemic areas [26-28]. Canine vaccination is a long-standing idea and has a proven role in controlling zoonotic diseases including rabies and leishmaniasis [58]. Reservoirs could conveniently be vaccinated by the oral route, and previous studies support the immunogenic potential of mucosally-delivered *T. cruzi* Ag [25, 50, 59-63]. It has also been shown that vaccination can generate effective immunity against mucosal *T. cruzi* challenge [61-63]. A key question that had not been addressed is whether a genetically attenuated strain of *T. cruzi* could be used as an oral vaccine. Here, we show that $ECH1^{+/-}$ $ECH2^{-/-}$ metacyclic trypomastigotes given by oral gavage provide protective immunity to WT *T. cruzi* challenge.

In our studies, protection was evident as early as 2 dpc, suggesting that vaccination generated *T. cruzi*-specific immunity that was rapidly effective at the challenge site (Fig 3.6*B*). Parasite burden at the challenge site was well controlled in vaccinated mice, whereas it dramatically increased in controls over the first 8 dpc (Fig 3.6*B*). Signal fell dramatically in the control group between 8 and 10 dpc, possibly representing trypanocidal activity of the nascent immune response at the infection site or dissemination of parasites to other tissues. Thus vaccination reduces the initial "take" of *T. cruzi* infection as well as replication of parasites that successfully invade host cells. Interestingly, the level of CD8+ T cell activation measured in peripheral blood prior to challenge predicted the graded amount of protection observed among vaccinated animals (Fig 3.6*C*). Using CD44 upregulation on T cells in peripheral blood as broad

measure of immunogenicity in combination with assays for Ag specific responses may be useful for screening vaccine candidates in experimental animals. Priming of *T. cruzi*-specific CD8+ T cells was evident in the two HR mice tested (Fig 3.5*B*), but LR mice clearly exhibit protection without any indication of immune stimulation (Fig 3.6*C*). Therefore, induction of parasite-specific immunity is a convenient indicator of vaccine effectiveness, but its absence should not absolutely discount a protocol. Although we have not yet formally demonstrated that orally vaccinated mice are also protected against oral challenge with WT *T. cruzi*, we would predict this to be the case. There is some uncertainty as to whether mucosal vaccination is absolutely necessary to induce protection at mucosal sites or if this could be accomplished by other means of vaccination [64, 65], but there is no doubt that mucosal vaccination can stimulate mucosal immunity [13]. Additionally, mucosal infection or immunization with *T. cruzi* parasites or Ag leads to IgA production [49, 50, 59, 60, 66] and confers resistance to mucosal *T. cruzi* challenge [61-63]. Thus, given the protection against systemic challenge observed after oral vaccination with *T. cruzi* in these studies, this immunization scheme is likely effective against mucosal challenge. While strategies such as heterologous prime boost regimens may maximize immunogenicity for a particular Ag [67], live parasites may best induce a broad-based, protective immune response [18] and be the practical choice to diminish the potential of reservoirs to infect triatomine bugs [26]. In conclusion, this work supports the development of attenuated parasite lines for vaccination against *T. cruzi* and defines a system in which to test vaccine candidates and further establish correlates of protection.

Figure 3.1. Oral infection with metacyclic trypomastigotes of *T. cruzi* leads to systemic infection. *A*, Metacyclic trypomastigotes delivered by either po or fp route are detectable in skeletal muscle. DNA was extracted from skeletal muscle mice 21-25 dpi and a 182 bp segment of satellite DNA was amplified by PCR. n = 10 fp and 7 po. *B*, Similar inflammatory infiltrates develop in skeletal muscle of mice infected fp and po. H & E sections of skeletal muscle are shown for mice in both groups at 35 dpi and are representative of at least 3 more experiments ended between 21 and 40 dpi. *C* and *D*, *T. cruzi* infection via the oral route induces a robust and lasting systemic T cell response. Representative flow plots show the frequency of CD8+ T cells specific for TSKB20 in PBMC at 14 dpi (*C*, n = 10) and at in spleens of two individual mice infected po at 140 dpi (*D*).

Figure 3.1

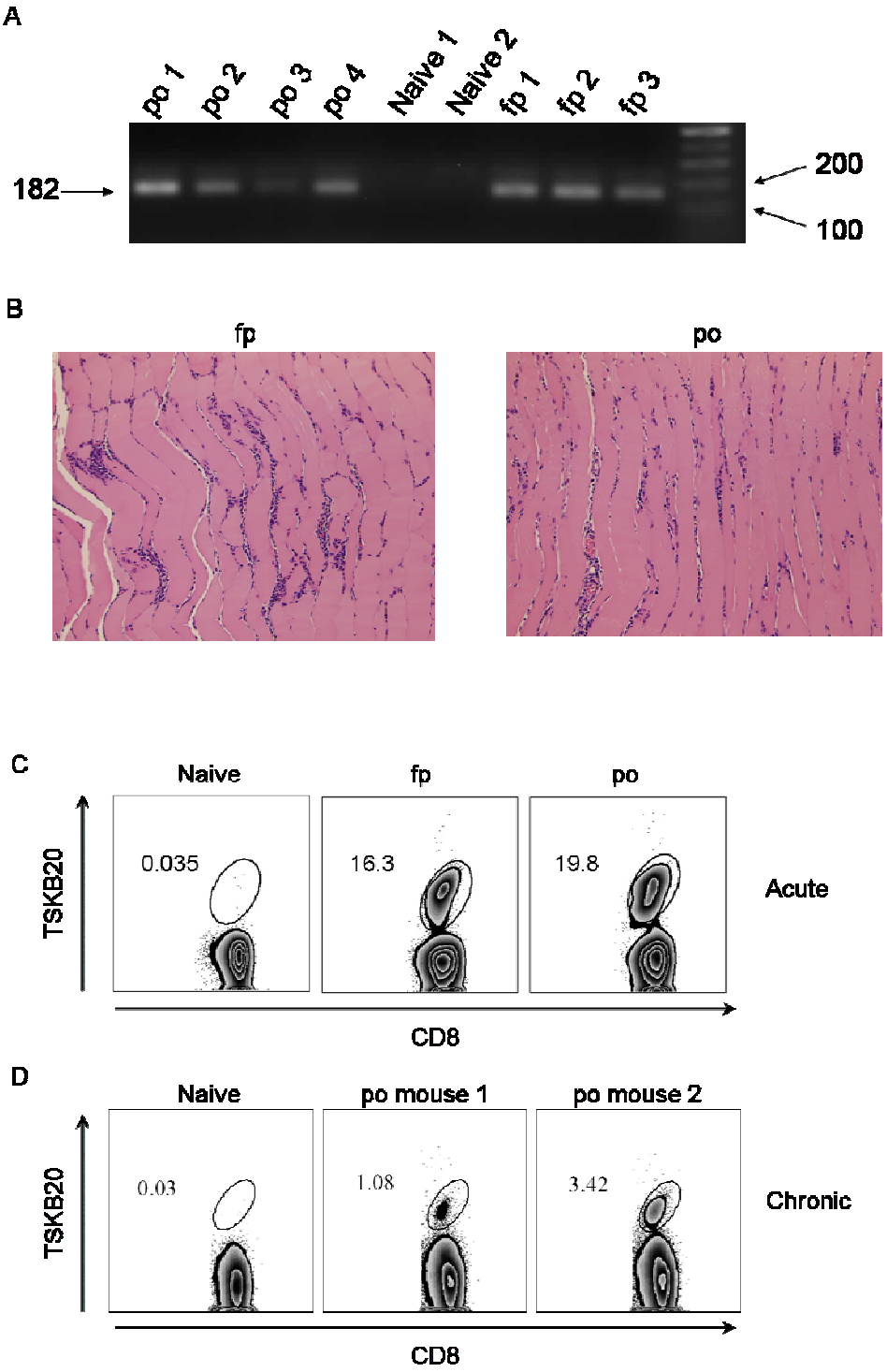

A
po 1
po 2
po 3
po 4
Naive 1
Naive 2
fp 1
fp 2
fp 3
182
200
100
B
fp
po
C
Naive
fp
po
0.035
16.3
19.8
TSKB20
Acute
CD8
D
Naive
po mouse 1
po mouse 2
0.03
1.08
3.42
TSKB20
Chronic
CD8

Figure 3.2. *T. cruzi*-specific CD8+ T cells traffic to peripheral tissues and are activated in sites of parasite persistence independently of infection route. *A*, Flow plots show the frequency of TSKB20-specific CD8+ cells in the indicated tissue of fp or po infected mice at 39 dpi. *B*, Recently activated *T. cruzi*-specific CD8+ T cells are present in sites of persistence. Flow plots are gated on CD8+ T cells isolated from spleen or skeletal muscle of fp or po infected mice at 39 dpi and show CD69 expression on TSKB20-specific cells. Numbers indicate the frequency of TSKB20+CD8+ (top right) or TSKB20-CD8+ (bottom right) T cells expressing CD69. Data are representative of at least 3 similar experiments.

Figure 3.2

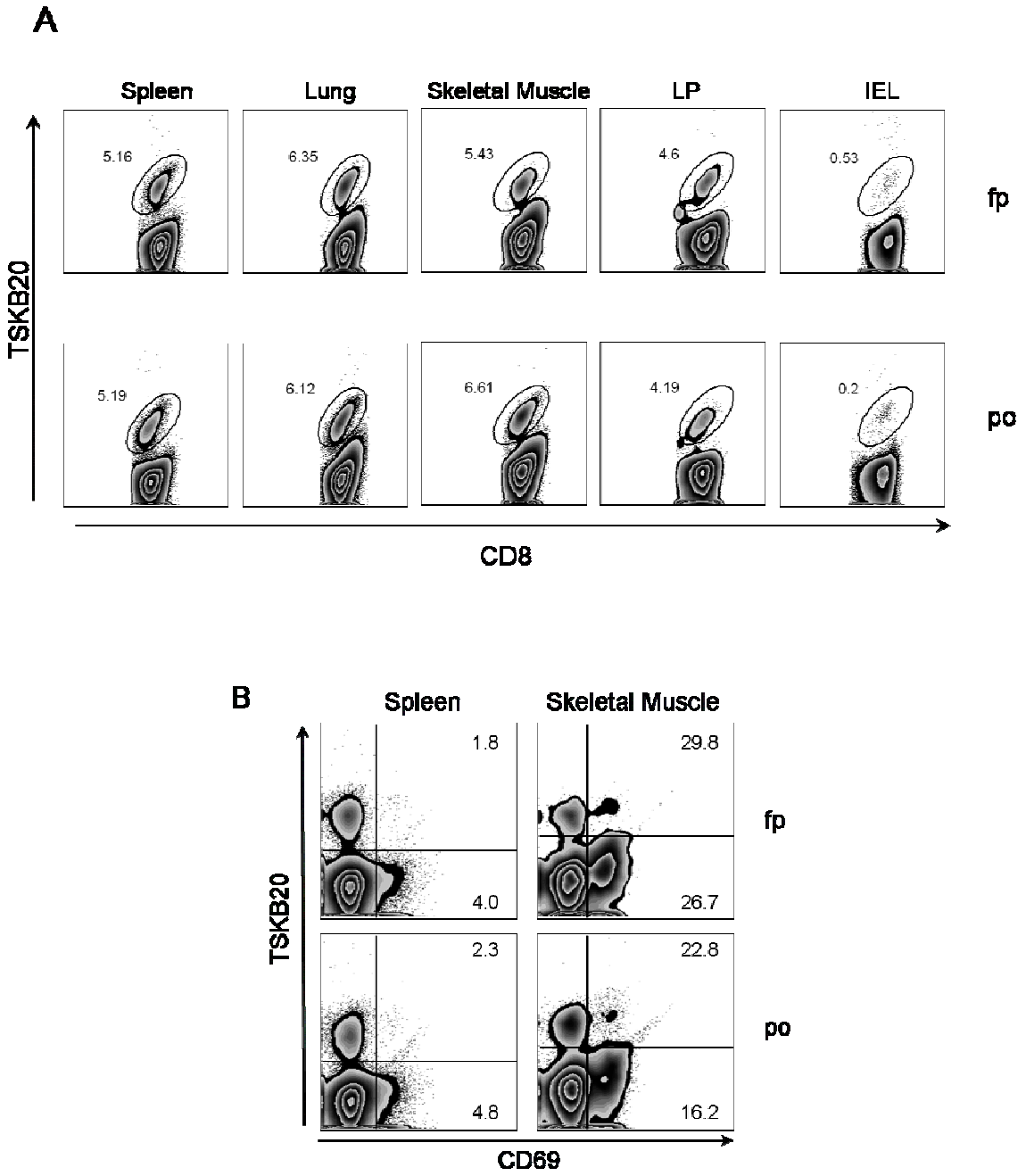
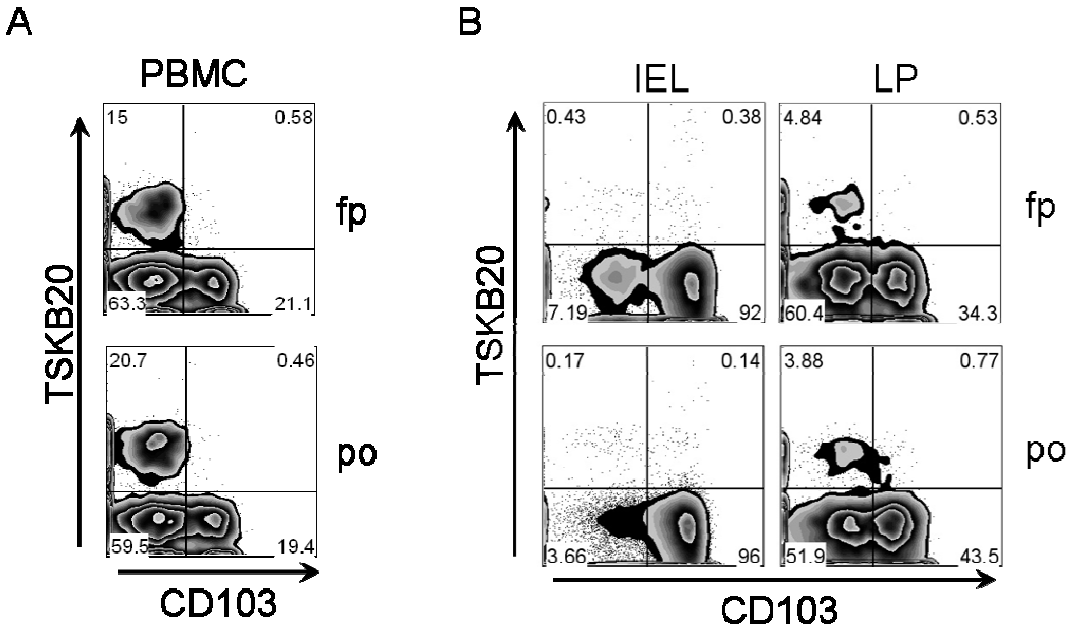

A
Spleen
Lung
Skeletal Muscle
LP
IEL
5.16
6.35
5.43
4.6
0.53
fp
5.19
6.12
6.61
4.19
0.2
po
TSKB20
CD8
B
Spleen
Skeletal Muscle
1.8
29.8
4.0
26.7
fp
2.3
22.8
4.8
16.2
po
TSKB20
CD69

Figure 3.3. *T. cruzi*-specific CD8+ T cells do not display a gut homing phenotype. *A*, Gut-imprinted CD8+ T cells specific for *T. cruzi* are not detectable in blood early in infection. PBMC from mice at 14 dpi (n = 10) were isolated and CD103 expression among TSKB20-specific cells was assessed by flow cytometry. Numbers in representative flow plots indicate the percentage of CD8+ T cells in each quadrant. *B*, CD103 is expressed by gut CD8+ T cells, but not *T. cruzi*-specific CD8+ T cells. IEL and LP lymphocytes were isolated from *T. cruzi*-infected mice at 39 dpi, and surface expression of CD103 was assessed by flow cytometry. Flow plots are gated on CD8+ T cells. Numbers indicate the percentage of CD8+ T cells in each quadrant. At least 2 other experiments yielded data similar to that shown in *B*.

Figure 3.3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0.58
63.3
21.1
fp
20.7
0.46
59.5
19.4
po
TSKB20
CD103
B
IEL
LP
0.43
0.38
7.19
92
4.84
0.53
60.4
34.3
fp
0.17
0.14
3.66
96
3.88
0.77
51.9
43.5
po
TSKB20
CD103

Figure 3.4. Route of infection does not affect the proportion of CD103+ or CD69+ cells in lymph nodes. *A*, Flow cytometry was used to assess the proportion of all CD8+ T cells expressing CD103 in pLN and mesLN of mice after fp and po infection with *T. cruzi*. Bars show average percentage of CD103+CD8+ cells ± SEM. *B*, The proportion of recently activated TSKB20+CD8+ T cells in different lymph nodes was assessed by flow cytometry. Bars show average percentage of CD103+CD8+ cells ± SEM. n = 3 mice per group. Similar data was obtained in another experiment.

Figure 3.4

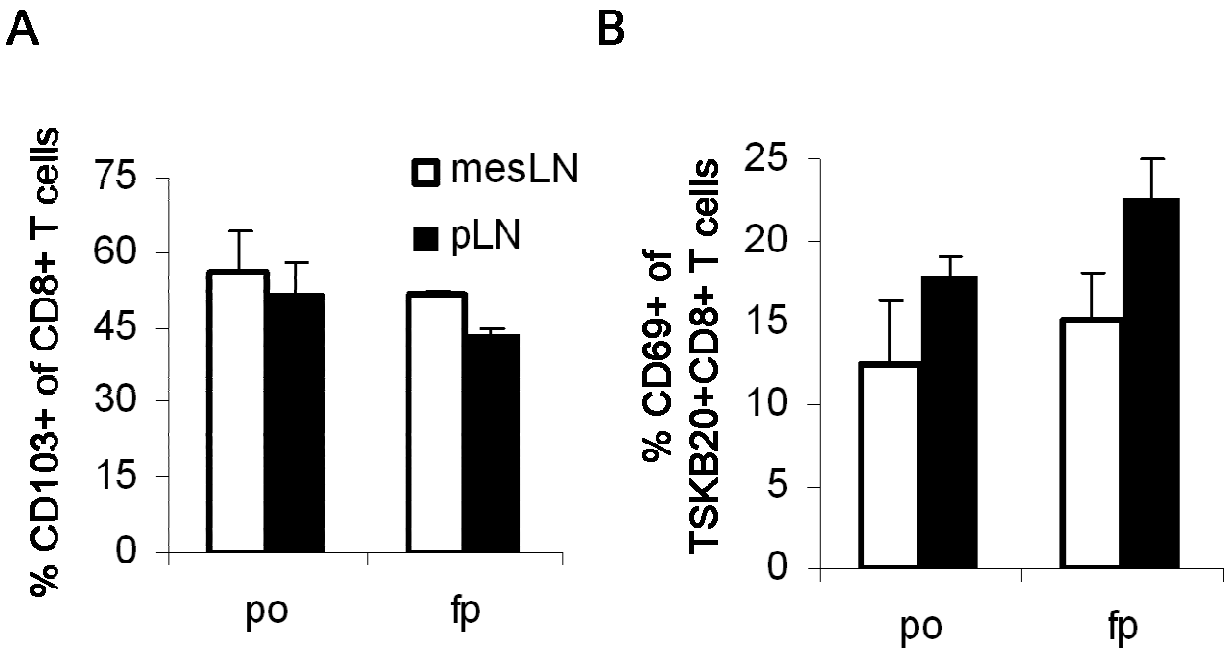

A
B
% CD103+ of CD8+ T cells
75
60
45
30
15
0
mesLN
pLN
po
fp
% CD69+ of TSKB20+CD8+ T cells
25
20
15
10
5
0
po
fp

Figure 3.5. Oral vaccination with attenuated parasites stimulates *T. cruzi*-specific CD8+ T cells. *A*, Variable degrees of T cell stimulation results from oral vaccination. CD44 expression was assessed 2 weeks after final vaccination on CD8+ T cells in PBMC. Flow plots are gated on CD8+ T cells, numbers and gates indicate the percentage of $CD44^{hi}$ cells. Of 10 vaccinated mice 3 were designated "low responders" (LR) and 7 were designated "high responders" (HR). *B*, Oral vaccination generates activated *T. cruzi*-specific CD8+ T cells. Flow plots show 2 individual mice chosen from HR group to screen for TSKB20+ responses and CD127 downregulation. All plots are gated on CD8+ T cells and numbers indicate the percentage of CD8+ T cells in a gate or quadrant.

Figure 3.5

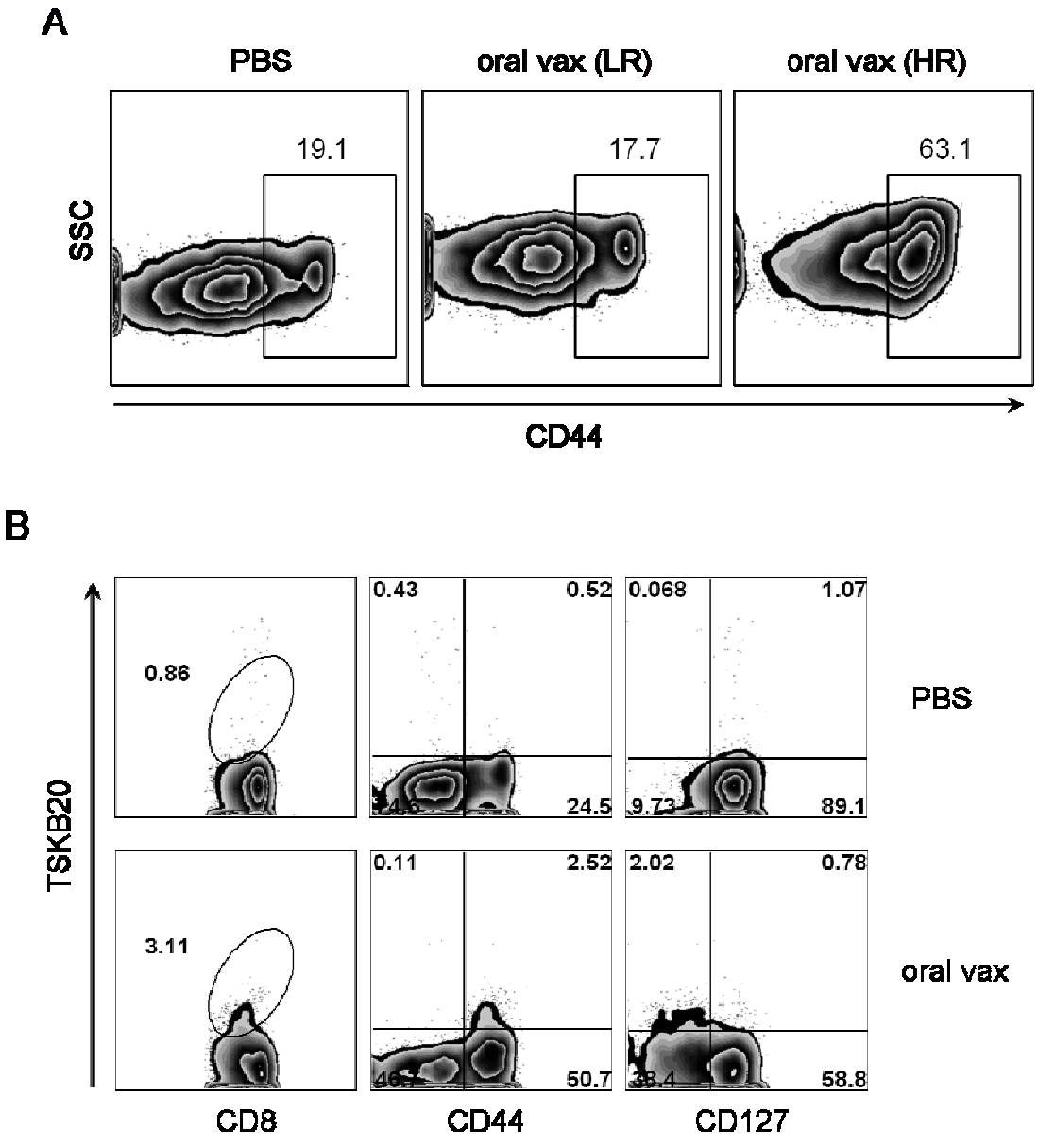
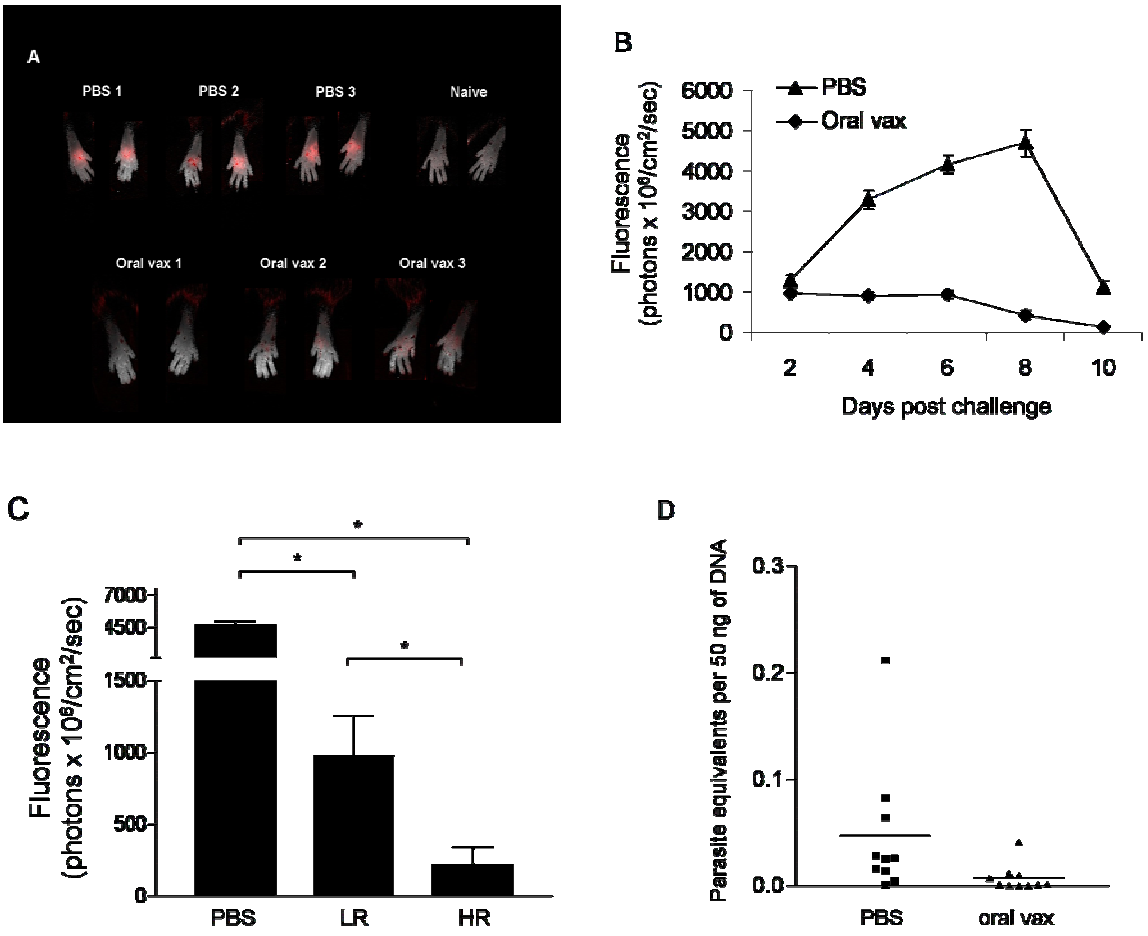

A
PBS
oral vax (LR)
oral vax (HR)
19.1
17.7
63.1
SSC
CD44
B
TSKB20
0.86
0.43
0.52
24.5
0.068
1.07
9.73
89.1
PBS
3.11
0.11
2.52
50.7
2.02
0.78
58.8
oral vax
CD8
CD44
CD127

Figure 3.6. Oral vaccination with attenuated parasites protects mice from WT *T. cruzi* challenge. *A*, Parasites are immediately controlled at infection site. Vaccinated mice (n = 10) and control mice (n = 10) were challenged with 2.5 x 10^3 WT fluorescent *T. cruzi* trypomastigotes injected in superficial subcutaneous tissue of each foot pad. Parasite load in fp was assessed by quantitating the fluorescent signal with an *in vivo* imaging system. Pictures show left and right feet of individual mice representative of the indicated group. *B*, Vaccine protection is evident at all time through the first ten days of *T. cruzi* infection. Graph shows the fluorescent signal of all feet in each group at indicated time points (average ± SEM, $p<0.05$ at all time points, n=20 feet (10 mice)). *C*, Extent of T cell activation is predictive of vaccine efficacy. Parasite load at 8 dpc is graphed for the 4 mice with the highest percentage of $CD44^{hi}$ CD8+ T cells (HR) and the 2 mice with the lowest percentage of $CD44^{hi}$ CD8+ T cells (LR) are graphed along with all mice from PBS group (average ± SEM, * indicates $p<0.0001$ by ANOVA analysis). *D*, Oral vax mice have lower parasite burden in skeletal muscle following WT *T. cruzi* challenge. Parasite load was measured in DNA samples extracted from skeletal muscle of mice 25 dpc by real time PCR. Bars show mean of group.

Figure 3.6


A
PBS 1
PBS 2
PBS 3
Naive
Oral vax 1
Oral vax 2
Oral vax 3
B
PBS
Oral vax
Fluorescence (photons x 10⁶/cm²/sec)
6000
5000
4000
3000
2000
1000
0
2
4
6
8
10
Days post challenge
C
Fluorescence (photons x 10⁶/cm²/sec)
7000
4500
1500
1000
500
0
PBS
LR
HR
*
*
*
D
Parasite equivalents per 50 ng of DNA
0.3
0.2
0.1
0.0
PBS
oral vax

3.5 References

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CHAPTER 4

CONCLUSION

Persistent infection by *Trypanosoma cruzi* leads to the pathologies of Chagas disease in many individuals who contract this parasite. Despite the chronic nature of human *T. cruzi* infection, few animal studies are carried out beyond the acute phase of infection where immune deficiencies such as the lack of CD8+ T cells are associated with early mortality or exacerbation of the acute infection [1]. The first aim of this project examined whether continuous actions of the immune system are critical to parasite control during chronic phase *T. cruzi* infection, particularly focusing on CD8+ T cell responses at peripheral tissue sites of persistence. The results confirm the hypothesis that optimal control of chronic *T. cruzi* infection is dependent on sustained immune activity, including actions of CD8+ T cells and IFN-γ. CD8+ T cells show evidence of recent activation selectively at sites of parasite persistence throughout chronic *T. cruzi* infection, and transient depletion of CD8+ T cells during the chronic phase led to loss of parasite control and increased tissue inflammation. Chronic-phase blockade of IFN-γ, a key effector molecule of CD8+ T cells, also led to increased parasite load in skeletal muscle. Finally, CD8+ T cells from spleen were readily incorporated into the ongoing peripheral immune response in nonlymphoid tissue, and inhibition of leukocyte extravasation by anti-VLA-4 treatment led to increased parasite load in skeletal muscle. Reliance on actively maintained *T. cruzi*-specific CD8+ T cells for parasite control is inconsistent with the T cell exhaustion that may be expected based on observations in several chronic viral infections [2]. Together, these findings suggest that a dynamic immune response, including active surveillance of peripheral

tissue by parasite-specific CD8+ T cells, must be constantly maintained to optimally control chronic *T. cruzi* infection. Additionally, this work further supports the central role for persistent *T. cruzi* infection of nonlymphoid tissue in the etiology of Chagas disease.

T. cruzi can be transmitted by introduction of parasites into the bite site of the triatomine vector, but also by contact with mucous membranes or ingestion [3]. The second aim of this work was to investigate the effects of infection route on the development of the peripheral CD8+ T cell response to *T. cruzi*. Following oral administration, *T. cruzi* still establishes a persistent infection in skeletal muscle, and CD8+ T cells exhibit a similar frequency, distribution, and surface phenotype independent of route of parasite introduction. Collectively, these data suggest that parasites disseminate early in the course of oral infection and that CD8+ T cells are stimulated by antigen produced distal to the initial site of infection. Having established that oral *T. cruzi* infection elicits robust T cell responses systemically, experiments were performed to test whether vaccination via oral infection with attenuated parasites could protect against *T. cruzi* infection at a distant challenge site. Orally vaccinated mice controlled footpad-injected parasites significantly better than control mice, a finding supporting potential oral vaccination of *T. cruzi* reservoir hosts as a strategy to limit human infection [4, 5].

One hundred years after its discovery, Chagas disease continues to be a detriment to human health, especially among individuals living in poverty in rural Latin America [6, 7]. Lack of a preventive vaccine and a need for improved and standardized diagnostics and prognostics are among the challenges confounding efforts to alleviate the burden of Chagas disease [8]. *T. cruzi* naturally infects both man and mouse, thus experiments in animals are useful in guiding and more thoroughly understanding human studies. The data reported here indicate that CD8+ T cells are a crucial and dynamically-maintained population of immune effectors throughout

chronic *T. cruzi* infection. When homing to sites of parasite persistence in peripheral tissue, these cells have a distinct phenotype and function compared to those in other lymphoid and nonlymphoid tissues. These findings should contribute to future work defining immunological parameters that can be incorporated into the clinical assessment of patients with *T. cruzi* infection. The CD8+ T cell response to oral *T. cruzi* infection is indistinguishable from that following other routes of infection. Oral administration could be a convenient way to immunize animals [9], and oral vaccine-mediated protection demonstrated here endorses pursuit of an attenuated parasite line to vaccinate reservoirs living in and around domestic areas and reduce *T. cruzi* transmission to humans.

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