THE ROLE OF ENDOGENOUS REGULATORY T-CELLS IN ACUTE AND CHRONIC IMMUNE RESPONSES TO EXPERIMENTAL MURINE *TRYPANOSOMA CRUZI* INFECTION

by

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(Under the Direction of Rick Tarleton)

ABSTRACT

Infection with the parasite *Trypanosoma cruzi* results in a robust and multi-faceted host immune response. The acute phase rarely results in death because the primary immune response is usually sufficient to control parasitemia. However, sterile cure of infection seldom follows, leading to a persistent infection by low numbers of parasites that live intracellularly in muscle and nerve tissue forming the basis for chronic disease. The exact mechanisms of initial or eventual immune evasion and parasite persistence in muscle tissue are unknown.

CD8+ T cells can kill host-cells harboring intracellular parasites, and are therefore important in immune control of *T. cruzi* infection. Recently, our lab reported that in *T. cruzi*-infected mice CD8+ T lymphocytes that infiltrate chronically-infected muscle tissue become dysfunctional as judged by their inability to secrete gamma interferon (IFN-γ) or lyse target cells *in vitro*.

Chronic or persistent parasite infections may be the result of the immune system mounting an incompetent immune response, or due to suppression of an appropriate

response, which can be induced by regulatory T cells and exploited by the parasite as an immune evasion mechanism. Understanding the mechanisms of initial *T. cruzi* immune evasion and CD8+ T cell dysfunction in chronically-infected muscle tissue could lead to new therapeutic strategies to combat Chagas' disease. The overall goal of this study is to investigate the role that "natural" CD4+CD25+ regulatory T cells play in modulating both acute and chronic immune responses to *T. cruzi* infection.

INDEX WORDS: *Trypanosoma cruzi*, Chagas' disease, Persistent Infection, CD4+CD25+ Regulatory T cells, Natural Treg, FoxP3, Scurfin, Interferon gamma, Intracellular cytokine staining, Dysfunctional CD8+ T cells, H2-k^b tetramers, DNA vaccination.

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B.S., Vanderbilt University, 1997

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2005

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December 2005

DEDICATION

To my wife, Carmen, without whom I could not have achieved this dream. I love you!!!

ACKNOWLEDGEMENTS

First I would like to thank my mentor, Rick Tarleton. He empowered me to become a better all-around scientist and critical thinker, and was always available for discussions with me. I also would like to thank Diana Martin, who taught me most of what I practically know about immunology. She was always so helpful and patient, and rescued me several times when I was in a pinch! She is also a very considerate friend. The knowledge that Todd Minning imparted to me was crucial to my success, and I always enjoyed having conversations with him. Todd is the man! Julie Nelson helped me with all aspects of flow fytometry, which comprised the bulk of my work. She is a delight to work with. I thank Angel Padilla for advice and musical gifts, as well as Juan Bustamante and Becky Bundy for helpful conversations. I would also like to thank my committee members, Dan Colley and Boris Striepen, for their mentoring and input into my research. I also appreciate the opportunity that Dr. Striepen gave me in allowing me to do a rotation in his lab, where he taught me how to clone genes, design primers, etc. Marcus Fechheimer and Ruth Furukawa offered me advice and encouragement just when I needed it, and for that I am grateful. The members of the Tarleton Laboratory brought out the best in me as a scientist, and I loved being a memebr of such a fun and diverse group. Brent Weatherly was like a beam of sunshine, always making the lab a fun place, even on Monday mornings. I don't know how he does it…pure genius. When I first joined the lab, I was delighted to find a kindred spirit in Drew Etheridge. He really helped me get through some rocky times. I have had many helpful conversations with

my fellow graduate students, Charles Rosenberg, Matt Collins, Lisa Bixby, and Xu Dan, and I wish them the best in their careers. I will miss being a part of such an intelligent-ragtag bunch. I also thank Charles, the braumeister, for providing that amber nectar, and for the good times. Courtney Boehlke was also a lot of fun in the lab and I will miss all my lab friends, like Gretchen Cooley and Angela Minning. I would like to pay tribute to all the people who got me here, starting first with my mom and dad, who always encouraged my pursuit of knowledge and biology, and they made me the naturalist that I am today. I thank all the scientific mentors of my past: Sean Boyd, Burton Bogitsh, Gary Polis (RIP), Todd Jackson, Renee Drucas, Nora Medrano, and Igor Nikoulin. I would like to thank Marian Thomas, Genia King, Kathy Vinson, and Brett Rudolph for all their support, as well as Tammy Andros and Donna Huber for their help. And last, but not least, I would like to thank all the friends I made in Athens, GA, as well as my old friends in San Diego, CA. Damon and Amanda Ely were good friends to Carmen and me. I have had many fun nights of making music with Paul Grifffin and Ryan Brown (PF2TN 4-ever!). All the friends I made in the department of cellular biology really enriched my experience here: thank you to Frank Hardin, Rich Davis, Engda Hagos, Danielle Webster, Jie Xu, Seonil Kim…(I know I am missing some). My old friends all gave me plenty of encouragement and kept the home fires burning: Craig Saxton, Mike Maass, Jeff Ludwig, Carl Haakmeester, Doug Hitchin, Jeremy Blough, Rob Robbers. I am indebted to my best friend and wife, Carmen, for making all this come to pass. I would finally like to thank the Almighty, for my life and free will.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1 *Trypanosoma cruzi*

1.1 Natural History

In 1909 Carlos Chagas, a Brazilian physician/scientist, discovered that houses in rural Brazil were infested with blood feeding insects that harbored flagellates in their digestive tracts. People who lived in the area were discovered to be diseased and were also found to harbor these flagellated organisms, later named *Trypanosoma cruzi* (*T. cruzi*) [1]. This stercorarian protozoan parasite is transmitted through the feces of triatomine vectors (Hemiptera: Reduviidae), regionally called “kissing bugs,” “vinchucas,” “barbeiros,” or “chinches”, to over 100 species of mammalian hosts, including rodents, sloths, armadillos, marsupials, dogs, and primates, including humans. The obligatory transmission between triatomine vectors and mammalian hosts is maintained in a sylvatic cycle that occurs between the 42nd North and South parallels, encompassing most of the American continent [2]. Transmission to the mammal via contamination of parasite-laden feces into broken skin or mucous membranes usually takes place during an insect blood meal.

The infective vector-borne metacyclic trypomastigote form of *T.* cruzi, as well as the vertebrate host blood-form trypomastigote, are highly motile and able to efficiently infect a wide variety of phagocytic and non-phagocytic cells [3]. After cell invasion, the trypomastigote resides for a short time in an endosome-

derived parasitophorous vacuole, but then escapes into the cytosol and converts to the amastigote stage, which is the replicative form in the mammalian host. Intracellular amastigotes divide by binary fission, eventually causing the host cell to rupture; during rupture amastigotes convert back into flagellated trypomastigotes which are released into the circulation, able to continue the cell invasion process. In the event that a triatomine ingests a circulating trypomastigote during a blood meal, the trypomastigote converts into an epimastigote form, which replicates in the gut of the insect, continuing the parasite lifecycle [3].

1.2 Chagas' Disease

1.2.1 Epidemiology

T. cruzi is the etiological agent of Chagas' disease in humans, also known as American trypanosomiasis. According to the WHO, approximately 17 million people in Latin America are infected. Another 120 million people are at risk of infection due to socio-economic and environmental factors which favor transmission, inadequate sustained vector-control measures, and lack of a successful vaccine. Furthermore, chemotherapy is toxic and not considered effective. In addition to vector-human transmission, the parasite can also be transmitted from human-to-human congenitally, through transplantation of contaminated blood or tissue, or through ingestion [4].

1.2.2 Infection and disease

Infection results in an acute phase which lasts 2-4 months, characterized by detectable parasites in the blood and widespread tissue parasitism. Death

rarely occurs during the initial acute phase of infection, which is usually asymptomatic or mildly symptomatic [5]. A chronic phase then ensues in which circulating parasites are virtually undetectable, but certain tissues, such as muscle and nerve tissues, remain persistently infected by low numbers of parasites for the life of the subject [6]. The majority of chagasic patients do not exhibit any severe disease symptoms, and are thus referred to as "indeterminate." However, over decades after initial infection, 30-40% of infected individuals develop a range of clinical symptoms of Chagas' disease such as cardiomyopathy, heart failure, digestive problems, and gut megasyndrome caused by denervation of the digestive tract. Chagas' disease causes 50,000 deaths annually, and is a leading cause of morbidity and mortality in many endemic regions (World Health Organization).

There is controversy regarding the underlying cause(s) of disease pathology. It is difficult to detect parasites in diseased tissues, and various disease symptoms usually occur later in life, several decades after the initial infection. For this reason, some groups argue an autoimmune basis of pathology (for review, see [7, 8]). However, immunosuppression exacerbates, rather than alleviates, disease symptoms and causes parasite recrudescence [9]. Strong evidence exists that disease is caused by parasite persistence in certain tissues, mainly in heart, gut, and skeletal muscle, leading to inflammation and tissue destruction (for review, see [6]). The correlation between parasite persistence and disease has been demonstrated in both *in vivo* experimental models [10] as well as in post-mortem studies involving chagasic cadavers [11]. Together,

these data support the theory that a misdirected or hyporesponsive (suppressed), rather than a hyperresponsive or autoimmune response is an underlying cause of Chagas' disease. Thus, in developing preventative or therapeutic strategies to combat disease, the focus should be on bolstering the proper immune compartments to heightened activities in the right location and time to decrease tissue-parasite load.

1.3 Immune Response to *Trypanosoma cruzi*

1.3.1 Cell mediated and humoral immunity

Given a lifecycle that includes antigenically-distinct intracellular (amastigote) and extracellular (trypomastigote) forms during infection of the mammalian host [12], a competent multi-faceted immune response is required to control infection in order to decrease morbidity and mortality. Since *Mus* species are natural hosts for *T. cruzi* infection, murine models are valid and useful tools to study the mammalian host-parasite biology. Cell-mediated and humoral adaptive immune responses are important in the immune response to *T. cruzi*, as disruption of either of these compartments during the acute or chronic phase can result in eventual failure to control infection. Extracellular trypomastigotes are susceptible to humoral components such as complement-fixing antibodies which lyse parasites [13] and opsonizing antibodies which lead to phagocytosis and killing by activated macrophages [14]. B-cell deficient mice eventually succumb to infection, although it is clear that antibody production is of secondary importance to cell-mediated immunity [15]. As an obligate intracellular parasite

T. cruzi is able to evade humoral immunity, and thus cell mediated immunity plays a major role in control of intracellular infection.

1.3.2 Helper T cells

CD4+ helper T (Th) cell-mediated immunity is important for immune control in both acute and chronic phases of *T. cruzi* infection. Animals deficient in class II MHC expression, which lack a functional CD4 compartment, display high parasitemias and 100% mortality in the acute phase of infection with the Brazil strain [16]. Depletion of CD4+ T cells during the chronic stage of infection results in exacerbation of disease [17]. Although mixed Th-type 1 and 2 cytokine responses are observed in both susceptible and resistant mice, a type-1 cytokine response is associated with resistance to disease. Th2-biased Stat4-/- mice are highly susceptible to infection with *T. cruzi*, while Th1-biased Stat6-/- mice survive infection and display reduced heart and skeletal muscle inflammation compared to Th2-biased mice [18]. Furthermore, antigen specific Th1 cells, but not Th2 cells, provide protection from lethal *T. cruzi* infection [19]. These studies highlight the roles of CD4+ Th1 cells in immune control and Th2 cells in parasite persistence and increased severity of disease.

1.3.3 CD8+ T cells

Cytosolic proteins found in nucleated mammalian cells are processed and presented to the immune system along the MHC class I pathway, which is surveyed by the MHC class I-restricted CD8+ T cell compartment. CD8 is the class I MHC co-receptor to the T cell receptor (TCR), which recognizes peptide-MHC complexes. Presentation of both endogenous and exogenous cytosolic

proteins requires targeting to and degradation by the proteosome followed by translocation of resulting peptide fragments into the lumen of the endoplasmic reticulum (ER). This movement is catalyzed by the transporter of antigenic peptides (TAP), an ATP-binding cassette (ABC) transporter protein family member. Once in the ER, peptide fragments of 7 to 14 amino acid residues are loaded into the peptide-binding groove of nascent MHC class I molecules and routed for display to the cellular surface. Upon recognition of MHC-class I-presented antigen by the cognate TCR, the effector mechanisms of CD8+ T lymphocytes include the killing of target cells via perforin/granzyme or Fas/FasL pathways (and are hence called CD8+ cytotoxic T lymphocytes, or CTLs) and the secretion of inflammatory cytokines, such as interferon gamma (IFN-γ). IFN-γ specifically induces the expression of several IFN-γ responsive genes, leading to the expression of co-stimulatory molecules, and the intracellular production microbicidal nitric oxide and reactive oxygen species.

The amastigote in the cytoplasm of infected mammalian cells. Since CD8+ T cells survey cytoplasm-derived microbial or self proteins displayed in the context of MHC-class I molecules, it is no surprise that CD8+ T cells play an important role in control of *T. cruzi*-infected cells. This conclusion was first suggested by the observation that CD8+ T cells are the major lymphocyte infiltrate in infected tissues [20]. β2-microglobulin deficient mice, which lack stable expression of class I MHC antigens and therefore lack mature CD8+ T cells and cytotoxic T cell activity, were found to be highly susceptible to infection and quickly succumbed to death, demonstrating the importance of CD8+ T cells

during the acute phase [21]. Furthermore, MHC class I gene knockout mice succumb to high parasitemias leading to 100% mortality in the acute phase following infection with the Brazil strain of parasite [16]. A model in which host cells were infected with *T. cruzi* transfectants that were engineered express secreted or GPI-anchored chicken ovalbumin (OVA) led to stimulation of OVA-specific CD8+ T cells by infected cells [22]. This suggested that secreted and GPI-anchored parasite proteins could be released into the host cytoplasm and become targets of CD8+ CTL responses. Later it was found that defined amastigote surface proteins were targets of CTL responses [23]. *T. cruzi* epitopes predicted to bind to class I MHC, based on the primary peptide sequence of regions of *T. cruzi* proteins, have since been identified that have been shown elicit CD8+ T cell responses [24]. Several of these epitodes are derived from GPI-anchored and secreted proteins belonging to the trans-sialidase (ts) protein family. The identification of immunodominant ts-derived epitopes has allowed us to use class I MHC ($H2k^b$) tetramers loaded with some of these peptides to monitor CD8+ T cell responses (Martin and Tarleton, unpublished). Furthermore, CD8+ T lymphocytes are also the primary cells in chronic human chagasic myocardial lesions [25].

The mechanisms by which CD8+ T cells mediate protection against *T. cruzi* are not yet completely defined. While CD8+ T cells from *T. cruzi* infected mice and humans are able to lyse target cells coated with parasite epitopes, our group has reported that mice lacking either perforin or granzyme can survive acute infection, indicating that these lytic pathways are not needed for control of

infection [15, 24]. However, some groups have reported that survival, or degree of pathology, are dependent on these mechanisms [26, 27]. These differences might be due to differences in parasite strain and infectious dose. CD8+ T cell production of IFN-γ is important in protection and resistance to *T. cruzi*. How the effects of IFN-γ mediated is somewhat unclear. IFN- γ knockout mice are highly susceptible to infection and neutralizing anti-IFN-γ antibodies administered in the acute phase led to higher parasitemias and higher mortality [28, 29]. IFN-γ induces the production of microbicidal nitric oxide (NO) in cells such as macrophages and muscle cells. However, the role that NO plays in protection against *T. cruzi* infection is not clear. While treatment with nitric oxide synthase (NOS) inhibitors to mice has been reported to lead to acute death, mice with knockouts in the inducible NOS and NOS2 enzyme isoforms respond to infection as wild type mice [30]. Since IFN-γ is a cytokine with pleiotropic effects, it is possible that it induces multiple, possibly undiscovered, anti-*T. cruzi* effects. In agreement with these *in vivo* animal models, a recent clinical study in humans reported that an increased frequency of *T. cruzi*-specific IFN-γ producing CD8+ T cells is correlated with a reduction in disease severity [31].

1.4 Immune evasion strategies of *T. cruzi*

1.4.1 Theories

Despite a robust and multi-faceted host immune response to *T. cruzi*, parasites are able to avoid sterile cure during the primary immune response, and persist in some tissues, forming the basis for chronic disease. Several theories have been suggested to explain mechanisms of initial immune escape,

subsequent inability to cure infection, or maintenance of parasite persistence. These include but are not limited to: subversion of the complement system [32], down-regulation of dendritic cell (DC) function [33], polyclonal lymphocyte activation [34], inability of lymphocytes to produce IL-2 [35], parasite-released immunomodulatory molecules [36], parasite expression of TCR-antagonistic altered peptide ligands (APLs) [24], parasite tropism to peripheral immune privileged-like tissue [37], and suppressor cells [38]. The role that CD4+CD25+ regulatory T cells play in suppressing immune responses during acute and chronic *T. cruzi* infection remains to be elucidated [39].

1.4.2 Dysfunctional CD8+ T cells and *T. cruzi* infection

When naïve CD8+ T cells are primed by professional antigen presenting cell, CD8+ T cells become activated, proliferate, differentiate, and perform their effector function. In this process they adopt a different surface phenotype that reflects function. Effector/memory CD8+ T cells ($CD8+T_{EM}$) that have differentiated from naïve cells promiscuously distribute throughout peripheral tissues and wait to encounter antigen [40, 41]. The surface phenotype of $CD8+T_{EM}$ reflects their status as well-positioned effector cells. For example: low expression of CD62L (L-selectin) allows the cells to leave the CD62-gated secondary lymphoid organs (spleen, lymph nodes) and travel via lymphatic vessels and the bloodstream to sites of inflammation in peripheral tissues. Expression of adhesion molecules such, as CD11a (an integrin) and CD44, can mediate extravasation and binding to antigen presenting cells, endothelial cells,

and components of the extracellular matrix. Antigen specific T cells from *T. cruzi* infected mice generally exhibit this effector/memory phenotype [42].

Upon cognate-antigen recognition/TCR stimulation, even in the absence of co-stimulation, CD8+T_{EM} perform their cytolytic and IFN-γ-producing effector function quickly. Of particular interest to uncovering the causes of parasite persistence is a recent study from our lab which described CD8+T_{EM} [$CD62L^{LOW}$, $CD44^{HIGH}$, CD11a+, Ly-6C+] cells isolated from liver and muscle tissues of chronically infected mice that exhibited dysfunctional responses to polyclonal TCR stimulation. This was in stark contrast to their CD8+ splenic counterparts, which also expressed an effector/memory phenotype but responded well to stimulation, as measured by interferon gamma production and cytotoxicity. The cause of the dysfunction was not found to be attributed to anergy, apoptosis, or expression of an inhibitory receptor (CD94), but seemed to be tissue specific. When functional donor splenocytes from a chronically-infect mouse were transferred to naïve or chronic congenic mice, those transferred cells that trafficked to the muscle and liver of the donor also became dysfunctional [37]. It is of particular interest to elucidate the causes of dysfunction in muscle-derived CD8+ T cells, as the muscle is a site of intracellular parasite persistence, and CD8+ T cells are required to kill infected host cells.

2 Peripheral Tolerance and Regulatory T cells

2.1 History

One of the cardinal features of a successful immune system is to discriminate between self and non-self. Immature T cells that exhibit high TCR avidities for self molecules presented by self MHC are deleted in the thymus, as these would have the potential to be strongly self-reactive. This deletion process, known as central tolerance, occurs during the clonal selection process. Autoimmunity would occur often and fatally if one's potentially self-reactive lymphocytes were not deleted in the primary lymphoid organs (bone marrow or thymus/fetal liver) before entering the periphery. However, central tolerance is incomplete and thus potentially detrimental self reactive T lymphocytes enter the periphery. The reactivities of these cells in the periphery are therefore modulated by peripheral tolerance mechanisms such as antigen ignorance, anergy, and deletion. These *"*passive" peripheral tolerance mechanisms can be induced when antigens are presented by resting/immature/quiescent dendritic cells or in environments poor in co-stimulatory molecules, leaving potentially reactive cells "ignorant." The long-term and repeated presentation/stimulation that occurs during antigen persistence can lead to an anergy or deletion of antigen-specific cells.

In addition to these examples of mechanisms of "passive" tolerance, different subsets of T cells have been shown to actively suppress activation and proliferation of lymphocytes *in vivo*. Several experimental murine models have demonstrated this. From the late 1960's into the early 1990's the concept of

“Suppressor T cells” evolved (for review, see [43, 44]). It was shown that either neonatal thymectomy or adult thymectomy followed by sub lethal irradiation led to the autoimmunity (for review, see [43]). Self tissues were attacked by the few lymphocytes that remained in the periphery in the absence of a thymus, as the lymphocytes homeostatically expanded to fill the immunological void. These lymphocytopenic mice could be rescued by early adoptive transfer of normal T cells, suggesting the existence a population of thymus-derived suppressors. Due to the knowledge and technology at the time, early efforts to characterize suppressor T cells were thwarted by the lack of an accurate, precise, and specific description [45]. This discredited the field of suppressor T cell-research until better markers for them were discovered, aided by improvements in technology.

In 1995 Sakaguchi and colleagues identified a subset of $CD45RB^{low}$ CD4+ T cells that constitutively expressed the IL-2 receptor α-chain (IL-2Rα, CD25) [46]. Comprising 5-10% of total CD4+ T cells, co-transfer of this CD25+ population could potently suppress the autoimmunity induced by transfer of CD4+CD25- T cells to a nude/SCID (lymphocytopenic) mouse [46]. Their discovery of a relatively small population of cells with regulatory potential that expressed a distinguishing marker revitalized the study of murine and human T cells with suppressive capabilities, now commonly called regulatory T cells (Treg).

Several subsets of Treg cells have been described in recent years, each which exhibit both distinct and overlapping phenotypic and functional characteristics. For example, CD4+CD25+ Treg mediate their effects by cell-cell

contact. Cytokines produced by Th1 and Th2 cells can cross-regulate immune responses. Some CD4+ Treg also mediate their effects through production of cytokines such as IL-10 and TGF-β, which have immunosuppressive properties and are produced in relatively large amounts by Tr1 and Th3 cells, respectively. Still other T cell populations such as CD8+, γδ T cells, and natural killer T (NKT) cells have been shown to suppress immune responses [44]

2.2 Natural and Adaptive Treg

2.2.1 CD4+CD25+ Treg and foxp3.

The desire to segregate the various Treg based on the aforementioned observations has led to the proposal that two categories of Treg exist, natural (or endogenous) and adaptive (or induced), which differ according to antigen specificity, manner of activation, and mechanism of effector function [47]. Generally, 'natural Treg' (nTreg) are thought to leave the thymus as self-antigen-activated regulatory cells and circulate throughout the periphery. Since they arise from this primary lymphoid organ and are present in all normal naïve individuals, they are called natural Treg. The CD4+CD25+ T cells described by Sakaguchi are synonymous with this definition of nTreg [46]. One of the primary functions of nTreg is to suppress cell-mediated autoimmunity, as in their absence autoimmunity intensifies, whereas in their presence it is controlled. nTreg require initial activation via the TCR and co-stimulation in order to be suppressive, and appear to mediate their suppressive effects via cell-cell contact, either by interacting with APCs and/or activated T cells.

The initial observations that an absence of CD4+CD25+ Treg cell activity allowed autoimmune pathology in mice led to the discovery that there was a common connection between CD4+CD25+ Treg function the Foxp3 (forkhead box p3) gene, which encodes a forkhead/winged-helix transcription factor known as scurfin [48]. Mice that exhibit a loss-of-function mutation in the Foxp3 gene product due to a frame-shift (known as *scurfy* mice) exhibit severe autoimmune pathology. Interestingly, IPEX (immunedysregulation, polyendocrinopathy, enteropathy, X-linked) is a severe autoimmune syndrome in humans which is also linked to a mutation in FOXP3 [48]. The facts that i) CD4+CD25+ T cells from *scurfy* mice display no suppressive activity, ii) CD4+CD25+ T cells from wild-type (*wt*) mice have increased foxp3 mRNA transcription/scurfin expression compared to CD4+CD25- T cells, iii) CD4+CD25+ T cells from *wt* mice can suppress the autoimmunity elicited by targeted deletion of the Foxp3 gene, and iv) transgenic expression of Foxp3 confers suppressive ability on CD4+CD25- and CD8+ T cells confirm that Foxp3 is necessary and sufficient for nTreg activity [49, 50]. Whether or not Foxp3 expression can be induced *de novo* in the periphery is a subject of controversy and is currently under investigation [51-54]. The recent development of mice containing a green fluorescent protein (GFP)-Foxp3 fusion protein may be used as a powerful tool with which to study nTreg development and function [51, 55]

Natural Treg submit to defined rules, express specific markers, and for this reason are the most extensively studied of the Treg [56]. As a result, nTreg have been shown to be key players in peripheral tolerance to not only self but also to

foreign antigens, including microbes. Furthermore, many of the findings from corresponding murine models can be applied human immune systems, allowing for potential clinical/therapeutic applications in the autoimmunity, transplant, tumor biology, and infectious disease research fields [56, 57].

2.2.2 Adaptive Treg

'Adaptive' or 'inducible' Treg (iTreg) describes a diverse group of T cells that are distinguished from nTreg by a requirement for further differentiation in the periphery based on the environment or immunological context in which they are exposed to their cognate antigen [47]. Such a context depends on the route of antigen administration, the costimulatory/inhibitory environment, and the cytokine milieu at the time of antigen priming. Regulatory Type 1 (Tr1) and T Helper Type 3 (Th3) cells are examples of regulatory cells induced under certain conditions in the periphery which subsequently mediate their suppressive effects through the immunosuppressive cytokines IL-10 and TGF-β. Induced Treg (iTreg) may or may not express CD25 (depending on their activation state), and may also suppress in a contact-dependent fashion [58, 59]. Much data regarding iTreg is based on experimental manipulation of *in vitro* or *in vivo* systems. However, naturally-occurring parasite antigen-specific CD4+ T cells that express IL-10 and have regulatory function have been described [60].

To separate Treg into "natural" and "induced" categories may prove to be arbitrary, as it is possible that Treg induced in the periphery as well as those arising from the thymus cooperate to regulate immune responses.

2.3 Characteristics of Natural Treg

2.3.1 Phenotypic markers

Aside from the discovery of Foxp3 expression as a well-defined marker for nTreg, other molecules associated with nTreg have been described. The expression of CD25 by nTreg has proven to be useful, both as a phenotypic marker and as a functional target. Lymphocytes express CD25 (the IL-2 receptor alpha chain, or IL-2Rα) during activation, which is required to form a trimeric complex with the IL-2Rβ (CD122) and common γ-chain (CD132), together constituting the high-affinity IL-2-binding receptor [61]. IL-2 is a cytokine produced by lymphocytes during activation and acts as a potent growth factor *in vitro*. However, IL-2 appears to maintain immune homeostasis and self-tolerance *in vivo*, as demonstrated by the severe lymphoproliferative autoimmunity that occurs in IL-2 or IL-2 receptor-deficient mice [55]. Interestingly natural Treg, which express CD25 and thus the physiologically relevant high-affinity IL-2 receptor, themselves produce very little IL-2. It appears the paracrine utilization of IL-2 secreted by non-regulatory cells is required for the maintenance of nTreg homeostasis *in vivo,* although a role for IL-2 in the development and activation of nTreg is controversial [55, 61-63].

2.3.2 *In vivo* manipulation of Treg function

The most widely used procedure to manipulate CD4+CD25+ nTreg in order to probe their function *in vivo* is via anti-CD25 monoclonal antibody-mediated depletion. This possibility was first demonstrated in a murine tumor model in which *in vivo* administration of anti-CD25 antibodies (7D4 or PC61

clones) led to reduction in numbers of CD4+CD25+ T cells and reduction of tumors, indicating that removal of Treg enhanced anti-tumor immune response. Administering depleting CD25 antibodies has also proven to enhance anti-microbial immune responses [56, 60]. Other functional markers for regulatory T cells have been identified. Differential expression analysis of CD4+CD25+ and CD4+CD25- T cells revealed elevated expression of glucocorticoid induced TNFα receptor-related protein (GITR). Importantly, anti-GITR antibodies abrogated nTreg suppression, suggesting a functional role for this molecule in Treg dependent suppression. GITR-specific antibodies (DTA-1 clone) administered *in vivo* have proven to enhance CD8+ T cell immune responses to chronic infections. Other described markers include CTLA-4, CD103, and LAG3. Treg must be activated to suppress T cell responses *in vitro*, requiring TCR-MHC and CD28-B7 signals, and suppress in a contact-dependent fashion. There are multiple mechanisms of nTreg suppression *in vivo*. They can act on the APC or on the effector cell, and may do so in both a contact-dependent and cytokine-dependent fasion [64].

2.4 Natural Treg: Roles In Infectious Disease

Chronic or persistent parasite infections may result from the immune system mounting an incompetent or inappropriate immune response, or due to an immune evasion mechanism developed by the parasite. Negative feedback mechanisms exist to suppress excessive inflammation and tissue damage that can occur during infection and restore homeostasis. One example of this is active suppression mediated by CD4+CD25+ Treg, which can act on both CD4+

and CD8+ T effector cells. While Treg can play a protective role in immune responses to infections by limiting collateral damage to tissues, Treg can also be exploited by certain pathogens, leading to immune evasion and the establishment of chronic infections (for review, see [56]).

One of the first examples of the role of Treg is demonstrated in the healing murine *Leishmania* model. C57BL/6 mice given low doses of parasites control, but do not clear infection: low numbers of parasites persist at distinct sites, and can recrudesce if immune deficiency occurs. Persistent parasites allow for concomitant immunity, and therefore these mice are resistant to re-challenge at distal sites. CD4+CD25+ Treg accumulate at sites of infection, and these Treg are responsible for the inability to achieve sterile cure of infection: removal of CD4+CD25+ Treg by administration of monoclonal antibodies directed against CD25 allowed effector CD4+ T cells to completely clear infection. In this case, concomitant immunity was also lost. Conversely, transfer of CD4+CD25+ T cells to a healed mouse with persistent infection resulted in over-regulation and inability to control infection [60, 65, 66].

Leishmania infect macrophages and reside in a phagolysosome; therefore the CD4+ T cell compartment is most important in control of this infection. Most of the early CD4+CD25+ Treg studies focused on their ability to modulate CD4+CD25- T cell responses. Several recent studies have also demonstrated Treg-mediated suppression of CD8+ T cell responses [67-71], which justifies investigating the influence of Treg in our chronic *T. cruzi* model.

CONCLUSION

The acute immune response to *T. cruzi* is usually not sufficient to achieve a sterile cure. Parasites somehow evade this robust acute-phase immune response, and continue to persist and cause Chagas' disease, which is characterized by long-term low-level inflammation in muscle and nerve tissue. CD4+CD25+ Treg function to curtail immunopathology caused by excessive inflammation. Several recent studies have demonstrated that Treg can be exploited by certain parasites in order to persist, leading to chronic infection. The role that CD4+CD25+ Treg play in the initial and/or subsequent escape from the anti-*T. cruzi* immune response remains to be elucidated.

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CHAPTER II

THE ROLE OF ENDOGENOUS REGULATORY T CELLS IN THE CHRONIC IMMUNE RESPONSE TO EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION[1]

[1] Kotner, J.S. and Tarleton, R.L. To be submitted to *Infection and Immunity*.

ABSTRACT

The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas' disease, a chronic inflammatory disease of humans that chiefly affects muscle, as well as neuronal and gut tissue. The severity of Chagas' disease is correlated with persistent parasitism of these tissues. In various experimental models of chronic disease caused by persistent infection, it has been demonstrated that CD4+CD25+ "natural" regulatory T cells (Treg), which comprise 5-10% of total CD4+ T cells and express the regulatory T cell lineage factor Foxp3, can modulate anti-microbial immune responses. In suppressing potentially pathological effector T cell responses during infection, Treg serve to limit collateral tissue damage, but may also ultimately prevent the clearance of infection by suppressing crucial immune responses. In this study we sought to elucidate the role of CD4+CD25+ regulatory T cells in suppressing CD8+ T cell effector responses in C57BL/6 mice chronically infected with the Brazil strain of *T. cruzi,* focusing particularly on the role of Treg in mediating a recently reported dysfunction of muscle-resident CD8+ T cells. It is reported here that frequencies of CD4+CD25+ T cells are often increased in the skeletal muscle tissue relative to the spleens of mice chronically infected with *T. cruzi*. However, neither FoxP3 mRNA nor its protein product scurfin could be readily detected in leukocytes isolated from chronically infected muscle tissue. *In vivo* depletion of Treg by treatment with anti-CD25 monoclonal antibodies failed to alter IFN-γ production by CD8+ T cells in peripheral tissues. Furthermore, lymphocytes isolated from the muscle of mice with chronic *T. cruzi* infection lacked the ability to suppress

proliferation of splenic T cells. Thus, Treg do not appear to play a major role in regulating CD8+ T cell effector responses in muscle and in the persistence of persistence of *T. cruzi* in this site.

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi* is naturally transmitted to mammals by certain vectors contained within the insect family, Reduviidae. The infective parasite may be transmitted to humans by inoculation of parasite-contaminated vector feces through an open bite-wound or mucous membrane, as well as through ingestion of infective forms, congenital transmission, or by receiving tissue transplants contaminated with parasites. Infection with *T. cruzi* results in an acute phase of detectable parasitemia and the systemic distribution of parasites to several types of tissue. Acute infection is rarely lethal in immune-competent hosts, and is usually controlled by a potent immune response. However, sterile cure of infection seldom occurs. Instead, parasites persist in muscle and nerve tissue, leading to the formation of Chagas' disease in humans. This disease, characterized by chronic inflammation and tissue destruction of persistently-parasitized tissues of the heart or digestive tract, causes 50,000 annual deaths. Of the estimated 18-20 million people that are infected, 30-40% will develop severe chronic disease symptoms [1, 2]. Currently, there are no completely effective chemotherapies or available vaccines. How the parasite is able to persist, despite a strong anti-parasite immune response, is unknown and is the focus of this research. Identifying the mechanisms of parasite persistence could lead to the development of effective preventative or improved therapeutic measures.

Two morphologically distinct forms of *T. cruzi* occur during parasitism of the mammalian host. The flagellated trypomastigote is the motile form, which

can travel via the bloodstream and can infect a variety of cell types. After cell invasion, the trypomastigote converts to the amastigote form, which resides intracellularly in the cytosol and replicates by binary fission. Amastigotes eventually burst the infected cell, convert to trypomastigotes, and repeat the invasion-replication process. CD8+ T cells are essential in the control of the intracellular stage of infection, as they are able to survey class I MHC molecules displaying antigens derived from the internal cytosolic environment of infected nucleated host cells. Mice without a functional CD8+ T cell compartment during any phase of disease, or lacking MHC-I, quickly succumb to and die of infection [3-5]. When antigen-specific CD8+ T cells detect their cognate *T. cruzi*-derived antigen in the context of MHC-I, they are induced to perform their effector function, which may include the secretion interferon gamma (IFN-γ) and the killing of target cells [6]. In murine models of Chagas' disease the inability to produce IFN-γ is lethal [7, 8]. In human Chagas' disease, it has been reported that the severity of chronic disease is inversely correlated to the ability of CD8+ T cells to secrete IFN-γ [9]. Thus, the CD8+ T cell production of IFN-γ is required for control of *T. cruzi* infection, and is one measure of CD8+ T cell effector function correlated with less severe disease in humans.

It has recently been reported that dysfunctional CD8+ T cells reside in the muscle of mice chronically infected with *T. cruzi*: While the surface phenotype of these dysfunctional cells shows they are effector/memory CD8+ T cells (CD11a+ CD44+ Ly-6C+ $CD62L^{low}$) and suggests they should secrete IFN-γ upon T cell receptor (TCR) stimulation, they are hyporesponsive in comparison to

effector/memory CD8+ T cells from the spleen of the same animal [10]. Since dysfunctional CD8+ T cells reside in muscle, a site of parasite persistence in chronically-infected mice, investigating the cause or maintenance of the observed CD8+ T cell dysfunction and potential correlation to parasite persistence is of great interest.

A variety of passive mechanisms exist which serve to regulate CD8+ T cell immune responses in peripheral tissues (for review, see [11]). Additionally, the 5-10% of total CD4+ T cells that constitutively express the IL-2 receptor α-chain (CD25) and FoxP3 exhibit potent regulatory properties. These cells, present in all normal individuals, and therefore known as “natural” or “endogenous” CD4+CD25+ regulatory T cells (Treg), are intensely studied and have been shown to modulate immune responses to self, tumor, and exogenous antigens [12-17]. Generally, Treg serve to suppress hyper-immune responses that may cause collateral tissue damage and harm to the host. However, the suppression of some immune responses may actually have a detrimental effect on the host by allowing certain infections to persist [18].

While much of the literature regarding Treg and infectious disease focuses on their suppressive effects against CD4+ CD25- T cells [19-21], accumulating evidence demonstrates that Treg also have the ability to suppress CD8+ T cell cytokine production and proliferation [22, 23], and that depletion of Treg before or during infection can lead to increases in CD8+ T cell cytotoxicity, IFN-γ secretion, clonal expansion, and enhanced formation of immunological memory [24-26].

Since induction of Treg function can sometimes favor the microbe rather than the host, this may be a mechanism of immune evasion by some parasites [18, 27]. To our knowledge, this is the first study investigating the role that endogenous Treg play in chronic *T. cruzi* infection. We used molecular and immunophenotypical approaches to characterize the regulatory potential of muscle infiltrating leukocytes, administered monoclonal antibodies proven to knock down Treg function *in vivo*, and employed an *in vitro* suppression assay to measure the suppressive potential of leukocytes derived from chronically infected muscle tissue.

MATERIALS AND METHODS

Mice and infection.

C57BL/6 (Ly5.2+) and B6.SJL (Ly5.1+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and bred and maintained in the AAALAC-approved University of Georgia animal facility in micro-isolator cages. At 6-12 weeks of age female mice were intraperitoneally infected with 10^3 blood form or tissue culture-derived trypomastigotes of the Brazil strain. Blood form trypomastigotes were maintained by serial passage through C3H/HeSnJ mice and tissue culture-derived trypomastigotes were created by passage through Vero cell monolayers cultured in RPMI supplemented with 10% FBS. Chronically infected mice were used at 5-15 months post-infection.

Lymphocyte isolation from secondary and non-lymphoid tissues.

In most experiments animals were exsanguinated and in some cases (as noted) were perfused with 10ml sterile Alsevers buffered salt solution (pH=7.2). Spleens and lymph nodes (LN, superficial inguinal, popliteal, mesenteric) were removed and sterile single cell suspensions were produced by mashing with the blunt end of a syringe, mechanical disruption using forceps, or by pressing between frosted slides. Red blood cells were lysed using sterile lysis buffer (10mM HEPES, 0.83% ammonium chloride) for 5 min at 25°C followed by filtering through a nylon mesh screen washing 2X using RPMI 1640 plus 10% FBS and centrifuging at 400 x g for 10 min. After removing popliteal lymph nodes, quadriceps and hamstring were collected. To aid in the isolation of lymphocytes from muscle tissue after mechanical disruption with forceps and mincing with blades, in most cases muscle tissue was then incubated in serum-free RPMI 1640 media containing [70 μg/ml] Liberase Blendzyme II (Roche Diagnostics, Indianapolis, IN) for 1 hr at 37°C. Muscle tissue was dissociated in RPMI 1640 containing 10% FBS and [80 U/ml] DNAse I (Roche) and supernatants were washed through 40 μm nylon cell strainers (BD Biosciences, Bedford, MA) to filter large debris from lymphocytes, followed by washing with media. The resultant pellet is a heterogeneous mixture composed of muscle fibers and lymphocytes. To enrich for muscle-resident lymphocytes, these pellets were subsequently stained with PE-conjugated anti-CD45 antibodies (BD Pharmingen) for 15 min on ice, followed by 2X washing with media. A MoFlo FACS was used to sort CD45+ cells. CD45 is a pan-leukocyte marker.

Immunofluorescent staining and analysis.

For direct *ex vivo* staining, 50-100 µl of whole heparinized blood or $1x10^6$ RBC-depleted single-cell suspensions from spleen, LN, or muscle (prepared as stated above) were first washed using PAB (1X PBS, pH=7.4; 0.05% azide; and 1% BSA (w/v)) and centrifuged at 400 x g for 10 min. Cells were stained in 100-200 µl PAB containing anti-FcRγIII/II antibodies and combinations of monoclonal antibodies diluted 1:100 against the surface markers CD3, CD4, CD8, CD25, CD45, CD45.1, and CD45.2 (BD, Caltag) for 20 min on ice in the dark, followed by 2 washes in PAB, and fixation in 2% formaldehyde for at least 15 min up to 20 hrs at 4°C. Whole blood was lysed in 500 ml lysis buffer followed by two washes in PAB prior to fixation. In some cases following surface staining, intracellular scurfin was stained using PE-conjugated anti-scurfin antibodies (1:50 dilution) and staining kit according to manufacturer's instructions (eBioscience, San Diego, CA). A Cyan (Dako Cytomation) or FACSCalibur (BD) flow cytometer and FlowJo software were used for cytometric analysis.

Cell stimulation, intracellular cytokine staining, and analysis.

For IFN-γ staining, $1x10^6$ cells were cultured overnight in 0.2 ml RPMI 1640 supplemented with 10% FBS in 96-well flat-bottomed plates that had been previously coated with 30 µg/ml anti-CD3. During the last 5 hrs of culture, Brefeldin-A (1 µg/ml) was added to block cytokine secretion. Cells were stained as above, and then intracellular IFN-γ was detected with antibodies and a Cytofix/Cytoperm kit (BD) according to manufacturer's instructions. In some

cases surface and intracellular staining were performed simultaneously. Cells were fixed and analyzed, as stated above.

RNA isolation, reverse transcriptase PCR, and qualitative PCR of cDNA.

Single-cell suspensions, prepared as stated above, were enriched for CD45+ cells to at least 95% purity using a MoFlo FACS (Dako Cytomation). Total RNA was isolated from $1x10^5$ to $1x10^6$ of FACS-enriched and also directly *ex vivo* isolated leukocytes using a HighPure RNA isolation kit and treated with DNAse I, according to manufacturer's protocol (Roche). RNA content was quantified using a spectrophotometer and adjusted to desired final concentration. First-round cDNA reactions from 500 ng purified total RNA were primed with a mixture of oligo-d(T)VN (5'-T_{24}VN-3') and a cDNA primer specific to murine 18S rRNA (5'-TAATGATCCTTCCGCAGGTTC-3'). Briefly, 0.5 µg oligo-d(T)VN and 5 pmoles of 18S rRNA primer were added to RNA samples, and the primer/template mixes were heated to 70°C for 10 min to denature. The samples were then cooled to 42°C, and 20 µl reactions were created which contained 1X first strand buffer, 10mM DTT, 0.5 mM dNTPs, 40 U RNAsin, and 200 U Superscript II reverse transcriptase (Invitrogen). The first-round cDNA reactions were incubated for 1.5 hrs at 42°C. For each RNA sample, parallel control cDNA reactions were prepared in which the reverse transcriptase was omitted. After first-strand synthesis, the reactions were inactivated by incubation for 10 min at 70°C, then treated with 2 U RNAse H (Invitrogen) for 45 min at 37°C to degrade RNA.

The following specific primers and conditions were then used to perform separate qualitative PCRs using equal amounts of first-round cDNA as a template: 18S, forward: 5'-GATGGTTTAGTGAGGCCCTCGG-3', reverse: 5'-ACCTACGGAAACCTTGTTACGACTTTTA -3', 60°C annealing; Foxp3, forward: 5'-CAGCTGCCTACAGTGCCCCTA-3', reverse: 5'-CATTTGCCAGCAGTGAG-3', 57°C annealing, according to [28]; and CD8α, forward: 5'-AGGATGCTCTTGGCTCTTCC-3', reverse: 5'-TCACAGGCGAAGTCCAATCC-3', 60°C annealing, according to [29]. PCR amplifications were carried out separately in 50 µl reaction mixtures containing 2 µl cDNA and final concentrations of 2.5 U Jumpstart Taq (Sigma), 200 µM dNTPs, and 0.5 µM specific primer mixes, utilizing the following program: 2.5 min at 94°C denaturation followed by 34 cycles of 30 s at 94°C, 30 s at indicated annealing temperature, and 30 s at 72°C. 10 µl of each reaction and 1 kb DNA ladder were loaded on a 1.4% agarose-TBE gel, electrophoresed at 100 V for 30 min, and stained using ethidium bromide.

Suppression Assays.

Splenocytes ($1x10^7$/ml) from a chronically infected (Brazil strain) SJL (CD45.1+) mouse were labeled in PBS containing 2 µM carboxy-fluorescein diacetate succinimidyl ester (CFSE) for 4 min, followed by quenching in serum for 1 min and two washes with RPMI 10% FBS. $4x10^5$ CFSE-labeled responders were seeded into 96 well plates (0.2 ml) containing media alone or pre-coated plate-bound anti-CD3ε (145-2C11 clone, 30ug/ml, 37°C, 60 minutes) and co-cultured with or without indicated titrations of CD45+ cells sorted from muscle

tissue (“suppressors,” prepared as stated above) or titrated with splenocytes (“mock suppressors”) from chronically-infected C57BL/6 (CD45.2+) mice for approximately 72 hrs at 37°C, 5% CO_2. Cells were then stained for CD45.2, CD4 and CD8, and proliferation was assessed by analysis of CFSE-dilution on a Cyan flow cytometer.

Treatment of mice with antibodies.

The anti-IL-2 receptor α-chain (CD25) hybridomas 7D4 and PC61.5.3 (PC61) were grown in protein-free hybridoma media II (Invitrogen). Supernatants were routinely harvested, spun at 400 x g to remove cells and debris, 0.45 μm filtered, and stored for a short term at 4°C. To precipitate antibodies, an equal amount of ice-cold saturated ammonium sulfate solution was added to the hybridoma supernatant, mixed at 4°C with a stir-bar for 12 hrs, then centrifuged at 1500 x g for 30 min at 4°C. Protein pellets were resuspended and dialyzed until isotonic with PBS, 0.2 μm filter-sterilized, and -20°C-stored at/greater than 1.0 mg/ml until use. Mice were depleted of CD25+ T cells by intraperitoneal (i.p.) injections of 250 μg -1 mg purified anti-CD25 monoclonal antibodies on days specified (noted in figure legends). Flow cytometric assessment of PC61-mediated mAb depletion was conducted using FITC-conjugated anti-CD25 mAb (7D4 clone, BD Pharmingen), and assessment of 7D4-mediated mAb depletion was conducted using APC-conjugated anti-CD25 mAb (PC61 clone, Caltag).

Statistical analysis

A students T-test was used to determine statistical significance.

RESULTS

CD4+CD25+ T cells are detected in lymphoid and non-lymphoid tissues of chronically-infected mice.

Our laboratory previously demonstrated that the majority of freshly-derived effector-memory CD8+ T cells derived from the skeletal muscle tissue of chronically-infected mice were dysfunctional with regards to production of IFN-γ and cytolytic activity in response to polyclonal TCR stimulation [10]. To examine the hypothesis that CD4+CD25+ natural Treg play a role in this suppression of CD8+ T cell effector function in muscle tissue, I sought to determine if CD4+CD25+ T cells were also detectable in muscle tissue, as infiltration into inflamed tissues is a requirement for Treg-mediated suppressive activity [30], and the accumulation of Treg at sites of parasite persistence and lymphocyte dysfunction has been reported [19]. The frequencies of CD4+ T cells that co-expressed CD25 were determined in blood, muscle tissue, spleen, and lymph nodes of naïve and chronically infected mice. CD4+CD25+ T cells were detected in all tissues analyzed, except in naïve muscle tissue, as lymphocytes are rarely found in naïve/uninfected muscle (Figure 1 A-E). The frequencies of CD4+ T cells that expressed CD25 were comparable in the blood and spleen, which comprised 3-10% of CD4+ T cells, regardless of infection status (Figure 1 A, B). Interestingly, up to a 2X higher frequency of CD4+CD25+ T cells was often observed in chronic muscle compared to chronic spleen. The calculated ratio of the frequency of CD4+CD25+ T cells detected in the muscle: spleen was usually greater than “1,” indicating a relative enrichment of CD4+CD25+ T cells in the

muscle tissue (Figure 1 D-F). This merited further investigation into the phenotypic and functional characteristics of the CD4+CD25+ T cells detected in muscle tissue of chronically-infected animals.

Treatment of chronically-infected mice with anti-CD25 antibodies depletes CD4+CD25+ T cells with varying tissue-specific efficiency and does not increase the frequency of IFN-γ producing CD8+ T cells in lymphoid or non-lymphoid compartments.

In studies of chronic experimental infections, the administration of anti-CD25 monoclonal antibodies to deplete CD4+CD25+ T cells has been used to elucidate roles for Treg in different infections (for review see [31]). Since an enrichment of CD4+CD25+ T cells was often observed in the muscle of chronically infected mice, the original hypothesis that depletion of CD25+T cells would lead to enhanced muscle-resident CD8+ T cell effector function was tested. In one experiment (experiment #1), after delivering (9 x 500μg) i.p. injections of anti-CD25 (7D4 for 8 days, PC61 for 15 days) or PBS (control) evenly over a 23 day period to chronically-infected mice (n=2/group), peripheral blood circulating CD4+CD25+ T cell frequencies were reduced by 91% compared to PBS-treated control mice. After 23 days of depletion, frequencies of CD4+CD25+ T cells were determined in selected lymphoid and non-lymphoid tissues of both depleted and PBS-treated mice. Reductions of CD4+CD25+ T cells were observed in the spleen (-67%), lymph nodes (-76%), and muscle (-28%), as compared to the frequencies of CD4+CD25+ T cells in the matching tissues of control mice (Figure 2A). *In vitro* polyclonal stimulation of lymphocytes

obtained from the spleen, lymph nodes, and muscle tissue of chronically-infected depleted and mock-treated animals indicated no differences in the frequencies of IFN-γ producing cells in antibody-depleted mice as compared to mock-treated controls (data not shown).

In a similar subsequent trial (experiment #2), after administering (3 x 1 mg) i.p. injections of anti-CD25 (PC61) or PBS (control) evenly over a 6 day period to chronically-infected mice (n=2/group), peripheral blood circulating CD4+CD25+ T cell frequencies were only reduced by 69% in antibody-treated mice compared to control-treated chronic mice (Figure 2B). Although peripheral blood circulating CD4+CD25+ T cells were not reduced to the same extent as in experiment #1, similar reductions of CD4+CD25+ T cells were observed in the spleen (-69%), inguinal lymph node (-79%), and politeal lymph node (-67%), as compared to the frequencies of CD4+CD25+ T cells in the matching tissues of mock-treated control mice. Additionally, CD4+CD25+ T cells were reduced by 76% in the muscle tissue of depleted mice compared to mock-treated control mice (Figure 2B).

In vitro polyclonal stimulation of lymphocytes obtained from the spleen, lymph nodes, and muscle tissue of chronically-infected depleted and mock-treated animals from experiment #2 indicated no significant differences in the frequencies of IFN-γ producing cells in antibody-depleted mice as compared to mock-treated controls (Figure 3).

One caveat of anti-CD25 mediated *in vivo* depletion of CD4+CD25+ T cells in mice chronically-infected with *T. cruzi* is that depletion may occur with differing efficiencies in different tissues, and total depletion may not be possible in the desired target organ (muscle).

Expansion of scurfin-expressing CD4+ T cells occurs in lymphoid organs of chronically infected mice, but is lower in muscle compared to spleen.

Since expression of CD25 is not exclusively limited to CD4+CD25+ Treg, but is also expressed on activated lymphocytes, it is not a definitive phenotypic marker for Treg. Scurfin, a forkhead box protein transcription factor that is the product of the Fop3 gene, is a more exclusive marker for natural Treg [12, 32]. Since anti-scurfin antibodies suitable for flow cytometry applications have recently become available [33], the intracellular expression of scurfin was utilized as a marker for T cells that could have regulatory potential. Scurfin was detected in approximately 2% of total lymphocytes from the spleen, inguinal, and popliteal LN of both naïve and chronically-infected animals, indicating that there is not an overall increase in the frequency of scurfin-expressing cells as a function of chronic infection with *T. cruzi* compared to naïve mice (Figure 4A). In some cases there was up to a 2X increase in the frequency of scurfin-expressing CD4+ T cells in chronic inguinal and popliteal LN compared to naïve LN (Figure 4B, C). The majority of scurfin expression was observed within the CD4+ T cell subset (Figure 4C). Surprisingly, very little scurfin was detected in CD4+ T cells derived from chronically infected muscle (Figure 4B, C).

FoxP3 mRNA is not detected in leukocytes derived from chronically infected muscle tissue.

Since scurfin was virtually undetectable in lymphocytes isolated from chronic muscle (Figure 4), a more sensitive approach was employed to qualitatively monitor the expression of the Foxp3 gene, which has recently been described as a natural Treg lineage factor [34]. Foxp3 mRNA was detected in all spleen and lymph node-derived total RNA samples from chronic mice, as well as in naïve splenocytes (Figure 5). However, three independent experiments (experiment #1-3) revealed relatively low to undetectable levels FoxP3-specific product in the CD45+ FACS-enriched (95% purity) samples prepared from chronically-infected muscle in comparison to their splenic counterparts, which is in accordance with the finding of lack of scurfin expression in lymphocytes isolated from the muscle tissue of mice chronically-infected with *T. cruzi* (Figure 4A,B. Figure 5). CD8α mRNA and/or 18S rRNA were used as loading/handling controls, and were detected in all samples indicating the presence of intact lymphocyte RNA and template cDNA in the samples. However, in experiment #2, a relatively weak CD8α mRNA signal was detected within the muscle-derived CD45+ total RNA sample compared to its splenic counterpart, although a strong 18S rRNA signal from the same muscle-derived CD45+ total RNA sample indicated the presence of cellular RNA. In experiment #3, a Foxp3-specific PCR product was detected in the muscle-derived CD45+ sample, although it was a relatively weak signal compared to its splenic counterpart.

CD8+ T cells derived from lymphoid compartments sufficiently secrete IFN-γ in response to anti-CD3 stimulation.

Since a modest increase in the frequency of CD4+ T cells that expressed scurfin was observed in lymph nodes of chronically-infected mice compared to those of naïve mice, the ability of CD8+ T cells from the inguinal, popliteal, or mesenteric lymph nodes of chronically-infected mice to produce IFN-γ was investigated. When freshly-explanted whole lymph node preparations from chronically-infected mice were cultured overnight in the presence of plate-bound anti-CD3 (a polyclonal TCR activator), intracellular staining for IFN-γ indicated that CD8+ T cells from these lymph nodes were able to produce IFN-γ in frequencies comparable to, although slightly lower than, their splenic CD8+ T cell counterparts. In comparison, the frequencies of IFN-γ producing CD8+ T cells isolated from the muscle tissue of chronically-infected mice were below par in comparison to both splenic and lymph node-derived CD8+ T cells, in response to polyclonal anti-CD3 TCR stimulation (Figure 6). Thus, severe suppression of CD8+ T cell IFN-γ production does not appear to be occurring in the inguinal or popliteal lymph nodes of chronically infected mice.

Muscle-derived CD45+ cells do not suppress anti-CD3-mediated proliferation of chronic CD8+ splenocyte responders.

While the effector functions of CD8+ T cells resident in the muscle tissue of mice chronically infected with *T. cruzi* are apparently suppressed, the relatively low frequencies of Foxp3-expressing lymphocytes in the chronically-infected muscle tissue suggest that natural regulatory T cells do not play a role in the

observed muscle-resident CD8+ T cell dysfunction. In addition to the subset of CD4+ T cells known as natural Treg that express CD25+ and FoxP3, additional populations of Treg have been described, including IL-10-secreting CD4+ T cells that do not express Foxp3 [35] as well as CD8+ Treg [36], which can suppress proliferation in a contact-dependent fashion. To determine if cells with regulatory activity are present in chronically infected muscle tissue, an *in vitro* suppression assay was developed, based on a previously described method [23]. CD45+ cells from the muscle tissue of C57BL/6 mice chronically infected with *T. cruzi* were FACS-sorted to 95% purity, and titrated into *in vitro* cultures of CFSE-labeled splenocytes ("responders") from a congenically-disparate SJL mouse that was also chronically infected with *T. cruzi*. Cultures were incubated for 3.5 days in the presence or absence of plate-bound anti-CD3 polyclonal stimulation, and the frequencies of proliferating cells, based on the dilution of CFSE, were determined flow cytometrically. As a negative control, splenocytes from a C57BL6/J mouse chronically-infected with *T. cruzi* ("mock suppressors") were titrated into responder cultures in amounts equal to those of the muscle-derived CD45+ cells. Of the responders, 10% of CD8+ and 7% of CD4+ T cells proliferated when cultured in media alone, which increased to 95% and 71% proliferation, respectively, in response to anti-CD3 stimulation (Figure 7A, B).

The addition of either muscle-derived CD45+ cells (experimental) or splenocytes (mock suppressors) to CFSE-labeled responders did not result in the suppression of CD8+ or CD4+ responder T cell proliferation at any suppressor: responder ratio, indicating that cells with regulatory function cannot be detected in the muscle mice chronically-infected with *T. cruzi.*

DISCUSSION

The role of CD4+CD25+ Treg in modulating immune responses during chronic Chagas' disease was investigated in this study. The impetus for this investigation was based on a previous report that effector CD8+ T cell responses in muscle are suppressed relative to the spleen in mice with chronic *T. cruzi* infection [10]. The knowledge that CD4+CD25+ Treg can both suppress CD8+ T cells and modulate anti-microbial immune responses in various chronic infection models stimulated me to examine the role of Treg in the regulation of CD8+ T cell responses in muscle.

Since the migration of regulatory T cells into sites of inflammation is required for their suppressive activity [30, 37], it was hypothesized that if Treg were suppressing CD8+ T cell responses in chronically infected muscle tissue, then Treg should be detectable in this tissue. An enrichment of CD4+CD25+ T cells was observed in chronically-infected muscle tissue compared to the spleen. However, CD25 is not exclusively expressed by natural Treg, but is also transiently expressed by other lymphoid cells upon activation during autocrine IL-2 signaling.

The observed expression of CD25 by MHC class II-restricted CD4+ T cells derived from chronically-infected muscle tissue may indicate these CD4+ T cells are activated effector cells. Class II MHC is expressed in inflamed muscle [38], chronically *T. cruzi*-infected muscle [39], and on professional antigen presenting cells that are found in the inflamed muscle of *T. cruzi*-infected mammals [40]. Thus, MHC-class II+ antigen presenting cells capable of activating CD4+ T cells are found in chronically infected muscle tissue.

Apparent roles for Treg in allowing parasite escape from host immunity have been demonstrated in various acute and chronic infection models by depleting Treg with anti-CD25 mAbs (for review, see [31]). Depletion efficiency after antibody administration can be monitored in peripheral blood and in peripheral tissues, such as the skin or spleen. In our hands, while we could deplete greater than 90% of CD4+CD25+ T cells from the blood after treatment, we were unable to completely deplete CD4+CD25+ T cells from other tissues analyzed, especially muscle tissue. This confounded interpretation of the results, which indicated that anti-CD25 depletion failed to alter CD8+ T cell responses.

Recently, the expression of the FoxP3 gene (and its product, scurfin) has been identified as a well-defined marker for natural Treg. Scurfin and FoxP3 mRNA were detected only in very low levels in lymphocytes derived from chronically infected muscle tissue. Because FoxP3 expression is detectable at very low levels in the muscle tissue of chronically-infected mice, it is concluded, at least using the Brazil strain/B6 mouse model, that Foxp3+ T cells are not

suppressing CD8+ T cells, and are unlikely to account for the observed CD8+ T cell dysfunction in the muscle.

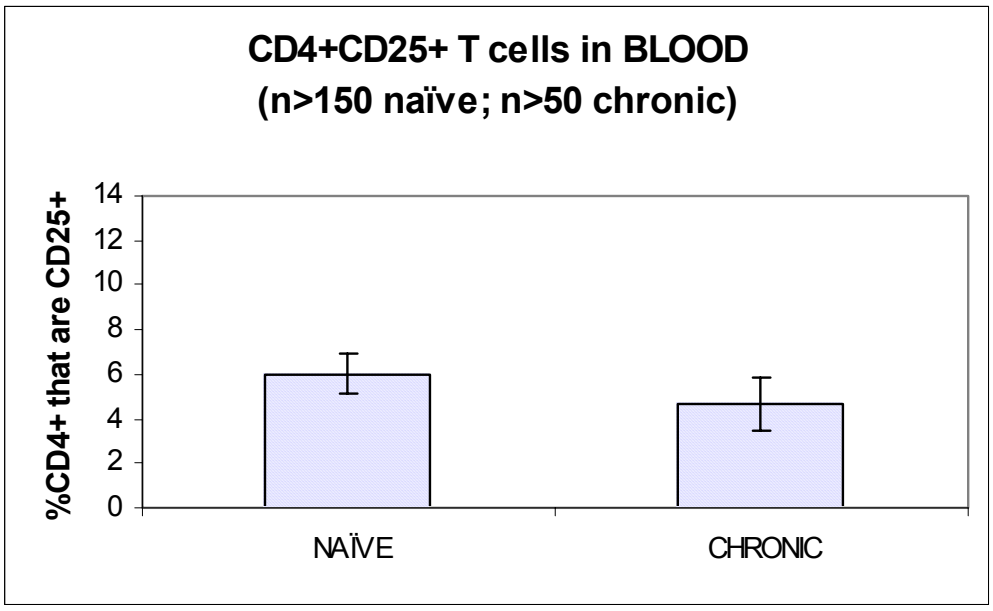
Despite a slight increase in the frequency of CD4+CD25+FoxP3+ Treg in the inguinal lymph nodes compared to the spleen, CD8+ T cells derived from these nodes of mice chronically infected with *T. cruzi* sufficiently responded to polyclonal anti-CD3 stimulation by producing amounts of IFN-γ comparable to splenocytes during overnight *in vitro* culture, indicating that suppression is not observed in these sites.

Based on these results, it is concluded that Foxp3+ “natural” Treg do not play a significant role in regulating CD8+ T cells responses that occur in chronically infected muscle, as their presence could not be detected in this particular site. We did not investigate other potential roles of natural Treg in different aspects of chronic Chagas’ disease in this study. The results reported here may indicate that future studies aimed at determining the causes of CD8+ T cell dysfunction and parasite persistence in the muscle of chronically-infected mice focus on possibilities other than natural CD4+CD25+ Treg, such as immunosuppressive cytokines (IL-10, TGF-β) or immunoregulatory pathways (e.g. PD-1/PD-L1).

Figure 1: CD4+CD25+ T cells are detected in peripheral tissues of naïve and chronically-infected mice. Blood and tissue lymphocytes were prepared as described in the methods section, stained with anti-CD4 and anti-CD25 antibodies, and analyzed on a Cyan flow cytometer (DAKO Cytomation). Average cumulative data is depicted with standard deviations. A-C) Calculated mean percentage of CD25+ expression among total CD4+ T cells in indicated tissues from naïve and chronically-infected mice. D) Pair-wise comparison of frequencies of CD25+ cells among CD4+ T cells between spleen and muscle of chronically infected mice. Significance ($p=0.001$) determined by a student's t-test. E) Muscle: Spleen ratio of frequencies of CD4+ cells that express CD25. F) Representative flow cytometry data from one experiment comparing frequencies of CD4+CD25+ T cells in spleen from one naïve mouse and spleen and muscle from one chronically-infected mouse. Numbers in upper right quadrant indicate percentage of CD4+ T cells that co-express CD25.

Figure 1

A


CD4+CD25+ T cells in BLOOD
(n>150 naïve; n>50 chronic)
%CD4+ that are CD25+
14
12
10
8
6
4
2
0
NAÏVE
CHRONIC

B

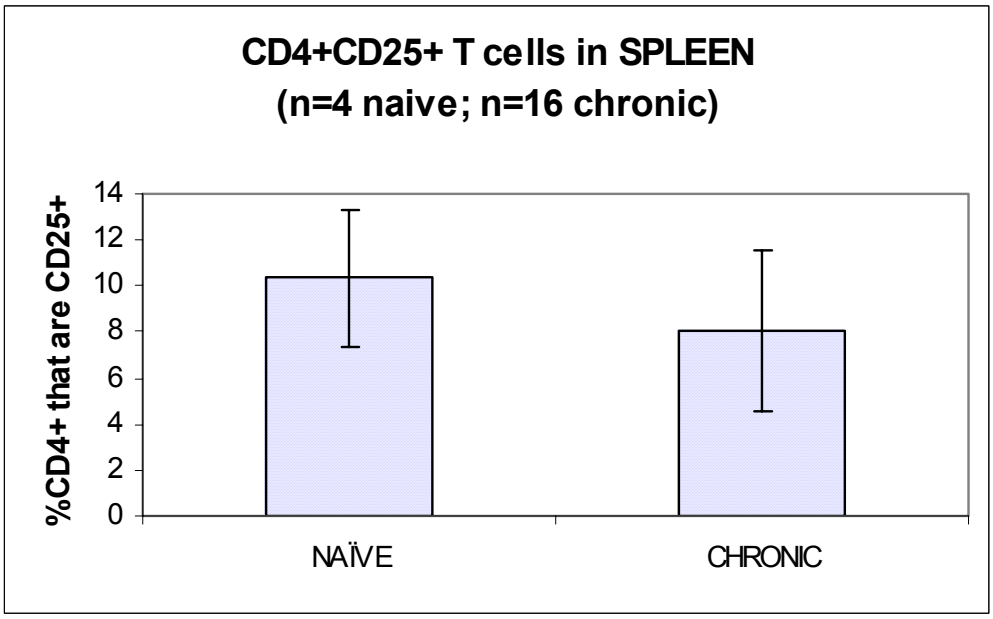
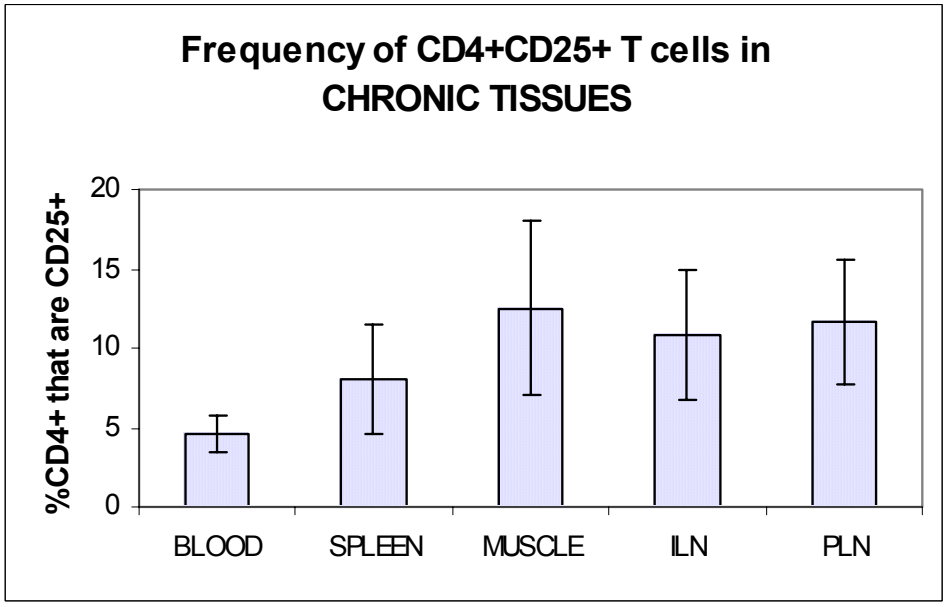

CD4+CD25+ T cells in SPLEEN
(n=4 naive; n=16 chronic)
%CD4+ that are CD25+
14
12
10
8
6
4
2
0
NAÏVE
CHRONIC

Figure 1

C


Frequency of CD4+CD25+ T cells in CHRONIC TISSUES
%CD4+ that are CD25+
20
15
10
5
0
BLOOD
SPLEEN
MUSCLE
ILN
PLN

D

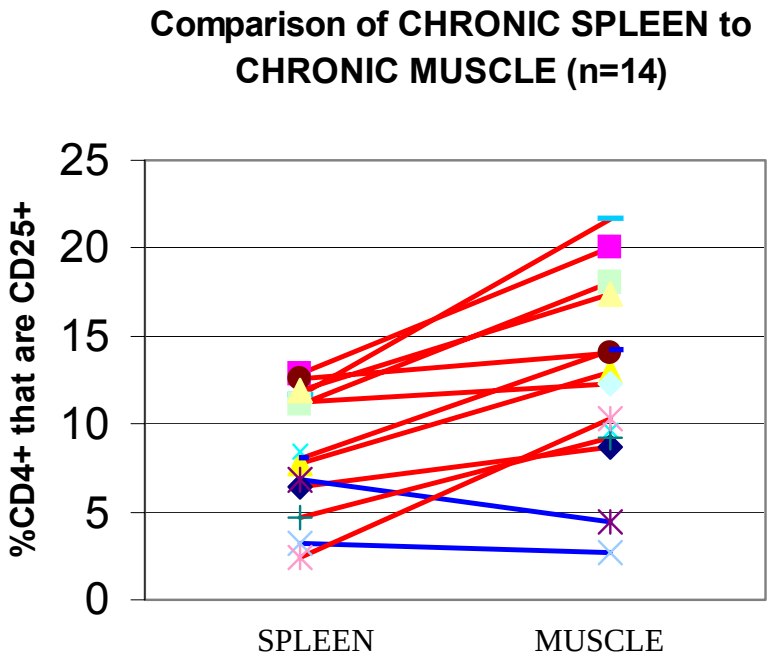
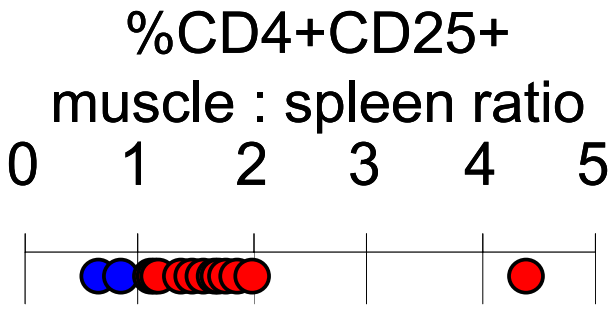

Comparison of CHRONIC SPLEEN to CHRONIC MUSCLE (n=14)
%CD4+ that are CD25+
25
20
15
10
5
0
SPLEEN
MUSCLE

Figure 1

E


%CD4+CD25+
muscle : spleen ratio
0 1 2 3 4 5

F

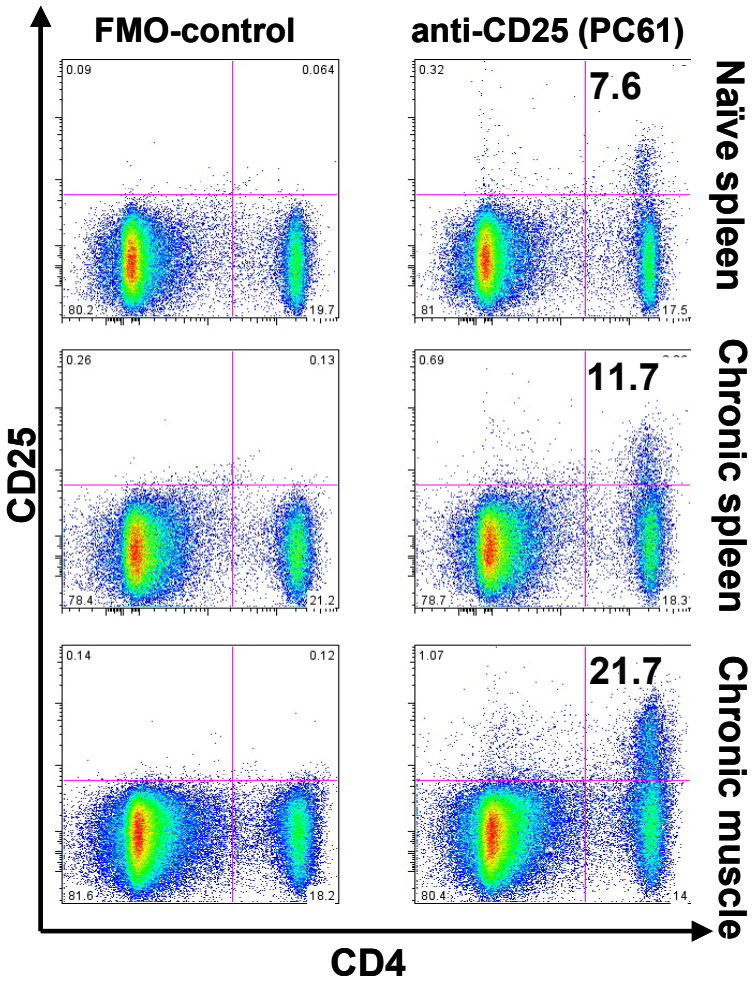
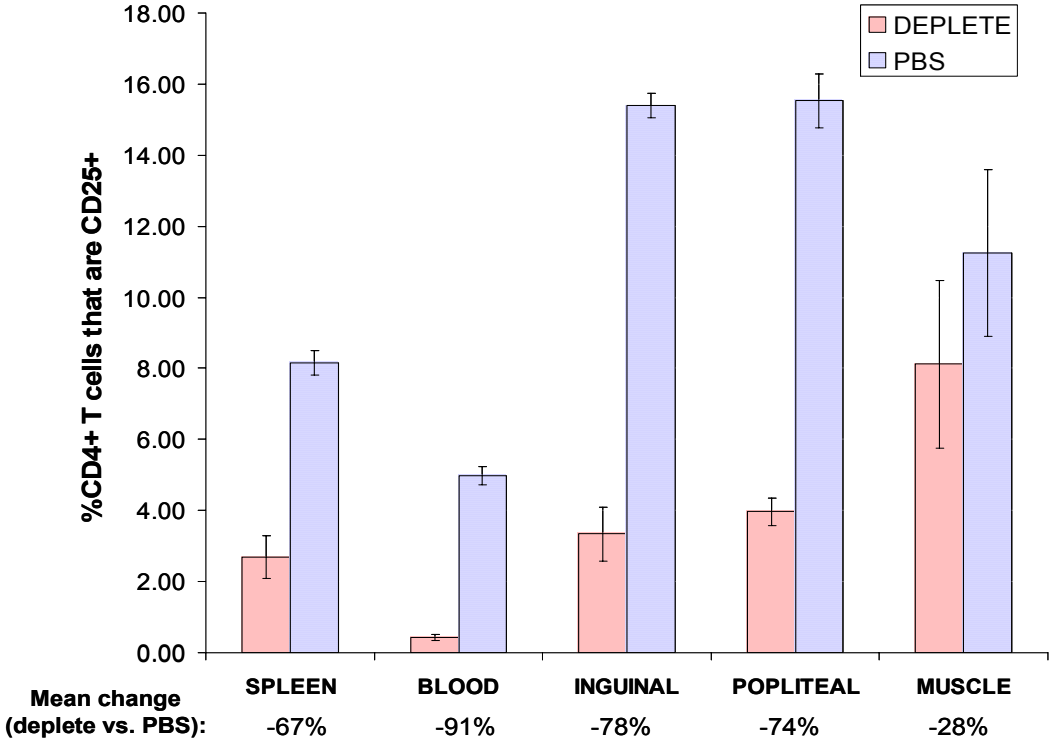

FMO-control
anti-CD25 (PC61)
Naïve spleen
Chronic spleen
Chronic muscle
CD25
CD4
0.09
0.064
80.2
19.7
0.32
7.6
81
17.5
0.26
0.13
78.4
21.2
0.69
11.7
78.7
18.3
0.14
0.12
81.6
18.2
1.07
21.7
80.4

Figure 2: **Treatment of chronically-infected mice with anti-CD25 monoclonal antibodies reduces frequencies of CD4+CD25+ T cells with varying tissue-specific efficiency.** Mice chronically-infected with Brazil strain *T. cruzi* were treated with anti-CD25 mAbs or mock-treated with PBS for indicated period of time. Lymphocytes were then isolated from the spleen, blood, lymph nodes, or muscle tissue, stained for extracellular expression of CD4 and CD25 (7D4 clone), and analyzed on a flow cytometer. Mean percent changes of CD4+CD25+ T cell frequencies, comparing the anti-CD25 treated (depleted) versus PBS control groups, are given below each graph. A) Experiment #1: Mice were treated with 9 x 400ug anti-CD25 mAbs (n=2) or PBS (n=2) over a 29 day period B) Experiment #2: C57BL/6 mice chronically infected with Brazil strain *T. cruzi* were given intraperitoneal injections of 1 mg depleting anti-CD25 mAbs (PC61 clone) 3 times spaced evenly over a 6 day period (n=2), or mock-treated with PBS as a control (n=2). In this experiment naïve mice were also treated with depleting mAbs (n=1) or mock-treated with PBS (n=1). Error bars represent standard deviation. In some cases samples from individual groups were pooled to obtain sufficient quantities of lymphocytes for analysis.

Figure 2

A


DEPLETE
PBS
%CD4+ T cells that are CD25+
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
SPLEEN
BLOOD
INGUINAL
POPLITEAL
MUSCLE
Mean change (deplete vs. PBS):
-67%
-91%
-78%
-74%
-28%

B

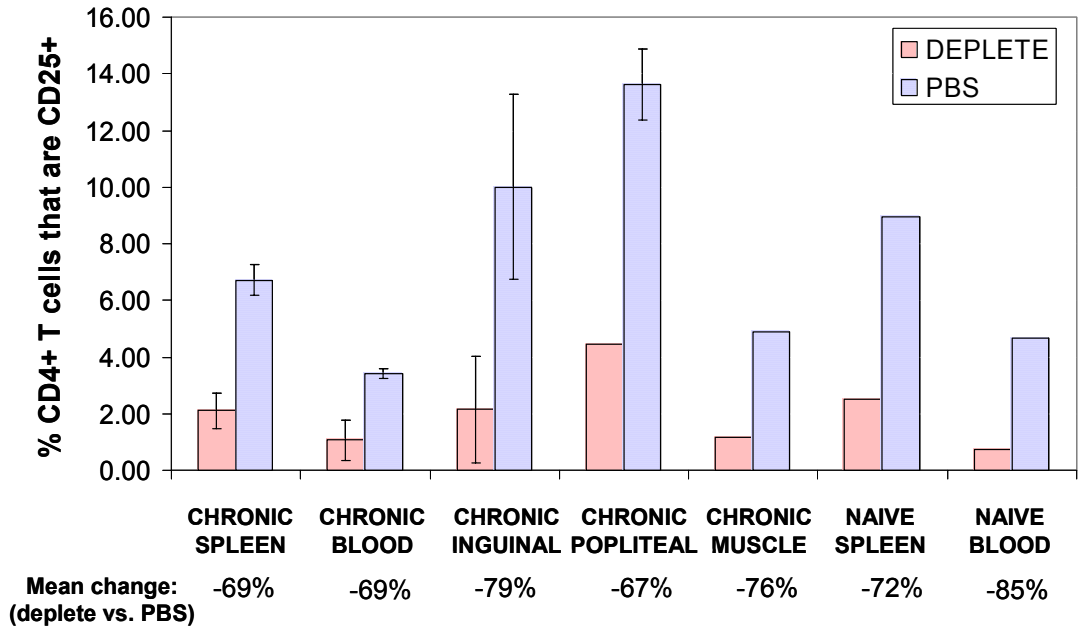

DEPLETE
PBS
% CD4+ T cells that are CD25+
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
CHRONIC SPLEEN
CHRONIC BLOOD
CHRONIC INGUINAL
CHRONIC POPLITEAL
CHRONIC MUSCLE
NAIVE SPLEEN
NAIVE BLOOD
Mean change: (deplete vs. PBS)
-69%
-69%
-79%
-67%
-76%
-72%
-85%

Figure 3: Administration of anti-CD25 mAbs to mice chronically infected with Brazil-strain *T. cruzi* does not increase the frequency of IFN-γ producing CD8+ T cells in lymphoid or non-lymphoid compartments. C57BL/6 mice chronically infected with Brazil strain *T. cruzi* were given intraperitoneal injections of 1 mg depleting anti-CD25 mAbs (PC61 clone) 3 times spaced evenly over a 7 day period (n=2), or mock-treated with PBS as a control (n=2). Single-cell suspensions were prepared from the spleen, inguinal lymph nodes, and popliteal lymph nodes; CD45+ cells from muscle tissue were FACS-enriched to >97% purity using a MoFlo cell sorter (Dako Cytomation) as described in methods section. Lymphocytes were cultured for 15 hours in the presence of plate-bound anti-CD3, PMA/ionophore, or in media alone. For the last 5 hours of culture, Brefeldin-A was added to block exocytosis. Extracellular CD8α and intracellular IFN-γ were detected using a Cytofix/Cytoperm kit and fluorescently-labeled antibodies (BD Pharmingen), and analyzed on a Cyan flow cytometer (Dako Cytomation). Error bars represent standard deviation. In some cases samples were pooled to obtain sufficient quantities of lymphocytes for analysis. Figure shows one of three representative experiments.

Figure 3:

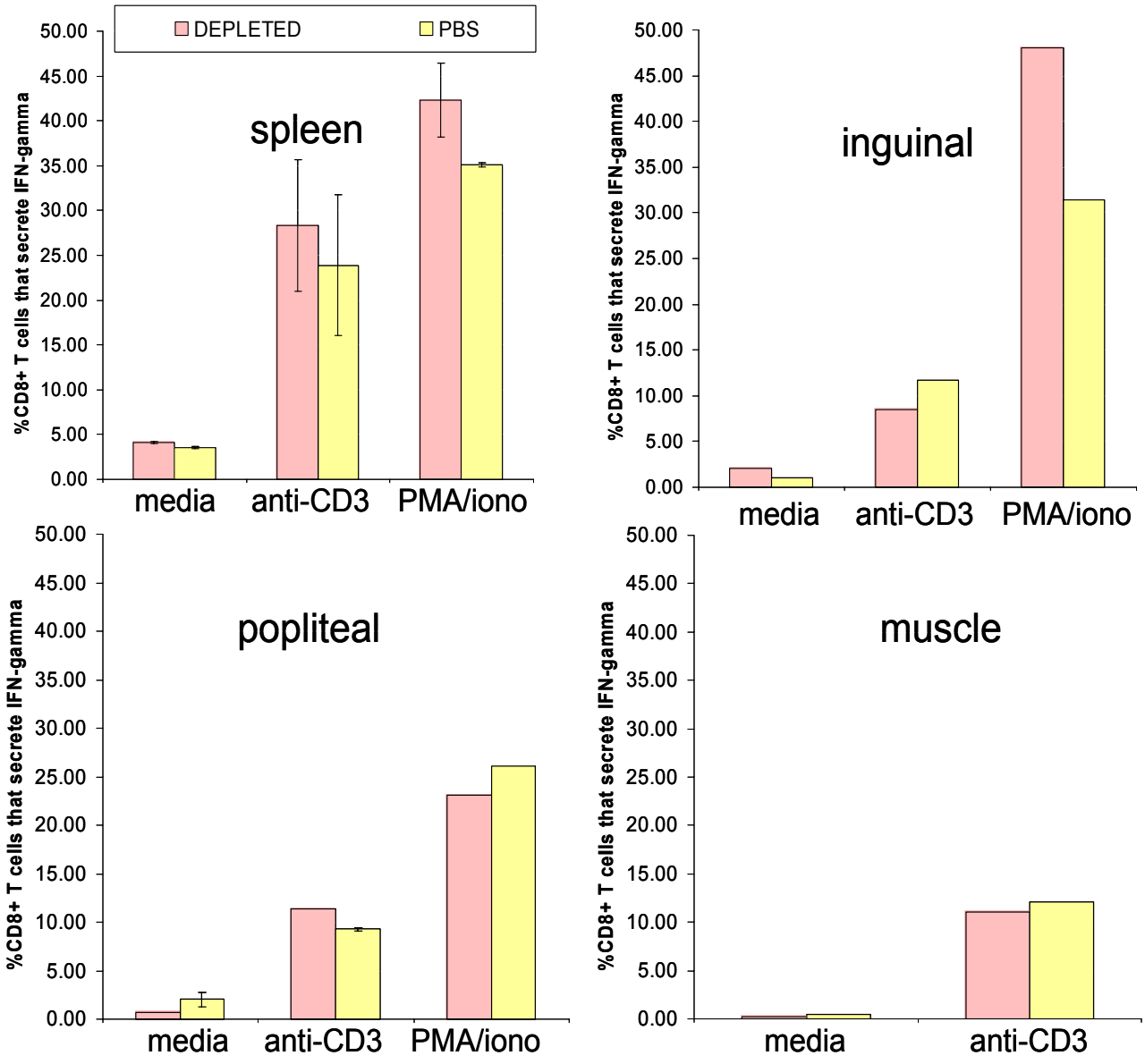

DEPLETED
PBS
spleen
inguinal
popliteal
muscle
%CD8+ T cells that secrete IFN-gamma
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
media
anti-CD3
PMA/iono

Figure 4: Analysis of scurfin expression. Lymphocytes were isolated from tissues as described elsewhere, and were stained for extracellular CD4 and intracellular scurfin using antibodies. Samples were fixed and analyzed on a flow cytometer. A) Representative flow cytometry data from one experiment depicting the frequency of scurfin-positive cells among total lymphocytes isolated from the spleen and indicated lymph nodes of a naïve and chronically-*T. cruzi*-infected mouse. B) Average cumulative data from two experiments representing the frequencies of CD4+ T cells that express scurfin in the indicated tissues of naïve and chronically-infected mice. Error bars represent standard deviation. C) Representative data from one experiment depicting the distribution of scurfin-expressing CD4+ T cells in indicated tissues from both a naïve and a chronically-infected mouse. Numbers in upper right quadrant depict percentages of CD4+ T cells that express scurfin.

Figure 4

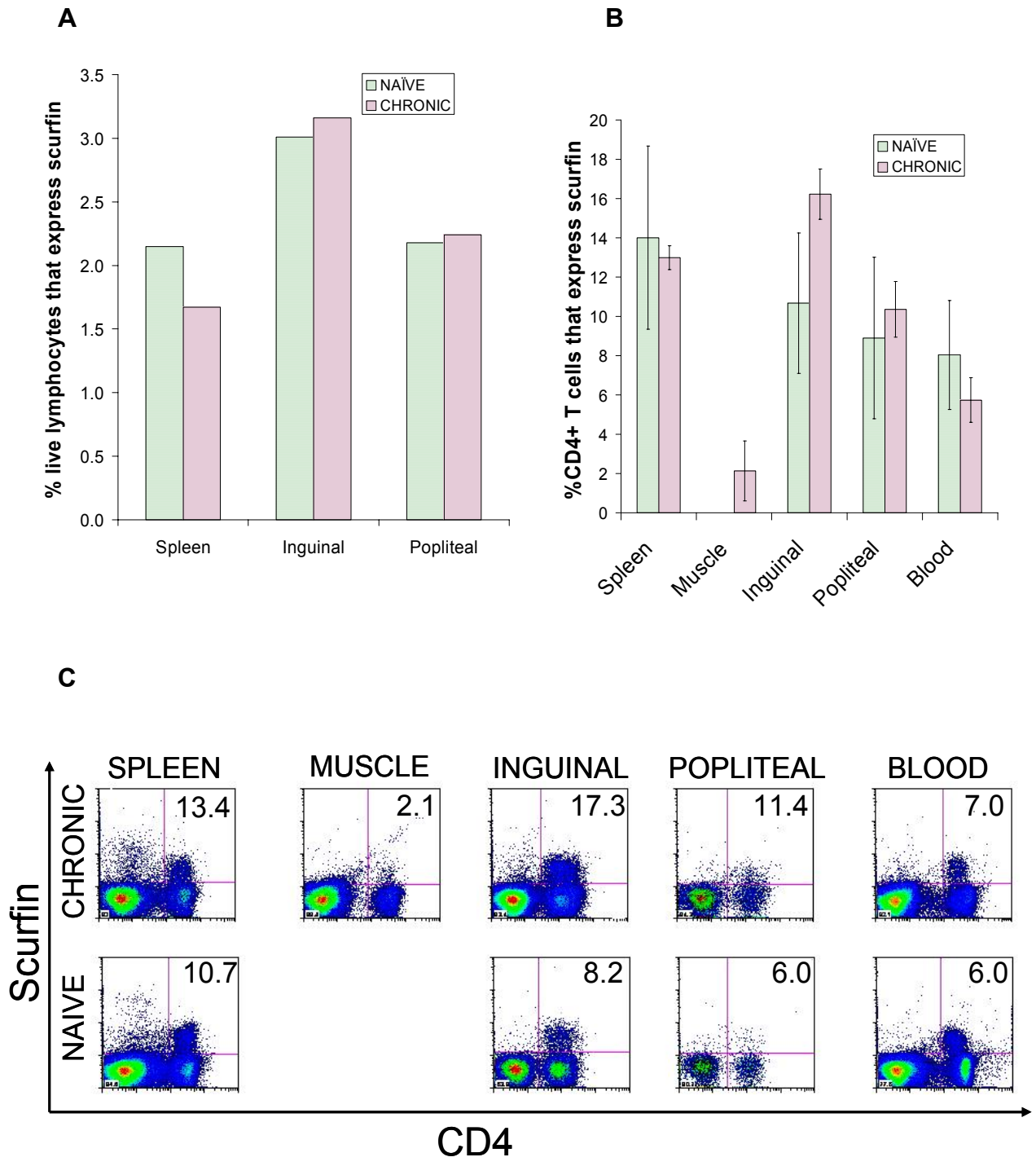

A
% live lymphocytes that express scurfin
NAÏVE
CHRONIC
Spleen
Inguinal
Popliteal
B
%CD4+ T cells that express scurfin
NAÏVE
CHRONIC
Spleen
Muscle
Inguinal
Popliteal
Blood
C
SPLEEN
MUSCLE
INGUINAL
POPLITEAL
BLOOD
CHRONIC
NAIVE
13.4
2.1
17.3
11.4
7.0
10.7
8.2
6.0
6.0
Scurfin
CD4

Figure 5: **Analysis of FoxP3 mRNA expression by reverse transcriptase PCR.** Total RNA was extracted from spleen, muscle, or lymph node-derived lymphocytes, as indicated. Utilizing oligo-d(T)VN and 18S rRNA primers, first round cDNA was reverse-transcribed using 500 ng total RNA of each sample. Subsequently, using first round cDNA as a template, standard PCR was performed using specific primers for FoxP3, CD8α (control), and 18S rRNA (control) cDNAs. PCR products were qualitatively analyzed by electrophoresing on a 1.4% agarose gel and staining with ethidium bromide. FoxP3, CD8α, and 18S rRNA cDNA from three similar independent experiments (Exp. 1-3) are depicted here. "Sp" and "Mu" refer to FACS-enriched CD45+ lymphocytes from the spleen and muscle, respectively, of mice chronically infected with Brazil-strain *T. cruzi*. "NSp" refers to splenocytes from a naïve mouse, while "CILN" and "CPLN" respectively refer to lymphocyte preparations from the inguinal and popliteal lymph nodes of one mouse chronically infected with Brazil-strain *T. cruzi*.

Figure 5

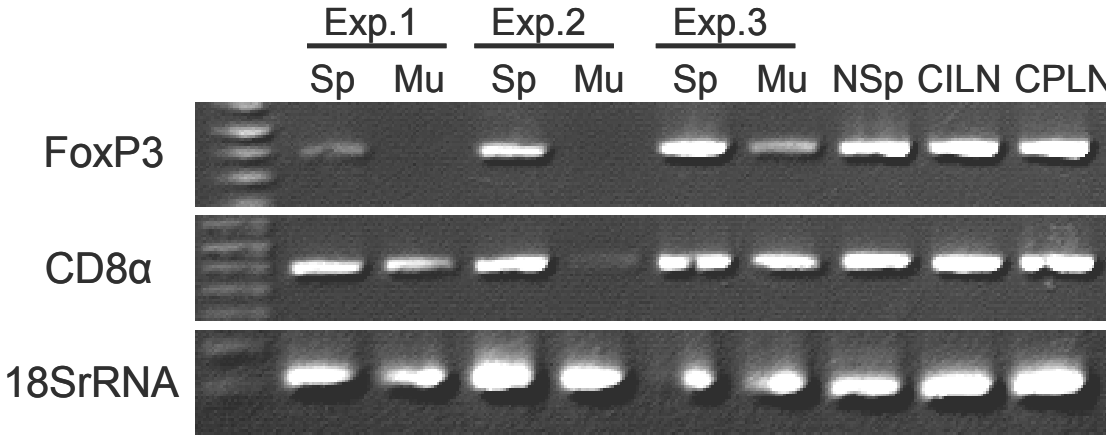

Exp.1
Exp.2
Exp.3
Sp
Mu
Sp
Mu
Sp
Mu
NSp
CILN
CPLN
FoxP3
CD8α
18SrRNA

Figure 6: **CD8+ T cells derived from lymphoid compartments of chronically-infected mice sufficiently secrete IFN-γ in response to anti-CD3 stimulation.** Lymphocytes were isolated from the spleen, lymph nodes, or muscle of mice chronically-infected with Brazil-strain *T. cruzi*, and incubated overnight in the presence of plate-bound anti-CD3 or media only. Brefeldin-A was added for the final 5 hours of culture. Extracellular CD8α and intracellular IFN-γ were detected using antibodies, and analyzed on a flow cytometer. This data represents mean cumulative data from four independent experiments depicting percentages of CD8+ T cells that express IFN-γ in response to anti-CD3 polyclonal stimulation. Error bars represent standard deviation. "ILN, PLN, and MLN" refer to inguinal, popliteal, and mesenteric lymph nodes, respectively.

Figure 6

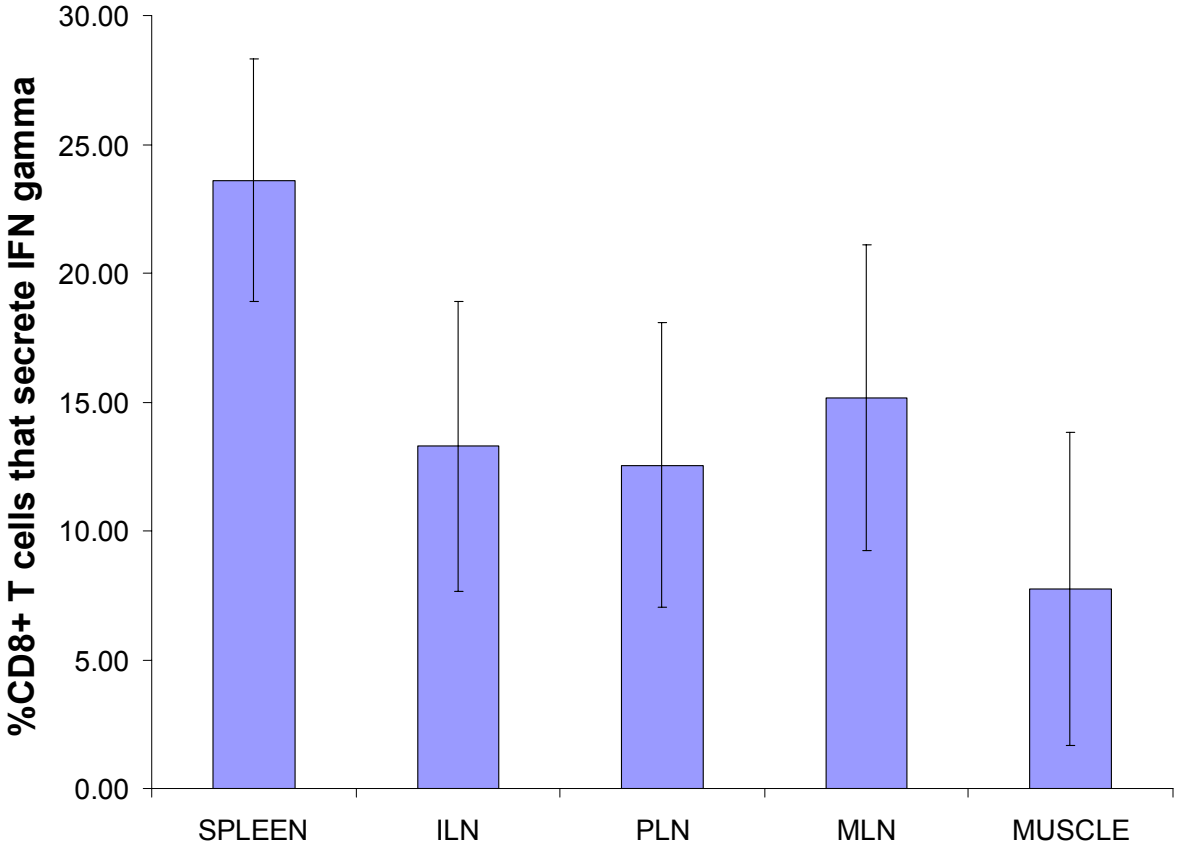
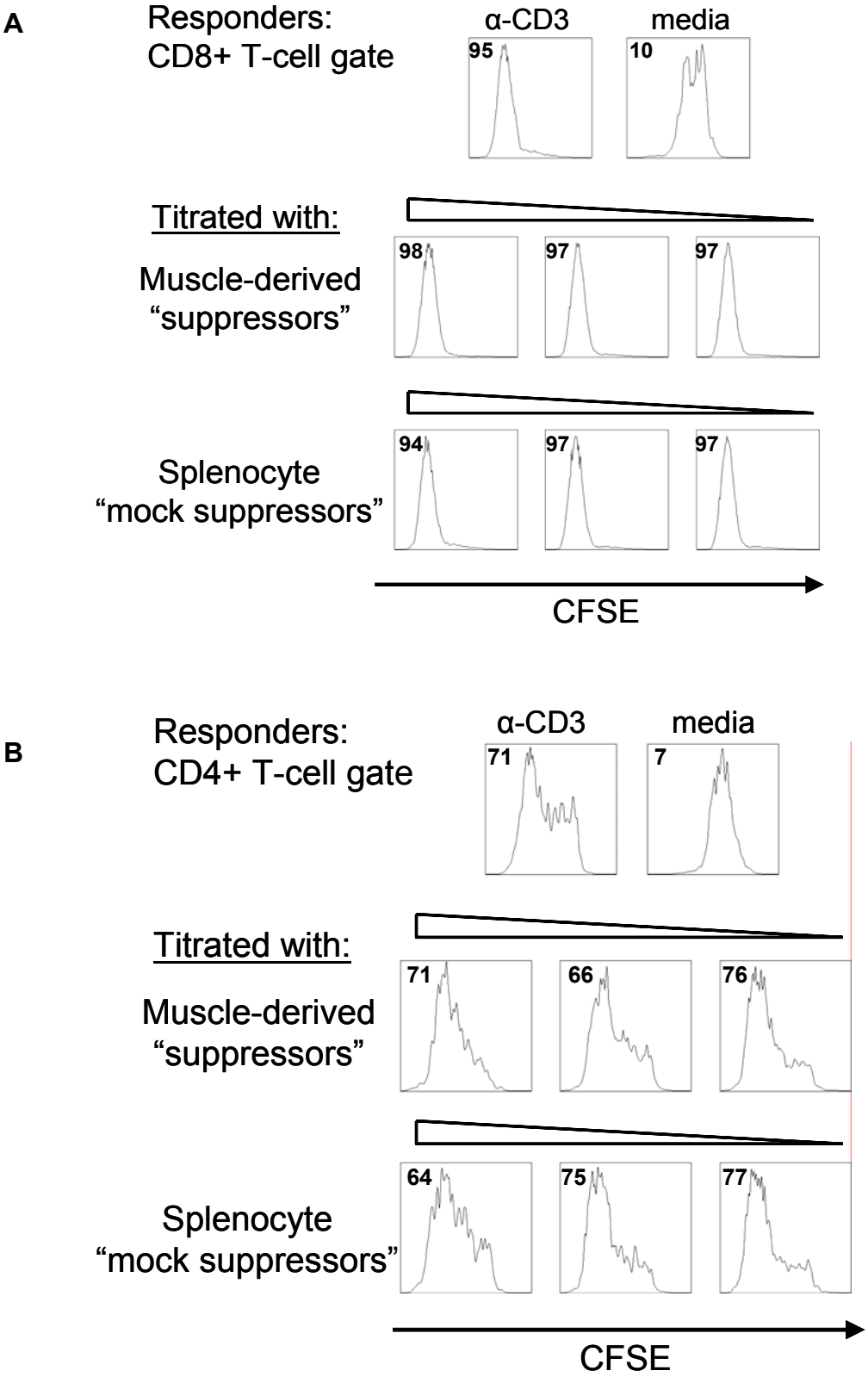

%CD8+ T cells that secrete IFN gamma
30.00
25.00
20.00
15.00
10.00
5.00
0.00
SPLEEN
ILN
PLN
MLN
MUSCLE

Figure 7: **Muscle-derived lymphocytes from chronically-infected mice do not suppress anti-CD3 mediated proliferation of CD8+ or CD4+ splenocytes from a chronically-infected mouse.** CD45+ cells from the pooled muscle tissue of 4 chronically-infected C57BL/6 (Ly5.1-) mice were sorted to >95% purity using a MoFlo FACS. Muscle-derived CD45+ cells ("experimental suppressors") or splenocytes ("mock suppressors") from chronically-infected Ly5.1- mice were titrated into responder cultures (using halving-dilutions to achieve a range of 1:1 to 1:32 suppressor: responder ratios). "Responders" consisted of $4x10^5$ CFSE (2 μM)-labeled splenocytes prepared from a congenically-disparate chronically-infected SJL (Ly5.1+) mouse. Responders were cultured for 80 hours in complete RPMI media the presence of anti-CD3 (positive control), in media alone (negative control), or in the presence of anti-CD3 with graded amounts of muscle-derived CD45+ cells (experiment) or splenocytes (mock). Cultures were stained with anti-CD45.2, anti-CD8, and anti-CD4 fluorescently-labeled antibodies. Proliferation was assessed in CD45.2-negative responders by the dilution of CFSE: Gates were arbitrarily set based on the $CFSE^{HIGH}$ parent peak from responders cultured in media alone such that proliferation was less than 10% of total gated cells in unstimulated cultures. Numbers in upper left indicate percentage of A) CD8+ T cells or B) CD4+ T cells divided past parent peak. Proliferation assays were conducted in triplicate. Representative data is shown here for single points reflecting suppressor: responder ratios of 1:1, 1:2, and 1:4 only.

Figure 7


A
Responders:
CD8+ T-cell gate
α-CD3
media
95
10
Titrated with:
Muscle-derived
"suppressors"
98
97
97
Splenocyte
"mock suppressors"
94
97
97
CFSE
B
Responders:
CD4+ T-cell gate
α-CD3
media
71
7
Titrated with:
Muscle-derived
"suppressors"
71
66
76
Splenocyte
"mock suppressors"
64
75
77
CFSE

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CHAPTER III

THE ROLE OF ENDOGENOUS REGULATORY T CELLS IN THE ACUTE IMMUNE RESPONSE TO EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION[1]

[1] Kotner, J.S. and Tarleton, R.L. To be submitted to *Infection and Immunity*.

ABSTRACT

The suppressive function of CD4+CD25+ regulatory T cells (Treg) to limit hyperactive immune responses can be exploited by microbes to evade host immune responses and establish infection. In humans, infection with the protozoan parasite *Trypanosoma cruzi* results in a robust and multi-faceted immune response that controls parasite load, but is unable to completely clear infection, resulting in parasite persistence and a chronic illness known as Chagas' disease. In this study, we show that Treg are not necessary for *T. cruzi* evasion of immune responses. *In vivo* depletion of Treg from naive C57BL/6 mice prior to challenge with the acutely lethal Tulahuen strain resulted in slightly increased antigen-specific CD8+ T cell expansion, but this did not improve the survival of depleted mice compared to controls. However, depletion of Treg prior to administering a DNA-based vaccine, followed by a lethal challenge, resulted in a slight improvement of vaccine efficacy versus vaccinated-only mice. Additionally, depletion of CD25+ cells prior to or during an acute infection with the non-lethal Brazil strain neither improved nor worsened the outcome of immune responses: Differences in parasitemia and kinetics of antigen-specific CD8+ T cell expansion were negligible between depleted and control groups. *In vivo* CTL responses to a subdominant epitope, as well as the *ex vivo* frequencies of antigen-specific interferon gamma-producing CD8+ splenocytes, were also of similar magnitude in both depleted and control groups.

Based on the results of these studies, it is concluded that CD4+CD25+ Treg do not a play a significant beneficial or detrimental role in modulating the primary immune response to experimental *T. cruzi* infection, although their potential to improve vaccine efficacy warrants further study.

INTRODUCTION

Naturally occurring CD4+CD25+ regulatory T cells (Treg) constitutively express IL-2Rα (CD25) and FoxP3, comprise 5-10% of human and murine peripheral CD4+ T cell pools, and serve to maintain immune homeostasis by preventing excessive immune responses such as autoimmunity (for review, see [1]). *In vitro* studies have shown that Treg can suppress proliferation and IFN-γ production of both CD4+ and CD8+ T cell subsets [2], and downregulate the activation and cytolytic ability of CD8+ T cells [3].

Treg are also implicated in the control of inflammation associated with infection. Treg may serve to limit immunopathological tissue damage, but may also allow parasite persistence by suppression of antimicrobial effector responses [4]. *In vivo* injection of anti-IL2Rα monoclonal antibodies depletes Treg, and thus abrogates their suppressive effects. This approach has been used to demonstrate a role for Treg in allowing *Leishmania* [5] and *Plasmodium* [6] parasites to escape from host immunity, as well as uncover Treg roles in establishing CD8+ T cell immunodominant responses [7] and improving vaccine efficacy [8] in response to microbial challenge.

Infection with *Trypanosoma cruzi*, the causative agent of Chagas' disease in humans, results in intracellular parasite replication by amastigote forms followed by host-cell rupture and dissemination of infective form trypomastigotes. As this iterative process occurs, the number of parasites circulating in the bloodstream increases, which allows for systemic infection and widespread tissue parasitism. Control of infection requires humoral as well as cell-mediated

immunity [9, 10] directed by a type 1 cytokine response [11]. While an optimal immune response controls parasitemia, sterile cure of infection is rare, and the resulting chronic infections often result in disease development due to lifelong infection. Various groups have reported different mechanisms of parasite escape from immunity, (for review, see [12-15]), although the ultimate causes of initial *T. cruzi* immune escape are unknown and debatable.

Here it is reported that in a rodent model from which CD25+ cells were depleted prior to parasite challenge that parasitemia, host survival, and *T. cruzi*-antigen-specific immune responses were not significantly different from non-depleted control mice. This suggests that CD4+CD25+ Treg are not required for *T. cruzi* immune evasion and parasite persistence.

MATERIAL AND METHODS

Mice, parasites, and infections.

C57BL/6 and C3H/HeSnJ mice were purchased from Jackson Labs and bred and maintained in the AAALAC-approved University of Georgia animal facility in micro-isolator cages. At either 7-10 weeks (depletion-only trials) or 14 weeks of age (depletion and vaccination trial), mice were infected intraperitoneally (i.p.) with blood form Tulahuen strain ($1x10^2$ to $5x10^3$, as indicated) or tissue culture-derived Brazil strain ($1x10^3$) trypomastigotes.

Blood form Tulahuen trypomastigotes were maintained via serial passage through C3H/HeSnJ mice and tissue culture-derived Brazil trypomastigotes were created by passage through Vero cell monolayers cultured in RPMI media supplemented with 10% fetal bovine serum (FBS).

Hybridomas and *in vivo* depletion of CD25 .

The anti-IL2Rα chain-specific B cell hybridomas (PC61.5.3 and 7D4) were grown in protein free hybridoma media II (Invitrogen, Carlsbad, CA). Supernatants were routinely harvested, centrifuged at 400 x g then 0.45 μm filtered to remove cells and debris, and stored for a short term at 4°C. To precipitate antibodies, an equal amount of ice-cold saturated ammonium sulfate solution was added to the hybridoma supernatant, mixed at 4°C with a stir bar for 12 hrs, and then centrifuged at 1500 x g for 30 min at 4°C. Protein pellets were resuspended and dialyzed until isotonic with PBS (pH=7.4), 0.2 μm filter-sterilized, and -20°C-stored at/greater than 1.0 mg/ml until use. Mice were depleted of CD25+ cells by i.p. injections of 250 μg-500 μg anti-CD25 monoclonal antibodies (mAb) at indicated times. Flow-cytometric assessment of PC61.5.3 mAb-mediated depletion was conducted using FITC-conjugated anti-CD25 (7D4 clone, BD Pharmingen) mAbs, and assessment of 7D4 mAb-mediated depletion was conducted using APC-conjugated anti-CD25 (PC61 clone, Caltag) mAbs.

Immunophenotyping, tetramer staining, and flow cytometry.

Blood was obtained via retro-orbital puncture using heparinized Natelson blood collection tubes (Fisher, Pittsburg, PA). For direct *ex vivo* staining, 25-100

µl of whole heparinized blood was first washed using PAB buffer [1X PBS, pH=7.4; 0.05% azide, and 1% BSA (w/v)] and centrifuged at 400 x g for 10 min (as for all additional washing steps). For MHC-tetramer staining, cells were first incubated for 30 min at 37°C with MHC class I ($H2K^b$) tetramers synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA) loaded with APL18 or APL20 peptides (for sequences, see Table 1), conjugated to the respective fluorophores, allophycocyanin (APC) or phycoerythrin (PE). Cells were then stained in 100-200 µl PAB containing anti-FcRγIII/II antibodies ("FcBlock," BD Pharmingen) and combinations of vendor-purchased fluorescently-labeled monoclonal antibodies, diluted 1:100, against the surface markers CD4, CD8, CD11b, CD25, and CD45R (BD Pharmingen, Caltag) for 20 min on ice in the dark, followed by 2 washes in PAB, and fixation in 2% formaldehyde for at least 15 min up to 20 hrs at 4°C. Whole blood was lysed in 500 ml lysis buffer (10mM HEPES, 0.83% ammonium chloride) for approximately 5 min at 25°C followed by two washes in PAB prior to fixation. A Cyan (Dako Cytomation) or FACS Calibur (BD) flow cytometer and FlowJo software (Tree Star, Inc., Ashland, OR) were used for cytometric analysis.

DNA vaccination.

4 days after i.p. administration of 400 µg of anti-CD25 (PC61) mAb to selected groups (n=5) of 8-week old female mice, the mice were vaccinated with 1.5 µg plasmid DNA comprised of a pool of *T. cruzi* genes ("Pool 4," see Table 2 for components) or multiple genes of the trans-sialidase family, each which contain the APL20 epitope (see Table 1 for amino acid sequence). Plasmid DNA

was coated onto 1.6 µm gold microcarriers (Bio-Rad Laboratories, Hercules, CA). Using a Helios Gene Gun (Bio-Rad), DNA was delivered to the dorsal auricular epidermis of ketamine-anesthetized mice. 2 weeks following the primary vaccination, mice were boosted in a similar fashion. 4 weeks following the boost, mice were infected with 5 x 10^3 Tulahuen strain blood-form trypomastigotes. As a control, one group of mice was not vaccinated, only infected.

***In vivo* cytotoxicity assay.**

Spleen cells from naïve mice were pulsed separately with 10µM cruzipain-9 (CRZP9) or APL20 peptides (for sequences, see table 2), or no peptide, for 1 hour at 37°C. Cells were washed twice in PBS, and labeled [1x10^7 cells/ml] with 5 µM, 1µM, or 0.2 µM (CRZP9, APL20, or unpulsed, respectively) of carboxy-fluorescein diacetate succinimidyl ester (CFSE) for 3 min at room temperature. CFSE labeling was quenched with an equal volume of cold FBS, and washed thrice in RPMI containing 10% FBS. The 5 µM (CRZP9), 1 µM (APL20), and 0.2 µM (unpulsed) CFSE-labeled cells were combined in equal quantities, and transferred intravenously to naïve and acutely infected CD25-depleted or control-treated mice. After 12 hrs recipient mice were sacrificed, single-cell suspensions were prepared from spleens and fixed. CFSE-labeled cells were detected using a Cyan flow cytometer and analyzed using FlowJo software. Percentage of specific killing for each peptide/condition combination was calculated using the formula: [1 – [(%$CFSE^{NO\ PEPTIDE}$ naive/%$CFSE^{PEPTIDE}$ naive)/(%$CFSE^{NO\ PEPTIDE}$ acute/%$CFSE^{PEPTIDE}$ acute)] x 100.

***In vitro* peptide stimulation of splenocytes and intracellular interferon gamma staining.**

Spleens were removed and sterile single cell suspensions were prepared by pressing between frosted slides. Red blood cells were lysed using sterile lysis buffer (10mM HEPES, 0.83% ammonium chloride) for 5 minutes at 25°C followed by filtering through a 70 μm nylon mesh screen, washing 2X with RPMI 1640 plus 10% FBS, centrifuging at 400 x g for 10 minutes between steps. $1x10^6$ RBC-depleted splenocytes were incubated for 5 hours in 96-well round-bottomed plates in a total volume of 0.2ml RPMI media plus 10% FBS containing a 1:1000 dilution of Golgi Plug (BD Pharmingen) and 2.5μM indicated peptide (see Table 1), PMA/ionophore ([50ng/ml]/[500ng/ml], respective) or media only. *In vitro*-cultured splenocytes were assayed for intracellular interferon gamma (IFN-γ) using the Cytofix/Cytoperm kit (BD Pharmingen) according to manufacturer's instructions. Briefly, FcRγIII/II were blocked using Fc-Block (anti-CD16, anti-CD32, BD Pharmingen) and cells were stained for surface expression of CD8 using PE-conjugated anti-CD8α (BD Pharmingen) for 30 minutes on ice. Cells were fixed using Cytofix/Cytoperm (BD Pharmingen) on ice for 15 minutes, washed 2X in PermWash (BD Pharmingen), and stained with anti-IFN-γ allphycocyanin (APC) for 20 minutes on ice in the dark. Cells were washed 2X and fixed in 2% formaldehyde for > 20 minutes, and then washed and resuspended in PAB for flow cytometric analysis on a Cyan flow cytometer.

Statistical methods. A student's T-test was used to determine statistical significance between appropriate groups.

RESULTS

Depletion of natural Treg prior to lethal challenge does not improve survival.

To examine whether CD4+CD25+ Treg contribute to the inability of C57BL/6 mice to survive a lethal parasite challenge, anti-CD25 monoclonal antibodies (mAbs) (7D4 clone) were administered intraperitoneally (i.p) to 6-week old female C57BL/6 mice both 7 days and 1 day prior to i.p. infection with either 10^2 or 10^3 Tulahuen (Tula) strain blood form trypomastigotes. Flow cytometric analysis of peripheral blood lymphocytes (PBLs), using an antibody (PC61 clone) that recognizes an epitope different than that of the depleting mAb, demonstrated that CD4+CD25+ T cells were reduced by over 90% compared to PBS-treated controls on the day of infection (Figure 1A, B). By day 6 post-infection, frequencies of CD4+CD25+ T cells were reduced by approximately 25% and 20% in depleted versus control mice (10^2 and 10^3 Tula-challenged groups, respective) (Figure 1B).

On day 15 post-infection, the frequency of circulating CD8+ T cells specific for the immunodominant *T. cruzi* epitope, APL20, was determined by MHC class I H2-K^b-APL20 tetramer staining. A modest (2X) increase occurred in the frequency of APL20-specific CD8+ T cells in the depleted group infected with 10^3 Tula compared to the control group ($p=0.013$). A non-significant increase was

also noted in the depleted group infected with 10^2 Tula compared to the control group (Figure 2). Regardless of the increase in APL20-specific CD8+ T cells in depleted compared to control groups, the groups exhibited similar mortality, according to the challenge dose (Figure 3A, B). The 10^2 Tula-infected groups (control and depleted) exhibited 100% mortality between days 24 and 28 post-infection (Figure 3A), with a single CD25-depleted mouse living until day 28. The 10^3 Tula-infected groups experienced 100% mortality between days 21 and 22 post-infection (Figure 3B). Similarly, there were no significant differences in parasitemias on day 16 post-infection between treated and control mice within each infectious dose group [10^2 Tula group: 8.55+/-4.77 (control) versus 7.20+/-4.59 (treated); 10^3 Tula group: 31.4+/-30.6 (control) versus 23.5+/-23.7 (treated). All values $x10^5$ parasites/ml of blood].

Depletion of natural Treg prior to vaccination may enhance vaccine-generated protection against a lethal challenge.

To examine the hypothesis that natural Treg regulate the vaccine-generated primary response against *T. cruzi* infection, nTreg were depleted prior to the start of a DNA vaccination prime-boost regimen employing either a mix of truncated *T. cruzi* trans-sialidase genes containing a common peptide (APL20) or a pool of *T. cruzi* genes empirically determined to be "partially protective" in response to a lethal parasite challenge ("Pool 4", see table 2 for components), followed by parasite challenge. Anti-CD25 mAbs (PC61 clone, 400 µg) were administered intraperitoneally to 8-week old naive mice 4 days prior to the priming vaccination. CD4+CD25+ T cells were depleted by 72% in treated mice

versus non-depleted control mice 2 days prior to vaccination (Figure 4A). 2 weeks after primary vaccination, mice were boosted in a similar manner. 12 days after boosting, low but detectable frequencies of CD8+ APL20-specific T cells were detected with MHC-Class I tetramers in the peripheral blood of both CD25-depleted and non-depleted control mice vaccinated with APL20. While the mean frequencies of APL20-specific cells in both groups were significantly elevated above the non-specific background of a naïve mouse (0.075%), mean frequencies of APL20-specific CD8+ T cells were similar between depleted and control mice (n=7, 0.432% and 0.408%, respectively). In order to determine whether depletion prior to vaccination improved the vaccine as measured by the ability to survive a lethal parasite challenge, mice were inoculated with a major lethal dose $5x10^3$ blood form trypomastigotes of the Tulahuen strain. The frequencies of APL20-specific CD8+ T cells were monitored on days 16 and 19 post-infection (Figure 5), and no significant differences were observed between the non-vaccinated, APL20 or pool 4-vaccinated, or depleted-then vaccinated groups. Non-vaccinated mice experienced 100% mortality by day 27 post-infection. While pool 4-vaccinated mice exhibited death-kinetics similar to non-vaccinated mice (i.e. the vaccine did not work), 40% of those mice first depleted of CD25 then vaccinated with Pool 4 survived beyond 40 days post-infection (Figure 6A). 20% of mice both vaccinated-only and depleted of CD25 prior to vaccination with APL20 survived beyond 40 days post-infection (Figure 6B).

Taken together, these results suggest that depletion of Treg prior to vaccination can lead to enhanced protection in some cases, specifically when pool 4 was used and seen ineffective on its own, but not in all cases. The protective effects of APL20 were, however, not enhanced by prior Treg depletion.

Depletion of natural Treg prior to or during non-lethal acute infection neither improves nor worsens the outcome of the immune response.

In some infectious disease models, depletion of CD4+CD25+ T cells prior to infection has demonstrated that Treg are necessary to allow parasite escape from the adaptive immune response [6]. One possible caveat in investigating the role of Treg in acute *T. cruzi* infection is that there is a relatively longer sub-patent period compared to other infections. To circumvent the possibility that CD4+CD25+ Treg, depleted prior to infection, are replenished during the early acute phase, administration of anti-CD25 mAbs prior to and throughout the acute phase of infection with the Brazil strain was employed. Using this approach, the frequencies of circulating CD4+CD25+ T cells could be depleted for a longer period, allowing for investigation of the effects of Treg reduction throughout the acute phase. Starting 2 days prior to infection, and on the day of infection, 400μg of anti-CD25 mAbs (PC61 clone) were administered intraperitoneally to two groups of mice ("deplete-infect"). Mice were then infected with $1x10^3$ Brazil strain tissue-culture trypomastigotes. As a control, another group of mice was not depleted of CD25, and was only infected ("infect"). To one group of mice that had been depleted with anti-CD25 mAbs prior to infection, additional 400ug

doses of anti-CD25 mAbs (PC61) were administered approximately every 4 days for one month during infection ("deplete-infect-deplete").

Compared to their respective pre-depletion frequencies, a reduction in the frequency of CD4+CD25+ T cells of approximately 90% was observed in both the "deplete-infect" and "deplete-infect-deplete" groups on the day of infection. The frequencies of CD4+CD25+ T cells in the "deplete-infect-delete" group remained reduced by at least 70%, compared to their pre-depletion CD4+CD25+ T cell frequencies, throughout the course of the experiment (Figure 7A). The kinetics of mouse weight can be used as a surrogate measure for the state of health [16]. In this experiment, no significant weight differences were noted between any of the infected mice, regardless of the administration of anti-CD25 depleting mAbs (Figure 7B).

Depletion of natural Treg prior to or during non-lethal acute infection does not modulate immunodominance hierarchies.

Previous studies have indicated that Treg can influence immunodominance hierarchies, as demonstrated by the increased magnitude of sub-dominant immune responses in the absence of Treg [7]. In order to determine if depletion of Treg would influence the immunodominance hierarchy of *T. cruzi*-specific CD8+ T cell responses, frequencies of peripheral blood-circulating CD8+ T cells specific for APL18 and APL20 epitopes were determined by class I MHC H2-k^b tetramer staining. The kinetics of the expansion and contraction of CD8+ T cells specific for the immunodominant APL20 epitope were very similar in all three infected groups, as measured on days 16, 20, and 28

post-infection (Figure 8A). Likewise, no significant differences were observed in the expansion of CD8+ T cells specific for the subdominant epitope APL18, as measured on days 16, 20, and 28 post-infection (Figure 8B). Thus, removal of Treg did appear to modulate the magnitude of the acute phase immune response as measured by clonal expansion of APL20 or APL18-specific CD8+ T cells.

During the acute phase of murine *T. cruzi* infection, CD8+ T cells kill target cells pulsed with the immunodominant peptide APL20 more efficiently than target cells pulsed with the sub-dominant peptide CRZP9, when equal quantities of each peptide-pulsed target cells are injected into the same animal (Martin and Tarleton, unpublished data). To investigate the role of Treg in modulating *T. cruzi*-specific cytotoxicity, an *in vivo* cytotoxic T lymphocyte (CTL) assay was performed to determine if the lytic activity of CD8+ T cells against CRZP9-pulsed target cells was increased when Treg were depleted during the acute immune response. The class I MHC H2-k^b –binding APL20 and CRZP9 *T. cruzi*-derived peptides were used to pulse spleen cells from naïve mice. After a 1 hour incubation with 2.5μM of either peptide, or with no peptide, these cells were labeled with 3 different concentrations of CFSE, mixed in equal amounts, and transferred to naïve, infected, deplete-infected, or deplete-infect-depleted mice. After 12 hours, splenocytes from each recipient mouse were analyzed for the presence of CFSE-labeled cells. Equivalent numbers of peptide-pulsed and unpulsed cells were recovered from the naïve mouse, as expected. In mice at 32 days post-infection, 96-97% killing of target cells pulsed with the immunodominant APL20 epitope was observed after 12 hours. In the same

mice, only 50-57% of target cells pulsed with the sub-dominant CRZP9 epitope were killed. 12 hours post-transfer, no significant differences were observed in the specific killing of APL20 or CRZP9-pulsed targets as a function of depletion of Treg (Figure 8C). Thus, Treg do not appear to suppress CD8+ T cell cytolytic activity directed against CRZP9.

To further investigate the effects of Treg depletion on the immune response of acutely-infected mice, the ability of CD8+ T cells to produce IFN-γ in response to a *T. cruzi* antigen-specific stimulus was assessed. IFN-γ production is crucial for control of *T. cruzi* infection [17] and in some infections is correlated with cytotoxicity [18]. Splenocytes from a naïve mouse and from infected, deplete-infect-depleted, and deplete-infected mice were cultured separately in the presence of single *T. cruzi*-derived immunogenic peptides (2.5 μM, each. See table 1 for sequences) and Brefeldin-A for 5 hours, then stained for intracellular IFN-γ production. Compared to the naïve and media-only controls, significant frequencies of CD8+ T cells from all infected groups produced IFN-γ in response to *T. cruzi*-derived peptides and PMA/ionomycin (Figure 8D). No significant differences were observed in the dominance hierarchies of antigen-specific CD8+ T cells as a function of depletion prior to or during infection.

DISCUSSION

Previous studies have demonstrated that CD8+ T cell-mediated primary immune responses are enhanced when CD4+CD25+ regulatory T cells are depleted prior to infection with *Plasmodium* [6] or vaccination then infection with HBV or HSV viruses [19, 20]. In this study similar experiments were performed

to determine the role of Treg in the acute immune response to *T. cruzi* infection. While depletion of Treg in the circulation prior a lethal parasite challenge did not enhance the survival of depleted versus non-depleted control animals, a modest difference in the magnitude of an antigen-specific CD8+ T cell clonal expansion was observed at a time of high parasitemia. Thus, while Treg may have played a role in modulating this immune response, *T. cruzi* has adopted a mechanism of immune evasion that is independent of Treg-mediated suppression of *T. cruzi*-specific immune responses.

Currently, no successful prophylactic Chagas' disease vaccine has been developed for clinical use, although some *in vivo* vaccine discovery trials have yielded protective vaccine targets [21-23]. Depletion of Treg can modulate the formation of a vaccine-induced primary immune response leading to an enhanced memory response [19, 20, 24]. To determine if depletion of Treg could improve the efficacy of a *T. cruzi*-DNA vaccine, Treg were depleted from the circulation prior to a prime-boost routine, followed by a lethal parasite challenge. In one trial using a vaccine candidate comprised of a pool of *T. cruzi* genes, depletion of Treg prior to vaccination resulted in an increased survival of mice after parasite challenge, compared to a non-depleted vaccinated-only control group. This indicates that Treg could be involved in the inhibition of T cell priming or memory formation during an immune response against *T. cruzi* antigens, and might be manipulated to improve the outcome of future vaccine discovery trials.

To further investigate the role of Treg in modulating various aspects of the acute immune response to *T. cruzi*, Treg were depleted prior to and during acute infection with a non-lethal dose/strain of parasite. In our hands, depletion of CD4+CD25+ had neither beneficial nor detrimental effects on the outcome of the acute immune response. Had Treg been suppressing the priming, activation, or effector function of CD8+ T cells during acute infection, it was hypothesized that their removal would lead to enhanced immunity, followed by decreased parasitemia and increased longevity. These outcomes were not observed. Conversely, if Treg were playing a beneficial role in the immune response by preventing immune hyperactivity, it was hypothesized that depletion of Treg would lead to earlier mortality, such as occurs during acute *T. cruzi* infection in the absence of the immunomodulatory cytokine IL-10 [25]. Early mortality or morbidity was not observed. Based on the parameters monitored during the course of infection in the aforementioned lethal and non-lethal parasite challenge models, no apparent roles were demonstrated for Treg as mechanism of parasite immune-evasion or as a critical modulator of hyperactive immune responses.

Table 1: Sequences of pertinent *T. cruzi*-derived peptides. APL18 and APL20 peptides were folded into class I MHC H2-k^b tetramers and fluorescently labeled with either allophycocyanin (APC) or phycoerythrin (PE), respectively, and used to monitor frequencies of CD8+ antigen-specific T cells during infection with *T. cruzi*. All peptides listed in this table were also used to stimulate splenocytes from acutely infected mice to measure frequencies of antigen-specific CD8+ T cells. "APL" signifies "altered peptide ligand" epitopes contained within trans-sialidase proteins. "GFT" denotes an epitope contained within a glucosyl-furanyl transferase protein, and "CRZP" denotes an epitope contained within a cruzipain protein.

Table 1

H2-K^b-binding peptide:	**Primary sequence**
APL18	ANYDFTLV
APL20	ANYKFTLV
APL60	LSHSFTLV
APL74	VNYDFTLV
APL80	ANYNFTLL
APL92	VGRPTTVV
GFT16	SVPIRLLVL
CRZP9	VPLNKCNRL

Table 2: Constituents of "Pool 4," a partially-protective DNA-based vaccine.

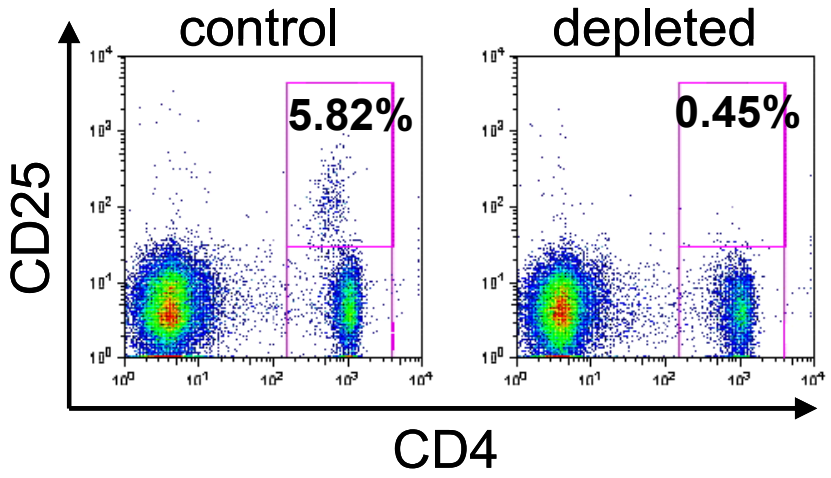
Table 2

Annotation:	**Gene ID:**	**Product Size:**
Tc cruzipain	Tc00.1047053507603.270	1416
AC113259.1.unk1	AC113259.1 (183 - 773)	591
AC113259.1.unk2	AC113259.1 (1453 - 1935)	483
putative ICAM-like molecule	AC093313.9 (109004 - 109288)	288
putative RAS family member	AC093313.9 (134185 - 134820)	636
similar to Tcc1j12.3 protein serine-alanine-and proline-rich protein	AC096945.2 (11389 - 12411)	1023
AC104502.1.199 probable protein	AC104502.1 (11866 - 12242)	377
possible cytomatrix protein	AC104500.1 (13564 - 14841)	1280
AC104500.1.unk4	AC103912.1 (98068 - 99249)	1182
AC104503.1.unk2	AC104503.1 (9969 - 10514)	549
Ankyrin Repeat Protein	AC105296.1 (55 - 654)	600
(PAR2) Paraflagellar Rod Protein	Tc00.1047053511215.119	1802
PAR1	Tc00.1047053506755.20	1787
PAR3	Tc00.1047053509617.20	1769
PAR4	Tc00.1047053510353.30	1731
LYT1	Tc00.1047053503829.50	1651
KMP11	Tc00.1047053510755.89	248

Figure 1: Depletion of CD4+CD25+ Treg prior to lethal challenge with Tulahuen strain parasites. Mice were given 400 ug anti-CD25 mAbs (7D4 clone) or PBS at 7 days and 1 day prior to infection, then infected with either 100 or 1000 Tulahuen strain parasites. To monitor frequencies of CD4+CD25+ T cells, blood samples were taken prior to depletion, immediately prior to infection after a week of depleting with anti-CD25 mAbs, and 6 days post-infection. Peripheral blood leukocytes (PBLs) were stained with CD4-PE and CD25 (PC61 clone)-APC, and analyzed on a Cyan (Dako Cytomation) flow cytometer. A) Representative flow cytometric analysis of PBLs obtained from one mouse of a PBS-treated group (control) and one mouse of an anti-CD25 mAb-treated group (depleted) immediately prior to infection. Numbers in upper-left corner represent the percentage of CD4+ T cells that express CD25. B) Cumulative data representing the mean frequencies of CD4+CD25+ T cells in control and depleted groups prior to depletion, immediately prior to infection, and 6 days after infection with either 100 or 1000 Tulahuen strain parasites. Each group (either control or depleted and then challenged with either 100 or 1000 parasites) consisted of 5 mice. Error bars represent standard deviation.

Figure 1

A


control
depleted
5.82%
0.45%
CD25
CD4

B

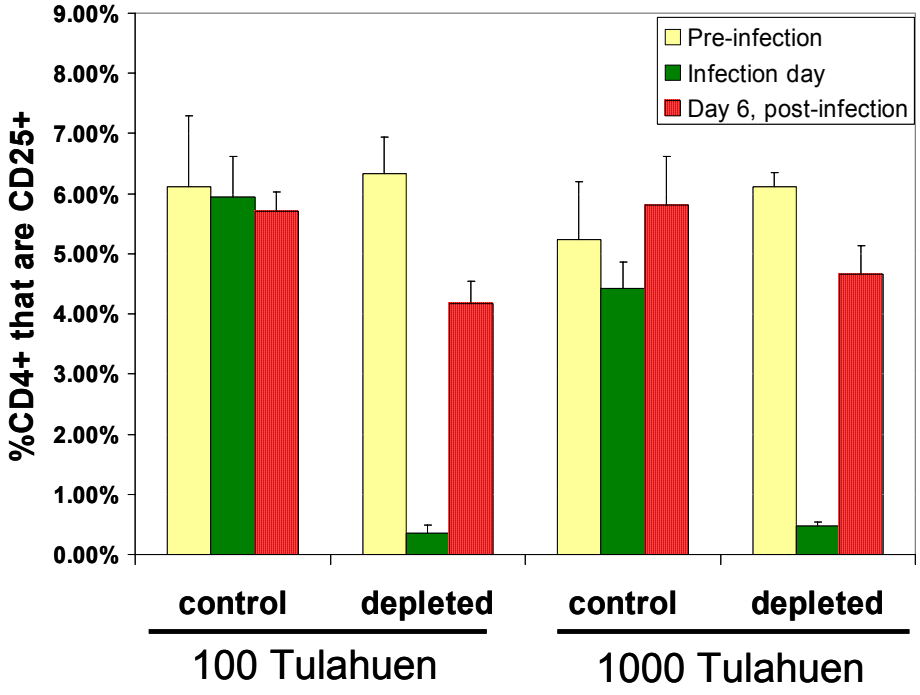

9.00%
8.00%
7.00%
6.00%
5.00%
4.00%
3.00%
2.00%
1.00%
0.00%
%CD4+ that are CD25+
Pre-infection
Infection day
Day 6, post-infection
control
depleted
control
depleted
100 Tulahuen
1000 Tulahuen

Figure 2: A 2X increase in the frequency of circulating APL20-specific CD8+ T cells occurs in mice treated with anti-CD25 mAb prior to infection with 1000 Tulahuen strain parasites. On day 15 post-infection, the frequency of APL20-specific CD8+ T cells was determined by staining PBLs with a PE-conjugated class I MHC H2-k^b tetramer loaded with APL20. Each bar represents 5 mice per group. A student's t-test revealed that there was a statistically-significant increase in the frequency of APL20-specific CD8+ T cells in the depleted, compared to the control group, of those mice infected with 1000 Tulahuen strain parasites ($p=0.013$). Error bars represent standard deviation. Each group consisted of 5 mice.

Figure 2

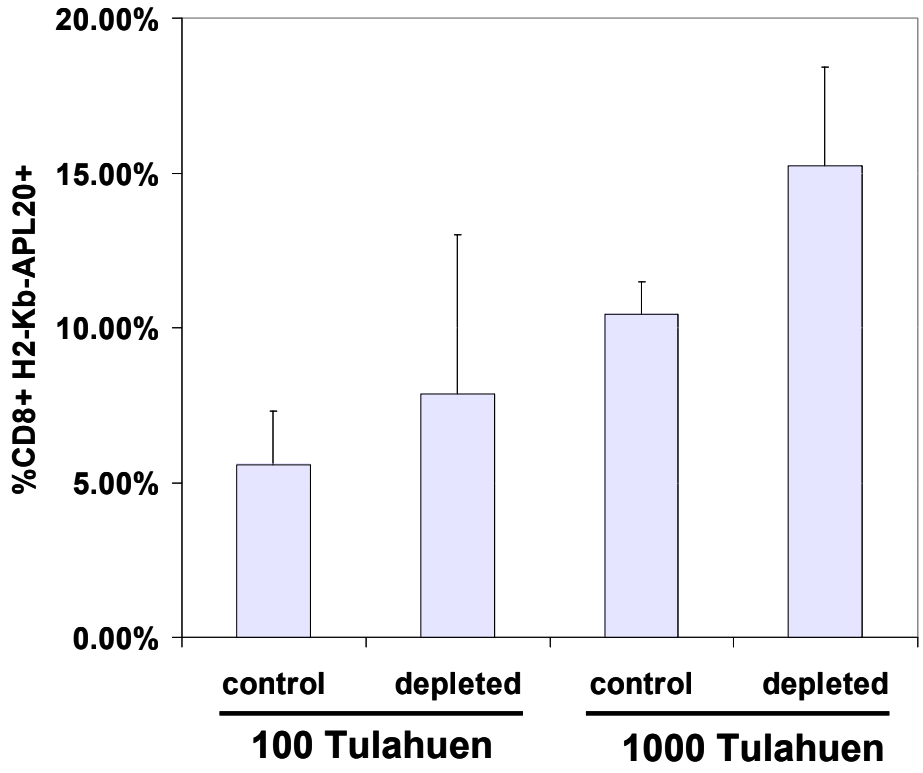
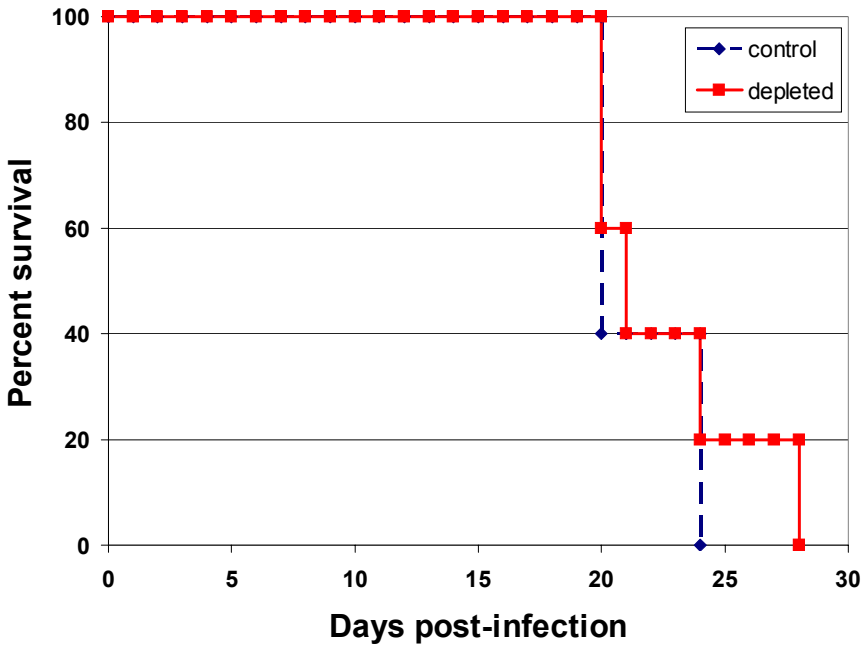

20.00%
15.00%
10.00%
5.00%
0.00%
%CD8+ H2-Kb-APL20+
control
depleted
control
depleted
100 Tulahuen
1000 Tulahuen

Figure 3: Depletion of CD4+CD25+ T cells prior to infection with the virulent Tulahuen strain of *T. cruzi* does not enhance survival in the face of a lethal parasite challenge. Mice were treated with anti-CD25 mAbs (red squares) or control-treated with PBS (blue diamonds), prior to infection with 100 (Figure 3A) or 1000 (Figure 3B) Tulahuen strain *T. cruzi* blood form trypomastigotes. Survival was monitored on a daily basis. Each group was comprised of 5 mice at the start of the experiment.

Figure 3

A


100
80
60
40
20
0
Percent survival
0
5
10
15
20
25
30
Days post-infection
control
depleted

B

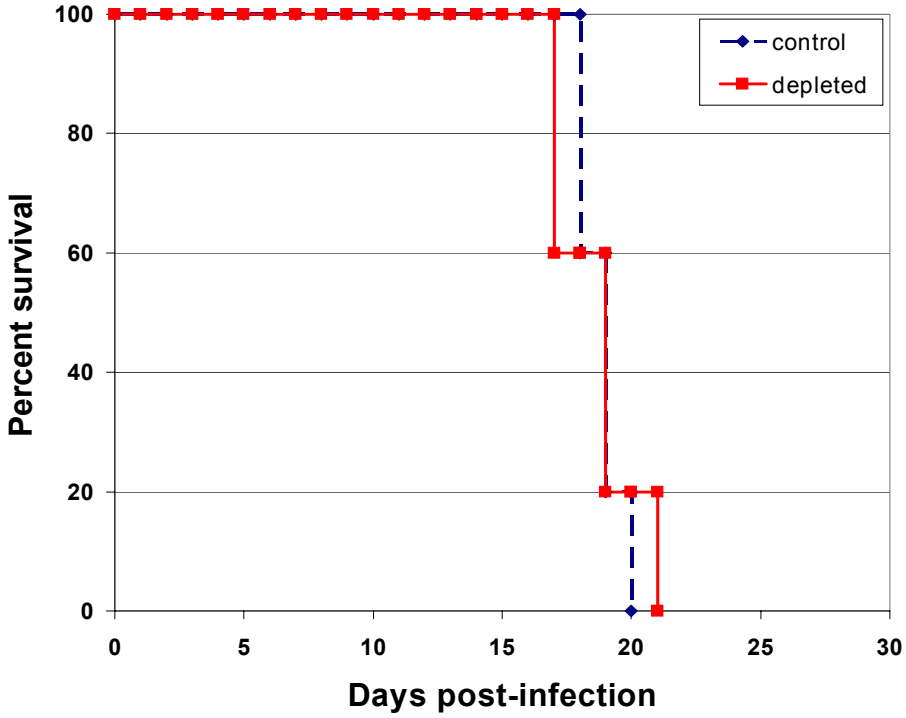

100
80
60
40
20
0
Percent survival
0
5
10
15
20
25
30
Days post-infection
control
depleted

Figure 4: Intraperitoneal depletion of CD4+CD25+ Treg prior to vaccination. Mice were injected with 400 µg anti-CD25 mAbs (PC61 clone) or PBS 4 days prior to vaccination with plasmid DNA encoding either APL20 or a pool of selected *T. cruzi* genes (see tables 1 and 2). To monitor frequencies of CD4+CD25+ T cells before vaccination, blood samples were taken prior to depletion (pre-depletion) and 2 days after administration with either PBS or anti-CD25 mAbs, which was 2 days prior to vaccination. PBLs were stained with CD4-PE and CD25 (7D4 clone)-FITC, and analyzed on a Cyan (Dako Cytomation) flow cytometer. "Pre-depletion" represents the cumulative mean frequency of CD4+CD25+ T cells in all groups prior to any treatment (n=25). "PBS" represents cumulative mean frequencies of CD4+CD25+ T cells in mock-treated mice destined for either non-vaccinated or vaccination only groups (n=15), whereas "anti-CD25" represents the cumulative mean frequencies of CD4+CD25+ T cells in those mice that were depleted and would then be vaccinated with either APL20 or pool 4 plasmid DNA (n=10). Error bars represent standard deviation.

Figure 4:

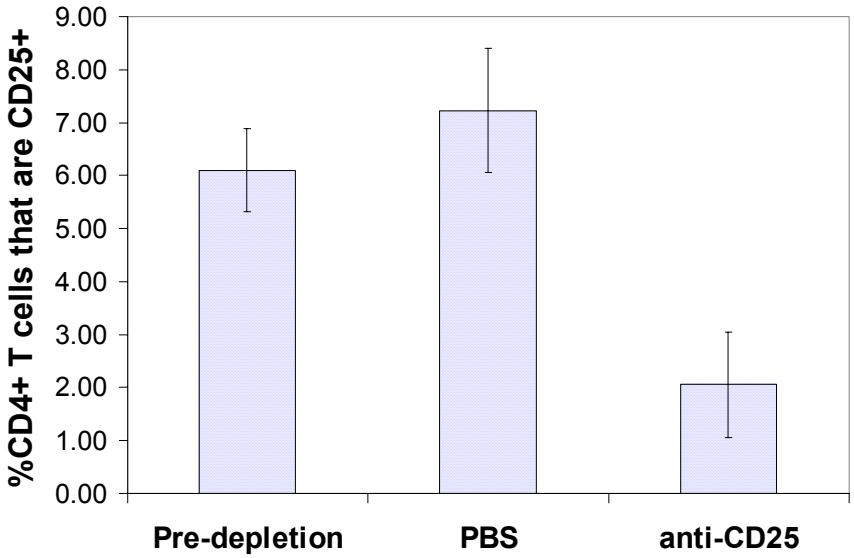

%CD4+ T cells that are CD25+
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Pre-depletion
PBS
anti-CD25

Figure 5: Anti-CD25 treatment prior to vaccination with plasmid DNA encoding APL20 does not lead to increased frequencies of APL20-specific CD8+ T cells during acute *T. cruzi* infection. 16 and 19 days post-inoculation with 5 x 10^3 Tulahuen strain *T. cruzi* blood form trypomastigotes, peripheral blood samples were taken from non-vaccinated (no vax), APL20-vaccinated (APL20) and anti-CD25 treated-then APL20-vaccinated mice (depl_APL20). PBLs were stained with CD8-APC and PE-labeled class I MHC H2-k^b tetramers loaded with APL20, fixed, and analyzed on a Cyan (Dako Cytomation) flow cytometer. On day 16 post-infection (light blue bars, left) each group consisted of 5 mice; "nd" signifies "no data." On day 19 post-infection (red bars, right), the "no vax" group consisted of 3 mice and the "APL20 vax" and "depl_APL20" groups each consisted of 5 mice. Error bars represent standard deviation.

Figure 5:

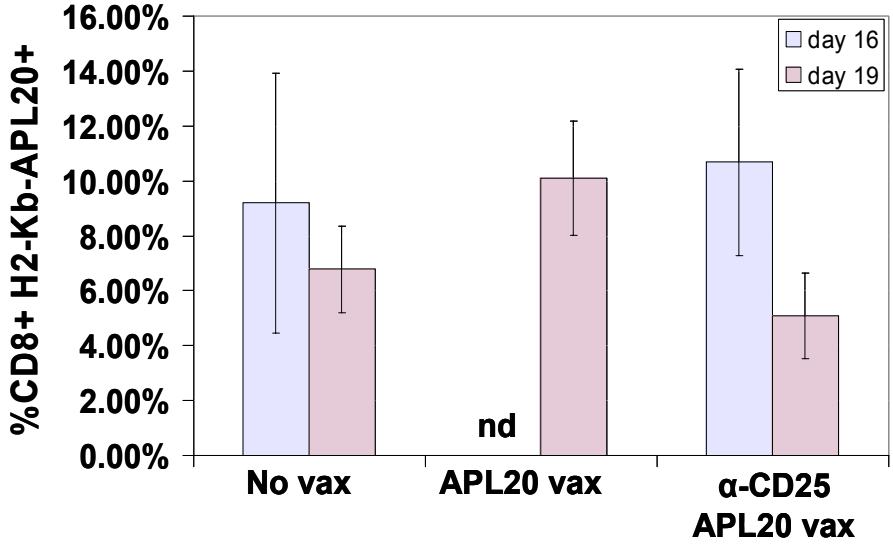
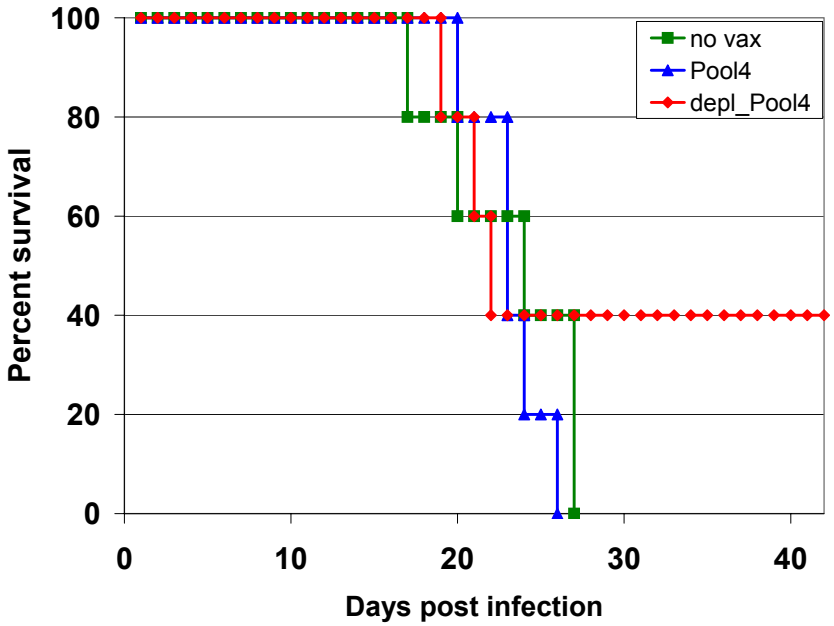

%CD8+ H2-Kb-APL20+
16.00%
14.00%
12.00%
10.00%
8.00%
6.00%
4.00%
2.00%
0.00%
day 16
day 19
nd
No vax
APL20 vax
α-CD25
APL20 vax

Figure 6: Depletion of CD4+CD25+ T cells prior to DNA vaccination can improve vaccine-generated protection following lethal *T. cruzi* challenge. Mice were treated with anti-CD25 mAbs (red diamonds) or control-treated with PBS (blue triangles), and vaccinated with pool 4 (6A) or APL20 (6B) plasmid DNA, or were not treated or vaccinated (green squares), prior to a challenge with 5000 Tulahuen strain blood form trypomastigotes. Survival was monitored on a daily basis. Each group was comprised of 5 mice at the start of the experiment.

Figure 6

A


100
80
60
40
20
0
Percent survival
0
10
20
30
40
Days post infection
no vax
Pool4
depl_Pool4

B

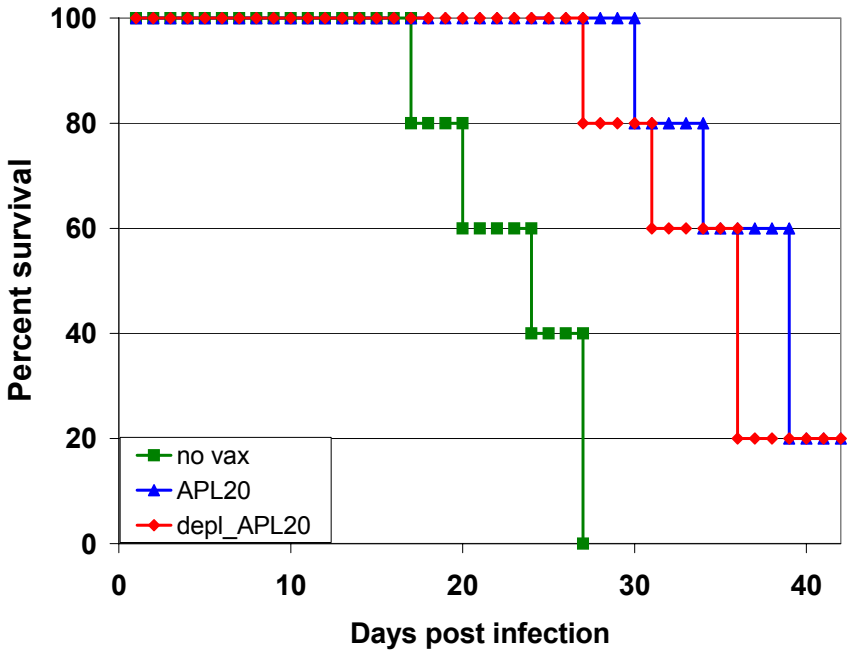
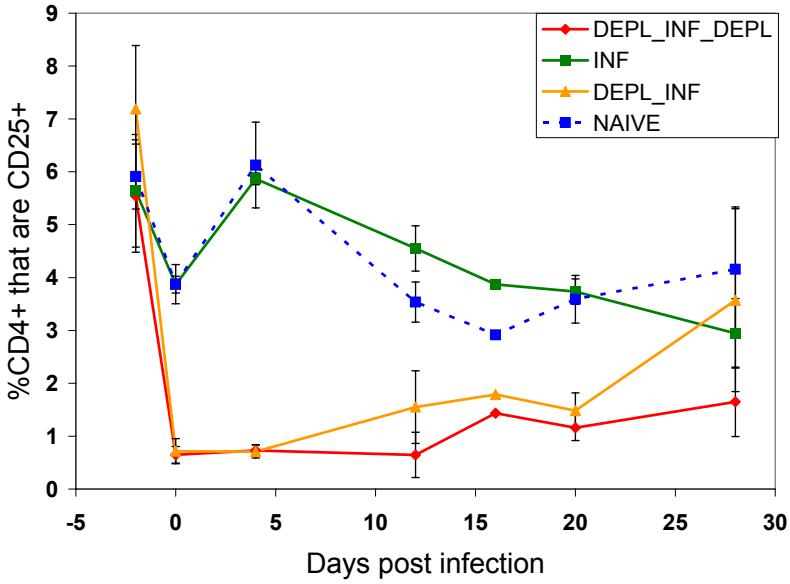

100
80
60
40
20
0
Percent survival
0
10
20
30
40
Days post infection
no vax
APL20
depl_APL20

Figure 7: Kinetics of CD4+CD25+ T cell frequencies and body weight during acute infection with Brazil strain *T. cruzi* in mice untreated or treated with anti-CD25 mAbs. 2 days prior to infection, and on the day of infection, 2 groups of mice (n=5, each) were i.p. injected with 400 µg anti-CD25 depleting mAbs (PC61 clone). As a control, one group of mice was left untreated (n=5). Mice were then inoculated i.p. with 1 x 10^3 Brazil strain tissue culture trypomastigotes. Additionally, 400 µg anti-CD25 mAb injections were continuously administered to one group of previously-treated mice ("depl_inf_depl," red diamonds, n=5) on days 7, 11, 14, 19, 24, and 29 post infection. After the day of infection, neither previously-treated mice ("depl_inf," orange triangle, n=5) nor untreated mice ("inf," green squares/solid line, n=5) received subsequent anti-CD25 mAb injections. As an additional control, one group of age-matched mice remained untreated and uninfected ("naïve," blue squares/broken line, n=3). 7A) To monitor frequencies of circulating CD4+CD25+ T cells, blood samples were obtained on days indicated. PBLs were stained with CD4-PE and CD25 (7D4 clone)-FITC, and analyzed on a Cyan (Dako Cytomation) flow cytometer. 7B) Body weights were measured on days indicated. For both graphs, error bars represent standard deviation.

Figure 7

A


%CD4+ that are CD25+
Days post infection
DEPL_INF_DEPL
INF
DEPL_INF
NAIVE

B

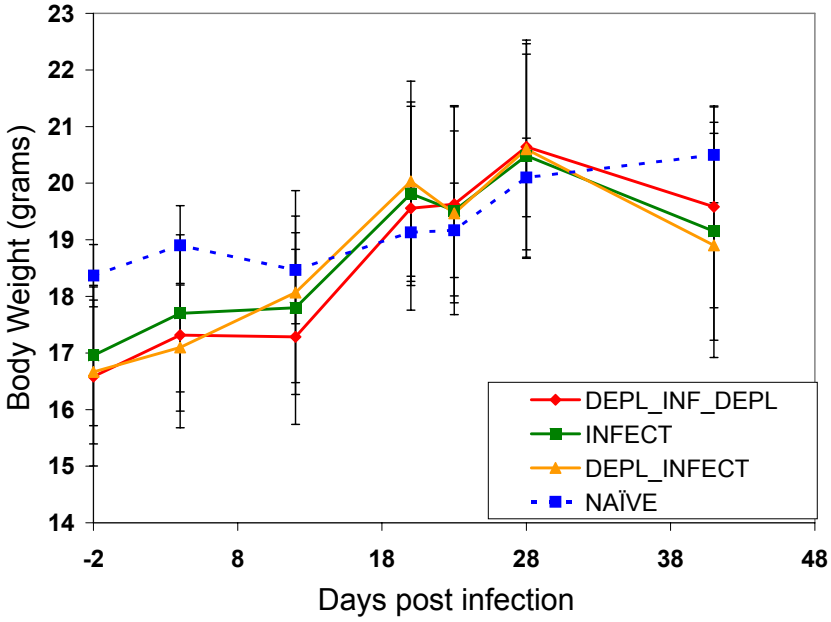
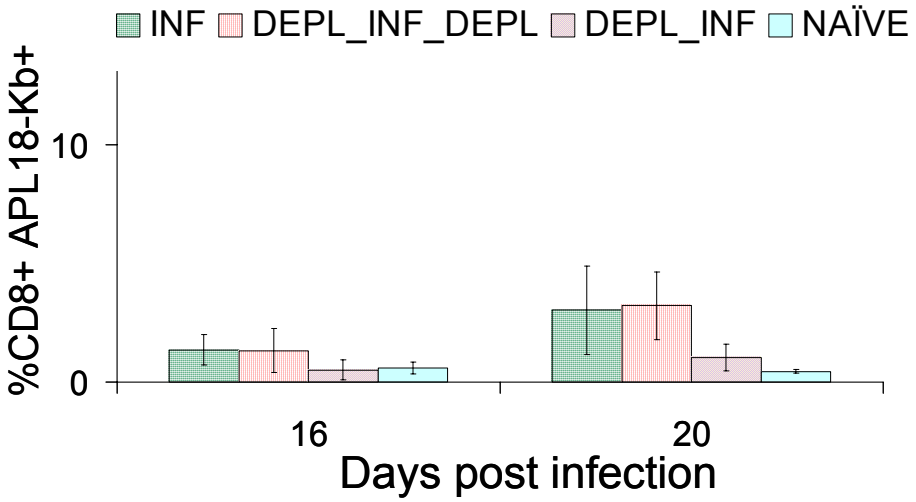

Body Weight (grams)
Days post infection
DEPL_INF_DEPL
INFECT
DEPL_INFECT
NAÏVE

Figure 8: Depletion of CD4+CD25+ T cells prior to or during non-lethal acute infection with Brazil strain *T. cruzi* has no effect on the magnitude of antigen-specific immune responses. 16, 20, and 28 days post-inoculation with 1 x 10^3 Brazil strain *T. cruzi* tissue culture trypomastigotes, peripheral blood samples were taken from mice depleted only prior to infection ("depl_inf," orange triangles), depleted both prior to and during infection ("depl_inf_depl," red diamonds), infected only ("inf," solid green squares), and non-infected ("naïve," open blue squares). PBLs were simultaneously stained with anti-CD8α FITC-conjugated antibodies and class I MHC H2-k^b tetramers loaded with either APL20 (n=7) (8A) or APL18 (n=7) (8B), conjugated to the fluorophores PE or APC, respectively. Naïve mice were included to determine non-specific background of tetramer-staining (n=3). PBLs were fixed and analyzed on a Cyan (Dako Cytomation) flow cytometer. Error bars represent standard deviation. 8C) 32 days after infection, an *in vivo* CTL assay was performed. Spleen cells from naive B6 mice were separately pulsed with APL20 or CRZP9 peptides, or no peptide ("unpulsed"), then labeled with CFSE, and adoptively transferred to one mouse of each indicated group, as described in the material and methods section. Splenocytes were recovered from naïve and acutely-infected recipient mice 12 hours post-transfer, fixed, and analyzed using a flow cytometer (Dako Cytomation). Numbers at the lower left and upper right indicate specific killing of APL20 and CRZP9-pulsed target cells, respectively. 8D) Splenocytes from naïve, infected only, deplete-infect-depleted, and deplete-infected mice (n=1, each) 32 days post-infection were cultured in the presence of indicated peptide (see Table 1 for sequences), media only ("no stim," negative control), or PMA/ionomycin ("PMA," positive control) for 5 hours in media containing Brefeldin-A. To determine the frequency of CD8+ T cells that produced IFN-γ, cells were stained with fluorescently-labeled anti-CD8α and anti-IFN-γ antibodies, as stated in the materials and methods section. Stained cells were then fixed and analyzed using a flow cytometer (Dako Cytomation).

Figure 8

A


INF
DEPL_INF_DEPL
DEPL_INF
NAÏVE
%CD8+ APL18-Kb+
10
0
16
20
Days post infection

B

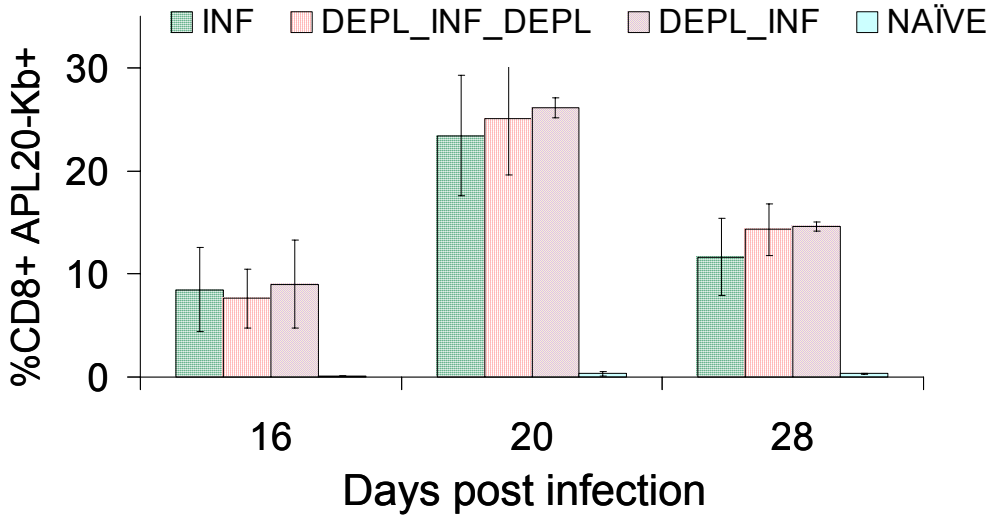
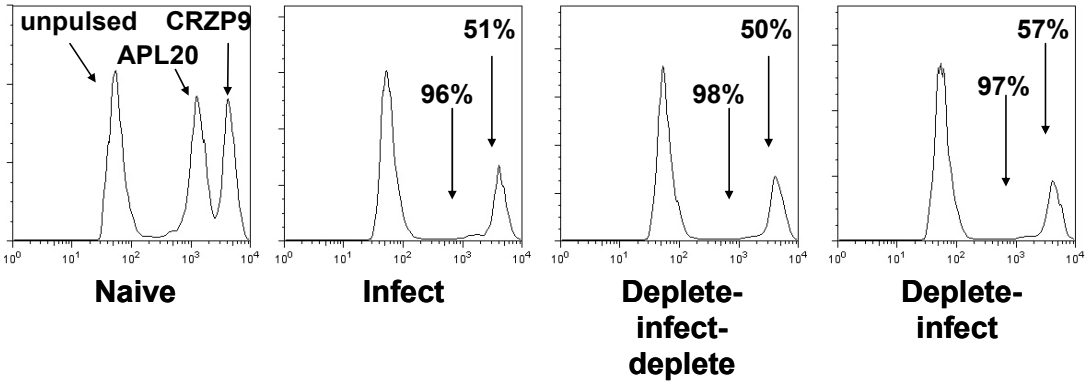
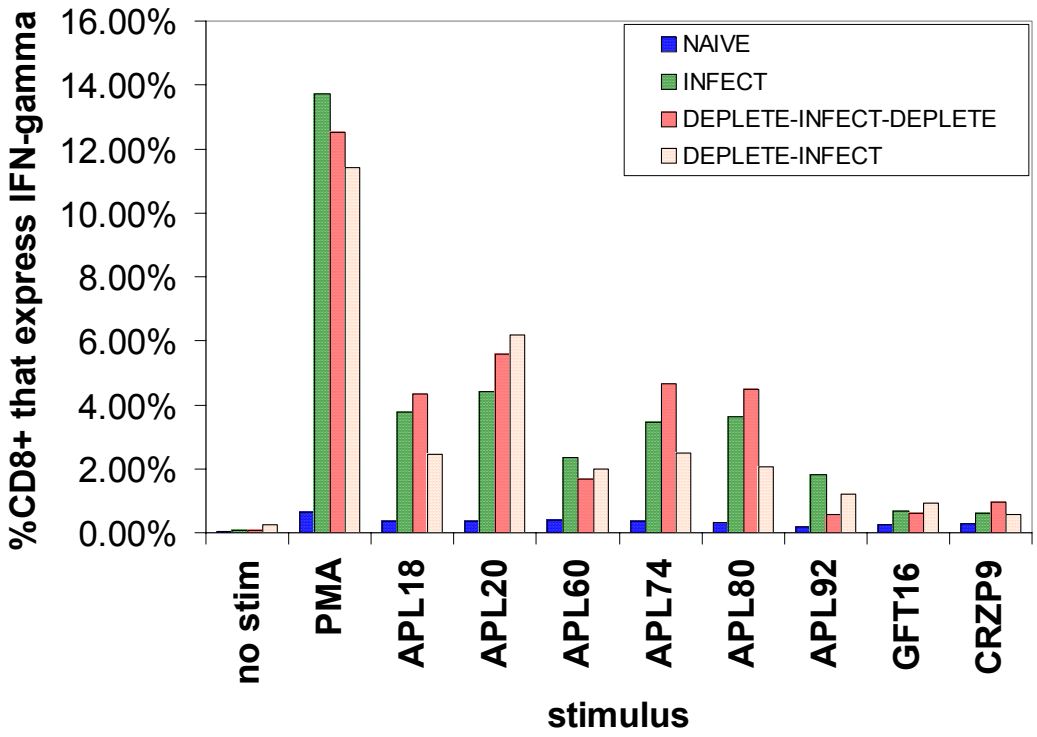

INF
DEPL_INF_DEPL
DEPL_INF
NAÏVE
%CD8+ APL20-Kb+
30
20
10
0
16
20
28
Days post infection

Figure 8

C


unpulsed
CRZP9
APL20
96%
51%
98%
50%
97%
57%
Naive
Infect
Deplete-infect-deplete
Deplete-infect

D


%CD8+ that express IFN-gamma
16.00%
14.00%
12.00%
10.00%
8.00%
6.00%
4.00%
2.00%
0.00%
NAIVE
INFECT
DEPLETE-INFECT-DEPLETE
DEPLETE-INFECT
no stim
PMA
APL18
APL20
APL60
APL74
APL80
APL92
GFT16
CRZP9
stimulus

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CHAPTER IV

CONCLUSION

CONCLUSION

Trypanosoma cruzi is the protozoan parasite that causes Chagas' disease. Infection with *T. cruzi* results in a complex immune response which controls parasite load, but is unable to achieve sterile cure of infection. Instead, parasites evade the immune response and persist in muscle, neuronal, and gut tissues, forming the basis for chronic disease. Successful vaccines and chemotherapies sufficient to cure infection are lacking. Thus, investigation into the mechanisms of parasite persistence is important to help develop novel therapeutic measures to combat the morbidity and mortality that *T. cruzi* infection causes.

A recent report described the presence of dysfunctional CD8+ T cells in the muscle compared to the spleen of mice chronically-infected with *T. cruzi* [1]. The molecular basis of this dysfunction is as yet unknown. Since muscle tissue is one preferred site of parasite persistence, and dysfunctional CD8+ T cells are found in the muscle of mice chronically infected with *T. cruzi*, one hypothesis is that there is a correlation between dysfunctional CD8+ T cells and the persistence of parasites in muscle tissue.

CD4+CD25+ regulatory T cells (Treg) are currently the subject of intense research. Not only have Treg proven to modulate autoimmune, anti-tumor, and allograft immune responses, they also can affect the outcome of the immune response to certain infections, playing both beneficial and detrimental roles to the host. Since Treg have been reported to suppress CD8+ T cell responses to certain pathogens allowing for persistent infection, the potential of Treg to

suppress CD8+ T cell responses in *T. cruzi* infection as a potential mechanism of parasite persistence needs to be investigated.

It was first hypothesized that Treg contributed to the observed dysfunction of CD8+ T cells in the muscle of *T. cruzi*-infected mice. If this hypothesis is correct, it was predicted that Treg would be readily detectable in chronically-infected muscle tissue, a site of both parasite persistence and dysfunctional CD8+ T cells. It would then follow that removal of Treg from chronically-infected muscle tissue would enhance CD8+ T cell responses, possibly reducing parasite load and disease. However, the best-defined marker for Treg, Foxp3 expression, was not readily detected in muscle-infiltrating lymphocytes. This primarily indicated that natural Treg may not be responsible for observed CD8+ T cell dysfunction. Two additional widely used methods to characterize the suppressive function of regulatory cells, *in vivo* administration of anti-CD25 depleting mAbs and *in vitro* suppression assays, were additionally employed to uncover possible roles for Treg in suppressing anti-*T.cruzi* immune responses. Administration of anti-CD25 mAbs did not improve the immune response compared to mock-treated controls, although complete depletion of CD4+CD25+ from the muscle could not be achieved. No suppressive properties were observed in cells isolated from chronically-infected muscle as measured using an *in vitro* suppression assay. Taken together, these results reject the hypothesis that natural Treg are responsible for the dysfunction of CD8+ T cells in chronically infected muscle and/or parasite persistence.

While Treg play roles in suppressing chronic immunopathology, they also have proven to suppress the quality and quantity of primary immune responses to acute infections, sometimes being necessary for the escape of pathogens from a protective immune response. It was hypothesized that if Treg were necessary for *T. cruzi* evasion of acute phase immune responses, that depletion of Treg prior to infection or vaccination would augment immune responses, possibility giving the immune response an extra edge to combat infection. Depletion of Treg prior to non-lethal or lethal parasite challenge did not yield any significant changes in the quality or quantity of the immune responses compared to mock-treated controls according to the parameters measured, including survival to lethal challenge, altered immunodominance hierarchies, increased pro-inflammatory immune responses, or highly improved vaccine efficacy. Therefore, it is concluded that Treg do not play a role in allowing *T. cruzi* to escape from the host immune response.

Thus, other immunoregulatory mechanisms which may aid in parasite immune evasion and persistence need to be investigated. For example, one recently described immunoregulatory ligand, PD-L1, can downregulate the ability of CD8+ T to perform effector functions [2], and could hypothetically explain the dysfunctional phenotype of CD8+ T cells observed in chronically-infected muscle tissue. Furthermore, public education, vector control, screening blood and organ supplies, and the eradication of poverty are all targets to curtail the spread of Chagas' disease.

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