THE ROLE OF CD8+ T CELL IMMUNODOMINANCE IN CONTROL OF EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION

by

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(Under the Direction of Rick L. Tarleton)

ABSTRACT

Trypanosoma cruzi infection drives the expansion of parasite-specific CD8+ T cells recognizing peptide epitopes encoded by *trans*-sialidase (TS) gene family members. The immunodominance of TS is remarkable; with up to 40% of all CD8+ T cells expressing T cell receptors recognizing the dominant TSKB20 and sub-dominant TSKB18 epitopes, these responses are among the strongest documented in any infection. However, mice fail to completely clear *T. cruzi* and subsequently develop chronic disease despite generating such highly focused T cell responses. Since *T. cruzi*'s genome encodes thousands of variant TS genes, we questioned the significance of these TS-specific CD8+ T cells. To determine the necessity of TS-specific CD8+ T cells for control of *T. cruzi* infection, we epitope-tolerized mice by injections of synthetic peptide epitopes. Mice tolerized to either the dominant or sub-dominant epitope, or both simultaneously, had transiently increased parasite burden though ultimately controlled acute infection, likely due to the activities of CD8+ T cells specific for unidentified parasite-derived epitopes. We hypothesized that these normally non-dominant CD8+ T cells could mediate long-term control of *T. cruzi*, so we developed transgenic mice

expressing the TSKB20 or TSKB18 peptides as self-antigen to ensure central tolerance of peptide-specific CD8+ T cells. Recapitulating our previous findings, mice deleted of CD8+ T cells specific for TSKB20, TSKB18 or both peptides, were resistant to *T. cruzi* and developed functional effector CD8+ T cells. Mice deleted of the normally dominant CD8+ T cells controlled *T. cruzi* infection, but developed chronic infection and similar disease as their wild-type littermates. Though immunodomination by TS-specific T cells interferes with the development of responses targeting other parasite-encoded epitopes, deletion of the described dominant CD8+ T cells did not appear to enhance protective immunity nor change the outcome of infection. These data do not support a major role for TS-epitope immunodominance as a mechanism exploited by *T. cruzi* to promote the parasite's persistence in the immune host, however, the data do indicate that strong responses against these TS-derived epitopes are non-essential. Furthermore, immunodominance by a particular CD8+ T cell population does not predict a critical role for that population in the control of *T. cruzi* infection.

INDEX WORDS: *Trypanosoma cruzi*, Chagas disease, CD8+ T cell, immunodominance, immune evasion, tolerance, transgenic mouse

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DEDICATION

This work is dedicated to my ever-supportive parents; their love, understanding, and encouragement have allowed me to realize this career in science.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Chagas disease and *Trypanosoma cruzi*

1.1.1 History of discovery and elucidation of T. cruzi's life cycle

American trypanosomiasis, referred to as Chagas disease in humans, is caused by infection with the protozoan parasite *Trypanosoma cruzi*. This zoonotic disease can be found in a large range of mammals in endemic countries of Central and South America where blood-feeding Reduviid bugs transmit the parasite to new hosts via contaminated feces. Infection can also be acquired by contaminated tissue donation and congenitally from mother to child. Evidence of human infection with *T. cruzi* dates back at least 9 centuries as the parasites DNA has been detected in the heart tissue of mummies in the Chilean Andes (1), so this new world parasite has likely afflicted humans since the earliest days of our arrival. Due to the rural nature of the transmission, Chagas disease impacts the poor the most, and recently has become an increasing public health risk in the United States due to immigration from Latin America (2) as well as autochthonous transmission cycles (3, 4).

The Brazilian medical officer, Carlos Chagas, is credited with describing *T. cruzi* in 1909, during the golden age of pathogen discovery. His individual accomplishments were quite remarkable as he described the parasite, its triatomine insect vector, and the disease manifestations caused by *T. cruzi* infection. He named the trypanosomatid parasite after Oswaldo Cruz, the director of the Instituto Oswaldo Cruz in Rio de Janeiro,

Brazil. In recognition of his seminal work, Chagas was nominated on two occasions for the Nobel Prize in medicine (1913 and 1921), though was never awarded the prize.

1.1.2 T. cruzi biology

T. cruzi is a single cell protozoan in the phylum Euglenazoa. Other related pathogens in the order Trypanosomatida include *Leishmania* spp. that cause a variety of leishmanial diseases of humans and mammals, *Trypanosoma brucei* spp. that cause African trypanosomiasis or Sleeping Sickness in humans, as well as other parasites of insects, amphibians, reptiles, fish, and plants (5). The "tri-tryps" causing disease in humans, *T. cruzi*, *Leishmania* spp. and *T. brucei* spp., often infect chronically, cause severe illness months to decades after their initial infection. Several unique organelles and enzymes were discovered in the trypanosomatids (6) and expression of the genome also appears to be unique compared to other eukaryotes (7), making these parasites interesting models for research in genetics, cellular biology and biochemistry.

T. cruzi has four distinct life stages that occur in either the insect's digestive tract or within cells or tissues of the mammalian host (6) (multiple intermediate forms have also been described (8)). The two forms capable of directly infecting nucleated host cells are the insect-stage metacyclic trypomastigotes that are released into the bug's feces during a blood meal, and the blood-form trypomastigotes that are released from infected cells after conversion from the replicating amastigote form. Both forms of trypomastigotes are flagellated and highly motile, and capable of infecting most-nucleated host cells including phagocytic and non-phagocytic cells. Several mechanisms of host cell invasion have been described for trypomastigotes, and amastigotes are thought to be able to infect new host cells, though not efficiently (9). Soon after cell

entry via generation of a parasitophorous vacuole, the parasite ruptures the vacuole and enters the cytosol where conversion to amastigotes occurs (in contrast to intracellular parasites that reside within a vacuole). The two replicating forms divide by binary fission and differ significantly in their morphology and metabolism due to their different lifestyles in distinct host environments (6, 10). Extracellular epimastigotes replicate in the insect's gut and convert to metacyclic trypomastigotes in the hindgut. Intracellular amastigotes replicate within the cytoplasm of nucleated host cells after conversion from the infecting trypomastigote form. After several division cycles (the parasite is reported to divide nine times (9)), the amastigotes convert back to tryposmatigotes and are released after rupturing the host cell. These released trypomastigotes can go on to infect local or distant host cells, or alternatively infect a new triatomine vector after being taken up in a fresh blood meal, thus continuing the parasite's relatively complicated life cycle. The amastigote and trypomastigote stages present in the mammalian host are most relevant for discussing the parasite in the context of chronic infection, which can last for decades within an individual host removed from endemic areas of transmission (11, 12). It is important to note that *T. cruzi* can be spread between humans through tissue transplants such as blood transfusion or organ transplantation, and can also be passed from congenitally from infected mothers to fetus. These man-made routes of transmission make disease spread outside of endemic regions a significant public health risk.

Contemporary analysis of *T. cruzi* and the related tri-tryp parasites has more recently relied on –omics level studies (13). The 2005 publication of the *T. cruzi* CL Brenner strain's genome sequence (14) allowed for the subsequent description of the

proteome (10) and transcriptome (15) of *T. cruzi*'s four main life-cycle stages. Combined, these studies reveal the complexity of *T. cruzi*'s genome arrangement (16) and give insights into how the parasite elaborates its stages-specific functions. Furthermore, genome level comparisons between isolated *T. cruzi* strains can now be made (17), providing new insights into the parasite's phylogeny and novel biology that we can take advantage of in efforts that help control infection and disease.

1.1.3 *T. cruzi evolved to expand genes encoding surface proteins*

Comparison of the genome sequences from *T. cruzi*, *T. brucei*, and *L. major*, revealed massive expansion in several gene families encoding surface proteins uniquely in *T. cruzi* (~18% of annotated genes) (13, 14) including: *Trans*-sialidase (TS), mucin, mucin-associated proteins (MASP), retrotransposon hot spot protein (RHS), and the surface protease gp63. Since the surface of the parasite is exposed to the host immune system, it has been suggested that immune selective pressures have influenced the evolution of these genes to dramatically expand in number. The need to interact with a diverse range of cell types within non-vertebrate and vertebrate hosts is also a likely evolutionary driving force for these genes to diversify.

The TS genes are an excellent example of *T. cruzi*'s surface gene expansion and are well studied; with over 1,430 annotated TS members this gene family represents a significant portion of *T. cruzi*'s genome (~12,000 genes) (14) and this number is likely an underestimate due to the collapsed assembly of near-identical sequences (16). Surface GPI-anchored TS transfers sialic acid residues from host glycoconjugates onto the parasite's mucin surface coat (18-20); a critical function since *T. cruzi* lacks the ability to generate sialic acid *de novo* and requires a sialidated surface for survival (21) and host

cell invasion (22, 23). TS gene expression (10, 24) and activities are differentially expressed by vertebrate stages of the parasite (19, 25-27), and are shed from the parasites surface during invasion and within the host cell cytosol (28).

Though several TS gene family members have been experimentally shown to be enzymatically active, the vast majority are thought to lack sialidase activity (14, 29-31) since they do not contain a sialidase superfamily motif (371 TS have the motif, VTVxNVxLYNR) and lack the SAPA epitope repeats (only 12 TS have SAPA repeats) that all confirmed active TS have (30). The simultaneous production of numerous TS products (26, 32, 33), and the assumption that surface expressed and shed TS are readily exposed to the host's immune system, indicate that the non-functional TS genes are likely involved in an immune evasion mechanism (30, 32, 34-37).

1.1.3 Disease: presentations, epidemiology, strategies for control and treatment

Chagas disease manifests over time in humans at acute, intermediate, and chronic phases of infection (11, 38). Acute infection is often unreported and defined as early infection up to 2-4 months. Clinical signs of initial infection are rare (described as flu-like symptoms), though parasite replication can induce swelling at the infection site such as the bite wound (chagoma) or the eye (Romaña's sign). Parasites can be readily detectable in a patient's blood smears, and parasites disseminate to all tissues via circulation and lymphatic drainage. Though acute death is rare, oral infections are an exception and can cause severe disease. Since most acute infections are unnoticed, it is assumed that individuals generate sufficient immune responses to readily control parasites without leading to severe disease. The majority of infected individuals can be classified as having the indeterminate stage, where disease symptoms are rarely reported

and circulating parasites are less common and difficult to detect. Unless acute symptoms were observed, infection status during this asymptomatic stage may be unknown. It is estimated that 20-40% of individuals go on to develop the severe complications of Chagas disease.

The disease that manifests during chronic Chagas disease likely depends on host and parasite factors as different forms of the disease appear to cluster geographically and are reported to depend on the strain (or strains) of parasite infecting the patient (39). Disease occurs mostly at sites of parasite persistence; persistence in heart leads to cardiomyopathy which can progress to heart failure (40); persistence in the gut leads to denervation and mega syndromes of the digestive tract including megaesophogous and megacolon, both causing severe digestive problems (41); persistence in nervous tissue is also reported to be associated with stroke risk (42, 43). Since its discovery, the etiology of Chagas disease has been controversial in part due to the technical difficulty of detecting parasites in the blood or organs of chronically infected individuals, and disease tends to manifest itself decades after infection. Arguments against the autoimmune hypothesis are that immunosuppressed individuals exhibit exacerbated parasite loads (44) and disease and AIDS patients have resurgence in parasites during HIV infection (45). Parasites can be detected in diseased patients' tissues using sensitive techniques like PCR for *T. cruzi* DNA (46-48). Furthermore, *T. cruzi*-infected mice do not reject syngenic heart transplants but rapidly reject *T. cruzi*-infected heart transplants (49), arguing against autoimmunity and proving that parasites are necessary and sufficient for disease during *T. cruzi* infection.

A recent report issued by the World Health Organization attributed 14,000 deaths annually due to Chagas disease out of a population of 8-11 million infected individuals, and an estimated 0.7 million disability-adjusted life year (DALY) as a result of disease (12). Control programs aimed at eliminating the major domestic insect vectors have been successful but ultimately failed to fully eradicate these sources of newly acquired infections in endemic areas (50). No vaccine exists for use in man or animals, due in part to lack of development by vaccine companies that have little economic incentive in this area, as well as the still prevalent controversy over the autoimmune etiology of disease. Anti-trypanosomal drugs developed for use in humans, nifurtimox and benznidazole, are rarely prescribed due to their toxic side affects and controversial effectiveness in the chronic phase.

1.2 Host immunity mediates control of *T. cruzi* infection and disease

The mammalian immune system is confronted with up to three of *T. cruzi*'s major life stages (two invasive trypomastigotes and the replicating amastigotes) having distinct extracellular and intracellular forms. The trypomastigotes and amastigotes express a diverse set of stage-regulated gene products allowing them to perform their stage-specific functions within their distinct niche (14, 24). This life cycle predicts numerous arms of immunity are activated and serve protective roles that keep both stages under control in resistant hosts. Here, I will briefly cover major themes in immunity to *T. cruzi*, and give a more in-depth review and discussion of literature on the role of parasite-specific CD8+ T cells in controlling infection and disease.

1.2.1 Innate immunity

Under normal circumstances, physical barriers (i.e. unbroken skin), prevent entry of parasites deposited in the insect vector's feces, however, wounds created from the triatomine's bite can serve as a portal for parasite entry and dissemination. Mucosal tissues, such as the eye conjunctiva or GI tract (orally ingested parasites), can also serve as entry points for the parasite to initially infect cells or disseminate into the blood stream (51, 52), and the parasite apparently has evolved mechanisms to overcome the physical barriers there (53-55). After several days, intense inflammation can develop at the initial site of infection and lead to the common clinical signs of acute infection: swelling at the bite site (chagoma) or of the eye (Romaña's sign) (11). This local inflammation, though delayed in comparison to more rapidly dividing pathogens, is evidence of the host's innate immune recognition of *T. cruzi*. The parasite also expresses surface proteins that confer some level of resistance to complement (56-59).

Cells of the innate immune system are of critical importance in controlling infectious organisms, and *T. cruzi* is no exception to this rule. Though *T. cruzi* invades a variety of phagocytic and non-phagocytic host cells (9, 60), the parasite can be actively engulfed by cells that include neutrophils, macrophages and dendritic cells (DC). These phagocytes each serve important roles by parasite killing, production of inflammatory cytokines like IL-12, and initiation of appropriate adaptive immune responses. Reactive oxygen species or nitric oxide, produced by macrophages or other infected host cells (61), are potent killers of the parasite and can be up-regulated by IFNγ produced by adaptive immune cells (62).

There has been recent interest in recognition of *T. cruzi*'s pathogen associated molecules (PAM) by innate germ line-encoded pathogen recognition receptors (PRR) (63-66). PRRs, like the well-studied toll-like receptor (TLR) system, sense PAMs expressed by the parasite and activate cells of the innate immune system to produce cytokines and perform functions that further potentiate development of adaptive immune responses (67). Though *T. cruzi* expresses several known ligands for TLR recognition (67), there appears to be an initial delay in this response that in turn contributes to the delayed generation of adaptive immune responses capable of containing initial infection (68). Overcoming this delay may be a key step towards complete parasite clearance by the host's immune system.

1.2.2 Adaptive immunity

The adaptive arm of the immune system is comprised of two major lymphocyte populations, B and T cells, that express unique germ-line rearranged antigen receptors. B cells produce surface-bound and secreted forms of antigen receptors referred to as antibodies that bind to specific three-dimensional antigens. Circulating antibodies bind to the surface of pathogens and target them for destruction by complement or facilitate uptake by phagocytes. CD8+ and CD4+ T cells recognize linear peptide epitopes in the context of class I major histocompatibility (MHC) (8-11 amino acid residues in length) or class II MHC (peptides of 10-30 amino acid residues in length), respectively, which are expressed on the surface of antigen presenting cells (APC). In most mammals, all nucleated cells express MHC-I, however, only professional APC like B cells, macrophages, and DCs can express MHC-II. Recognition of specific epitopes in the context of MHC activates the T cells to perform effector functions that lead to

elimination of antigen. Both humoral- (provided by B cells) and cell-mediated- (provided by T cells) immunity are critical for control of *T. cruzi* since the parasite circulates in the blood and extracellular tissues as well as infects professional and non-professional APCs that differ in their capacity to present antigen to CD4+ and CD8+ T cells. CD4+ T cells are often referred to as "helper" T cells since they interact with professional APC and help coordinate the development of adaptive immune responses through production of canonical cytokines.

Evidence for the relative importance of lymphocyte subsets that eliminate the parasite from most tissues (69) is provided by studies in rodent models by infection of knock-out (KO) strains of mice, or using antibody-based depletions to eliminate lymphocyte subsets or components of their effector functions (70). Mice deficient for B cells control early parasite replication, though they succumb at two months post-infection (71). Mice depleted of, or genetically deficient for, CD8+ T cells and components of MHC-I presentation are highly susceptible to acute infection and succumb within a month of infection (71-75). Interestingly, though mice genetically deficient for CD4+ T cells generate *T. cruzi*-specific CD8+ T cell subsets (76), depletion of these helper T cells also results in uncontrolled acute infection (76, 77) likely due to the ability of these cells to program appropriate antibody production, activate phagocytes to kill intracellular parasites, and also regulate the ensuing acute parasite-specific response. A T helper 1-polarized cytokine response is necessary (78, 79), and antibody depletion of IFNγ (80) or deletion of the IFNγ gene (81) enhances susceptibility to infection. Depletion of CD8+ or CD4+ T cells reverses protection afforded by prior vaccination (72, 82). Thus, the coordinated efforts of humoral and cell-mediated immunity are necessary for control of

T. cruzi, and manipulations to enhance these adaptive immune cells are a possible means towards tipping the balance of control in favor of the host during chronic infection.

T. cruzi replicates within a variety of hosts cells and persists within muscle, fat, and nervous tissues. Parenchymal cells in these tissues express MHC-I so are surveyed by parasite-specific CD8+ T cells and are likely targeted for destruction. Evidence for this is the observation that amastigote pseudo-cysts are rarely observed in chronically infected tissues containing inflammatory infiltrates (69) which are primarily composed of CD8+ T cells (83). Different strains of mice infected with certain *T. cruzi* strains develop distinct disease in heart or skeletal muscle, and inflammation is primarily restricted to the sites where the parasite or its products are found (69). Confirming the importance of CD8+ T cells in parasite control, antibody depletions of CD8+ T cells exacerbates parasite load at sites of persistence leading to enhanced disease at those sites (84).

A longstanding question has been what are the critical functions performed by effector CD8+ T cells that limit parasite numbers in non-lymphoid tissues? Infected humans that maintain IFNγ-producing parasite-responsive CD8+ T cells are less likely to progress to severe Chagas disease (85, 86). Genetically deficient mice lacking IFNγ cannot control *T. cruzi* and infected mice treated with IFNγ depleting antibodies have enhanced parasite loads and disease (70). Administration of recombinant IFNγ further enhances parasite control (87). Though few CD8+ T cells stain for (88) or *ex vivo* produce (89) IFNγ in chronically infected tissues, cytokine producing CD8+ T cells are readily detected during acute infection and are maintained in lymphoid tissues throughout the infection (89-91). The observation of reduced numbers of cytokine producing cells has been proposed as a mechanism accounting for parasite persistence (89), though this is

currently under debate. Both perforin and granzyme B KO mice control *T. cruzi* infection (71), suggesting that direct cell killing by parasite-specific CD8+ T cells is not required, however, multiple redundant pathways exist that mediate cytotoxicity and the absence of one pathway might be compensated for by another. Whatever the means used to eliminate the infected host cell, the ultimate fate of the released parasites is currently unknown, though local phagocytic cells within the inflamed tissue likely take up the majority of these parasites.

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

1.3.1 TS-genes as sources of vaccine targets

An elegant study by *Garg et al* (92) investigated the cellular properties of *T. cruzi*-derived proteins that enter the MHC-I presentation pathway. *T. cruzi* parasites were engineered to express the model antigen ovalbumin (OVA) targeted to distinct cellular compartments. Host cells infected with OVA-expressing parasites that expressed secreted or glycosylphosphatidylinositol (GPI)-anchored versions of OVA could stimulate OVA-specific CD8+ T cells, whereas parasites expressing cytoplasmic or trans-membrane forms of OVA did not. These results indicated that secreted or shed surface proteins entered the cytosol of infected host cells and were subsequently degraded to MHC-I-restricted epitopes and presented on the surface. With the knowledge that *T. cruzi*'s GPI-anchored trans-sialidase (TS) proteins were shed into the cytosol during invasion (20, 22, 28), *Wizel et al* (93) tested infected mice for recognition of epitopes predicted to bind to mouse H-2K^b or H-2D^b MHC-I complexes that were encoded by cloned TS genes. The previously studied TS gene, TSA-1 (94-96), was found to encode the VDYNFTIV epitope (Pep77.2) that was recognized by CD8+ T cells from *T. cruzi*-

infected mice (93). Furthermore, adoptive transfer of cloned lines of CD8+ T cells specific for Pep77.2 conferred significant protective immunity to naïve mice prior to *T. cruzi* challenge (93), indicating epitopes encoded by TS genes were recognized by CD8+ T cells during infection and likely an excellent source of protective antigens for vaccination.

Trypomastigotes express significant levels of TSA-1 mRNA (94) and immunization with plasmids bearing TSA-1 DNA induced protective immunity to *T. cruzi* (97). Further identification and cloning of TS genes with evidence of expression by amastigotes (98, 99) showed that epitopes encoded by the ASP-1 and ASP-2 TS clones were also immune targets of protective parasite-specific CD8+ T cells (100). Numerous studies have since supported the protective role of TS-specific CD8+ T cells responses in experimental vaccinations against *T. cruzi* infection and have been reviewed in depth elsewhere (101-103). It appeared that these TS-specific T cells were a minor population within the total CD8+ T cell compartment of *T. cruzi*-infected mice (91), possibly reflecting the unfocused nature of *T. cruzi*-specific immune responses potentially responding to thousands of epitopes encoded by the parasite's genome. Importantly, though TS vaccine studies often report protection from challenge infection in individuals immunized against TS, no report has definitively shown that TS vaccines induce immunity capable of sterile control of the parasite, a clear goal of vaccines that protect from infection and subsequent disease in humans (104).

1.3.2 Parasite-specific CD8+ T cells are highly focused on peptides derived from the variant trans-sialidase gene family

The minimal MHC-I-restricted epitopes encoded by TSA-1 (Pep77.2, VDYNFTIV), ASP-1 (P14, VNHDFTVV), and ASP-2 (P8, VDYNFTIV) are encoded at overlapping regions of TS and the sequence of these epitopes bears striking resemblance to one another. Using the location and sequence of these known targets as a template, we expanded the list of potential MHC-I epitopes recognized by screening *T. cruzi*'s genome for peptide homologues encoded by the 1,430 annotated TS family members (105). Of the 324 overlapping H-2K^b-restricted peptides present at the same position in raw sequence reads with homology to published TS genes, 156 were present in annotated TS genes (including TS pseudogenes) (105). Subsequent testing for IFNγ produced by CD8+ T cells in response to these TS-derived H-2K^b-restricted (referred to as "TSKB") epitopes indicated that numerous TS-encoded epitopes were recognized by *T. cruzi* infected mice. Several strong inducers of IFNγ production were peptides with similar amino acid sequences: TSKB18 (ANYDFTLV), TSKB20 (ANYKFTLV), TSKB21 (ANYNFTLV), TSKB74 (VNYDFTLV), TSKB80 (ANYNFTLL), TSKB89 (VNYDFTIV), and TSKB260 (ANYKFTLL). Furthermore, *T. cruzi*-infected mice retained peptide-specific responsiveness towards these peptide targets during chronic infection as measured by cytokine production and *in vivo* killing of peptide-loaded APC (105). Staining with fluorescently labeled recombinant MHC-I tetramers individually loaded with the recognized TSKB peptides (thus useful for marking CD8+ T cells bearing peptide-specific TCR for detection by flow cytometry) revealed an unappreciated expansion of these epitope-specific CD8+ T cells. During acute Brazil strain infection,

the TSKB20-specific CD8+ T cells represented 20-30% of the CD8+ T cell population, and the TSKB18-specific population 4-10% (105). It was also subsequently determined that these CD8+ T cells recognized distinct sets of over-lapping cross-reactive peptides, perhaps an unsurprising finding given the sequence similarity shared by these epitopes. Thus, the TSKB20-specific CD8+ T cells respond to TSKB20, TSKB21, and TSKB260 whereas the TSKB18-specific CD8+ T cells respond to TSKB18, TSKB74, TSKB80, and TSKB89 peptides, so > 300 TS family members encode potential targets of parasite-specific CD8+ T cells.

Concomitant with parasite load reduction, the TSKB20-specific and TSKB18-specific CD8+ T cell populations remained detectable at a reduced, yet, relatively high number into the chronic phase of infection and remain at similar proportions towards each other. Since TS-genes sequences are variable between *T. cruzi* strains, the recognition of TSKB20 and TSKB18 was analyzed after infection with distinct strains which showed that the dominance of the CD8+ population by these peptide-specific CD8+ T cells varied depending on the infecting strain (105). Furthermore, some strains of *T. cruzi* induce an immunodominant ASP-2 (P8)-peptide-specific response (106). Thus, similar to contemporary analysis of viral (107, 108) and bacterial infections (109), CD8+ T cell responses to *T. cruzi* are characterized by focused immunodominance hierarchies (discussed in more detail below) despite the possibility of recognizing thousands of possible parasite-derived epitopes, and the dominance generated is likely dependent on the complement of TS-genes encoded by the infecting *T. cruzi* strain (105, 106).

T. cruzi-infected humans also respond to TS-derived epitopes (110, 111), albeit at a reduced magnitude than observed in certain experimental mouse models of infection. Individuals that maintain CD8+ T cells producing IFNγ in response to stimulation with TS-derived peptides or whole parasite antigen preparations are less likely to develop severe disease (85). This supports the idea that these parasite-specific CD8+ T cells are important controllers of Chagas disease in humans.

1.3.3 Non-TS-encoded CD8+ targets

Though TS-specific responses are immunodominant during infection, other parasite-derived antigens have been assessed for recognition by CD8+ T cells after vaccination and/or *T. cruzi* infection including: paraflagellar rod proteins (PAR-1, -2, -3, and -4) (112-119), flagellar calcium-binding protein (120), KMP-11 (121-128), Cruzipain (Crz) (105, 129-134), LYT1 (120, 135), mucins (105, 120), mucin associated proteins (MASP) (105), gp63 (105), retrotransposon hot spot protein (RHS) (105), and β-galactofuranosyl transferase (Gft) (105). Similar to TS, these antigens are also expressed on, or released from, the surface of the parasite's mammalian stages, likely making them available for MHC-I presentation (92). Though KMP-11 does appear to be recognized by CD8+ and CD4+ T cells at detectable frequencies in chagasic individuals (128, 136), the majority of non-TS antigens listed here induce CD8+ responses in mice that are at the edge of detection by immunological assays (even after boosting by prior immunization). This is perhaps unsurprising considering some of these antigens are not encoded by large gene families, and likely are expressed at relatively low levels compared to the high copy TS family (7, 14, 17, 24, 137). However, several of these proteins are encoded in large numbers of genes, some comparable in size to the TS family (1,430 annotated members)

including the MASPs (1,377 annotated members), mucins (863 annotated members), RHP (752 annotated members), and gp63 (425 annotated members) (14). Though experiments that tested recognition of these non-TS gene family members focused primarily on predicted peptides capable of binding H-2K^b with high affinity, many of the sequences encoding these epitopes were present at higher abundance than the dominant TS-encoded epitopes (105). Furthermore, previous studies found low-level responses targeting several Crz- and Gft-encoded epitopes (105, 138, 139), and these gene families are amongst the smallest in the genome (105), suggesting copy number in the genome may not be the only factor influencing the recognition of these sub-dominant epitopes. Thus, high copy number TS genes appear to encode the optimal targets of CD8+ T cells responses in mice, and vaccinations against non-TS antigens can induce protective immunity despite their sub-dominant status, although none confer sterilizing immunity.

1.4 CD8+ T cell immunodominance

Immunodominant is a term describing the relative strength of an immune response towards a given antigen (140). Any reproducibly measured set of antigen-specific responses can be described as an immunodominance hierarchy, with the strongest responses referred to as dominant (or co-dominant with another response) over weaker responses that are referred to as sub-dominant (sometimes designated in decreasing order as α-, β-, γ-…). Antigens that are at the limit of detection by immunologic assays are referred to as cryptic, and often are directly measurable only under certain conditions (140). In reality, this collection of terms allows for the sorting and description of immunologic information, and is based on reproducible experimental results that are measurable, statistically significant, and reportable in scientific literature. Any antigen-

specific response can potentially be referred to as immunodominant as long as it is detectable. Whether or not immunodominance in immune responses towards pathogens is biologically important is debatable (as I attempt to do in the next section), however, this importance is only rarely discussed (or directly tested for that matter) in the literature. Here, I will briefly review the major mechanisms that account for generating immunodominance hierarchies, review studies that test for the importance of immunodominance during control of infections, and discuss the implications of immunodominance during T cell responses towards parasitic pathogens.

1.4.1 Mechanisms

Immunodominance occurs during antigen-specific responses mounted against infections, cancer, autoimmunity, as well as immunization. For the sake of clarity, mechanisms that account for immunodominance in CD8+ T cell responses will be discussed here with an emphasis on contemporary studies relevant to *T. cruzi* and intracellular parasites. Though largely based on observations in inbred animal models (aka mice), humans sharing HLA-alleles also generate reproducible immunodominance hierarchies (141).

Depending on their size, protein antigens may encode many 8-10 amino acid peptides that bind to a given MHC-I (mice have three loci H-2K, H-2D, and H-2L, and humans have three loci HLA-A, HLA-B, and HLA-C). A single viral, bacterial, or protozoal pathogen can contain on the order of ~10s-10,000s of unique antigens for further processing into these minimal epitopes. The epitopes are generated by the concerted actions of the proteasome and peptidases that further trim peptides for loading onto nascent MHC-I molecules (142). Though numerous epitopes may be encoded in a

protein, the likelihood that a given epitope is properly processed and binds with high enough affinity to stabilize the surface expression of a given MHC-I is actually quite low (143) and likely the major factor that determines the immunodominance of a given antigen (141).

There are several described pathways utilized by professional APC (cells specialized to prime naïve T cells and initiate immune responses) (144) that include: direct presentation by infected APC, cross-presentation of acquired extracellular antigens, and the more recently described cross-dressed APC can tragocytose or 'steal' peptide-MHC from the surface of adjacent APC and directly present this complex to T cells (145). The distinction between modes of antigen presentation for parasite-derived antigens is important (146, 147) since viral pathogens utilize host machinery for protein production, whereas parasites utilize their own cellular machinery for protein production. Thus, though directly presented or cross-presented parasite-derived antigens are available for priming T cells (146, 147), infected host cells must directly present antigen for parasite-specific CD8+ T cells to be effective. Along these lines, bacteria that invade host cells are perhaps a better comparison to parasites than virus, as these intracellular pathogens also independently produce their gene products. Similar to several intracellular parasites (92, 148-152), secreted proteins by intracellular bacteria make optimal targets for priming of and recognition by effector CD8+ T cells (153-155). It is likely that bacterial and protozoal pathogens have evolved to balance the necessary functions of their secreted proteins with the possibility of them serving as immunodominant antigens.

If a pathogen expresses a sufficient amount of a given antigen, that in turn is broken down by lymphoid-resident professional APC into peptides that bind with high affinity to MHC I, and the sufficient inflammatory conditions exist for T cell priming (156), the development of an immunodominant response ultimately depends on the presence of T cells bearing high affinity T cell receptors that recognize the given peptide-MHC-I complex (157). Though the need for a naïve T cell bearing a clonally rearranged TCR recognizing a pathogen's epitope in complex with MHC-I is obviously important for generating immunodominant responses, it is a process that is not completely understood. The thymus is the location for positive selection of bone-marrow-derived immature T cells that express rearranged TCRs capable of interaction with self-MHC-I, yet do not react strongly with self-peptide-MHC-I complexes (negative selection) (reviewed in (157)). The result of thymic selection is referred to as central tolerance and is critical for preventing the development of autoimmunity, yet some self-specific CD8+ T cells escape this selection process and are further kept in check by peripheral tolerance mechanisms (158). Further complicating matters is the observation of degeneracy in TCR recognition of peptide-MHC-I (159, 160), resulting in cross-reactive T cell populations (161, 162) that recognize epitopes of homologues sequence and/or shape when bound within the MHC-I cleft via differing TCR. TCR cross-reactivity increases the potential number of T cells clones able to participate in a response and can provide an otherwise naïve host with protective memory T cells providing heterologous immunity to newly encountered infections. In turn, T cells bearing these promiscuous TCR can be activated and participate in pathogenic roles or even trigger autoimmunity (163, 164). The frequency of naïve precursors bearing rearranged epitope-specific TCR influences

the magnitude of their expansion, as well as effector and memory phenotype attained (165, 166) after an immune response is initiated.

By analogy, the tissues and molecules of the immune system serve as an ecosystem for T cells, and these cells compete with one another for limiting space and resources during stages of an immune response (167). Competition between T cells expressing the same TCR or different TCRs recognizing similar epitopes is termed immunodomination. T cells recognizing different epitopes present in the same antigen (or pathogen) also exhibit competition, which is referred to as cross-competition. Immunodomination is likely an important mediator of immunodominance and impacts protective immunity (168). In essence, the previously mentioned mechanisms which account for generating an immunodominant response influence the ability of CD8+ T cells to compete with another (TCR affinity, amount of epitope presented, type of APCs, etc…). Though this is somewhat a circuitous defining mechanism of immunodominance, the next section will describe studies that demonstrate that T cell competition is a factor in CD8+ immunodominance during infection.

1.4.2 Implications of immunodominance for control of viral, bacterial and protozoal pathogens

The observation that immunodominance is common to T cell responses implies, but does not prove, the necessity of immunodominant responses for immune protection. Since the description of T cell recognition of infected cells via MHC-I-restricted peptide presentation (169), the role of dominant and sub-dominant T cells has been studied in a variety of models including: adoptive cell transfer or vaccinations to boost specific CD8+ T cells, infections with escape mutants or genetically modified pathogens lacking

expression of immunodominant epitopes, and the induction of tolerance to specifically ablate T cells recognizing specific antigen.

Transfer studies have indicated that immunodominance generated during viral infection does not predict protective roles (170). Though infection of mice expressing transgenic TCR recognizing pathogen encoded epitopes has demonstrated the ability of a single specific T cell population to control a complex pathogen (171), the immune selective pressure of such focused responses can select for epitope escape mutants (172-175) that are no longer recognized by the dominant T cells. Infections using viral escape mutants (176) or engineered viruses and bacteria that lack the epitope of interest (170, 176-184) impact immunodominance differently depending on the infection. Furthermore, induction of tolerance to deplete mice of epitope-specific CD8+ T cells during viral infection (185-192) also gives mixed results. Loss of the immunodominant CD8+ T cell population sometimes impaired viral control (179, 181, 182, 184, 185, 187-191) or enhanced disease without affecting viral load (182), while in other situations, pathogen load was not affected (178, 180, 183, 186) or disease was ameliorated (181, 186, 189, 192, 193). Compensation in the absence of immunodomination by dominant T cells occurred in many of these infection models (170, 177, 178, 180-182, 184), however, the altered immunodominance observed was often characterized by enhanced T cell responses to minor epitopes instead of sub-dominant responses (177, 180, 181, 184). Thus, the effects of altering immunodomination are not simple to predict, and likely depend on the pathogen and antigen in question.

The relative importance of a particular epitope-specific T cell population is especially complicated during parasite infections where there are potentially more

epitopes encountered by the immune system compared to infections with virus or bacteria. These eukaryotic pathogens have larger genomes and often express stage-regulated proteomes within their vertebrate host. Parasites often infect chronically (i.e. *T. cruzi*, *Leishmania* spp., and *Toxoplasma gondii*) or can subsequently infect an immune host (ie *Plasmodium* spp.), suggesting immunity to these parasites is somewhat lacking.

Immunodominant antigens recognized by parasite-specific CD4+ and CD8+ T cells have been described for *T. cruzi* (TS), *Leishmania* spp. (LACK (171, 194-196)), *Plasmodium* spp. (CS (197-201)), and for *Toxoplasma gondii* (SAG1, GRA4, GRA6, ROP7 (202-206)); tolerance to deplete these T cell responses has been employed to study some of their roles during infection. The response towards *L. major*'s LACK, though protective (171, 195), has also been associated with diverting the immune phenotype of the response in favor of parasite persistence (207). Deletion of LACK-specific CD4+ T cells can redirect the immune response to *L. major* (208-210) (though not always (211)), however, it did not affect the responses to the related *L. mexicana* (212) (which also expresses LACK). Transgenic expression of *T. gondii*'s SAG-1 (204, 213-215) demonstrated a crucial role of CD4+ T cell mediated immunity during control of virulent challenge, though mice lacking SAG-1 responses could be protected by prior vaccination with less virulent parasites. Similarly, CS-transgenic mice were less protected in the absence of CS-immunodominance (201), though these mice were protected after numerous vaccinations with irradiated sporozoites or genetically modified avirulent parasites (216). Though these transgenic mice showed decreased immunity in some situations, protection could be induced towards normally non-dominant T cell antigens (201, 204, 216). Furthermore, mice expressing immunodominant parasite antigens

appear to be less affected, in comparison to viral antigen transgenics that exhibit severe immune deficits (187, 189-191), likely due to compensating T cell responses specific for the numerous possible antigens expressed by these parasites.

1.5 Conclusion and experimental plan

The immune system's recognition of intracellular protozoan parasites, like *T. cruzi*, is not fully understood. Functions of effector CD8+ T cells are critical for control, and improving their ability to target parasite-infected host cells is a promising avenue for better controlling chronic infection and disease or preventing long-term infections all together. *T. cruzi*-infected mice generate significant immunodominant parasite-specific CD8+ T cells populations, yet cannot fully clear infection despite maintaining these responses throughout infection. Furthermore, provision of memory CD8+ T cell populations recognizing these dominant targets by vaccination (217) or drug-induced cure (218) also fails to prevent parasite persistence after infection. Therefore, the biological significance of generating these immunodominant CD8+ T cells responses is questionable.

We hypothesize that *T. cruzi*'s variant TS genes are involved in an immune evasion mechanism that promotes the parasite's persistence in its host. We propose that the strong immunodominance observed by TS-epitope-specific CD8+ T cells inhibits the generation of alternative parasite-focused responses. Furthermore, these normally out-competed CD8+ T cells may recognize targets that allow for better control of the parasite. To test the role of these immunodominant CD8+ T cells, we have induced immune tolerance to the TS-derived epitopes during *T. cruzi* infection and determined the consequences for parasite control in the absence of these responses. Determining why

these immunodominant CD8+ T cells fail to fully eradicate parasite infected cells will inform us how to better develop immunotherapeutics aimed at altering the course of infection in favor of complete parasite control.

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CHAPTER 2

CD8+ T CELLS SPECIFIC FOR IMMUNODOMINANT *TRANS*-SIALIDASE EPITOPES CONTRIBUTE TO CONTROL OF *TRYPANOSOMA CRUZI* INFECTION BUT ARE NOT REQUIRED FOR RESISTANCE [1]

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2.1 Abstract

CD8$^+$ T cells are essential for controlling *Trypanosoma cruzi* infection. During Brazil strain infection, C57BL/6 mice expand parasite-specific CD8$^+$ T cells recognizing the dominant TSKB20 (ANYKFTLV) and sub-dominant TSKB74 (VNYDFTLV) *trans*-sialidase gene (TS)-encoded epitopes with up to 40% of all CD8$^+$ T cells specific for these epitopes. Though this is one of the largest immunodominant T cell responses described for any infection, most mice fail to clear *T. cruzi* and subsequently develop chronic disease. To determine if immunodominant TS-specific CD8$^+$ T cells are necessary for resistance to infection, we epitope-tolerized mice by high-dose intravenous injections of TSKB20 or TSKB74 peptides. Tolerance induction led to deletion of TS-specific CD8$^+$ T cells but did not prevent the expansion of other effector CD8$^+$ T cell populations. Mice tolerized against either TSKB20 or TSKB74, or both epitopes simultaneously, exhibited transient increases in parasite loads, though ultimately they controlled the acute infection. Furthermore, BALB/c mice tolerized against the TSKD14 peptide effectively controlled acute *T. cruzi* infection. These data are consistent with the hypothesis that development of high frequency CD8$^+$ T cell populations focused on TS-derived epitopes contributes to optimal control of acute infection, but is not required for the development of immune resistance.

2.2 Introduction

$CD8^+$ T cells are critical for adaptive immune control of intracellular pathogens by virtue of their ability to produce a variety of cytokines and to directly target infected host cells for destruction. Pathogen-specific $CD8^+$ T cells recognize foreign peptide epitopes presented in the context of surface bound class I major histocompatibility complexes (MHC I) using clonally diverse T cell receptors (TCR). During the course of infection with viral, bacterial, and protozoan pathogens, clones of pathogen-specific $CD8^+$ T cells expand in number, providing the host with the effector cells capable of controlling pathogen load (1, 2). A focused, reproducible hierarchy of epitope-specific $CD8^+$ T cells often occurs in which certain clones are represented at higher numbers (dominant) than other pathogen-specific T cells (sub-dominant), a phenomenon termed immunodominance (3).

Much of our knowledge concerning the role of dominant and sub-dominant $CD8^+$ T cells in control of infection has been derived from mouse infections with model bacterial (e.g. *Listeria monocytogenes*) (4) and viral (Lymphocytic Choriomeningitis virus, Influenza virus, and Vaccinia virus) (5) pathogens. For these less complex viral and bacterial pathogens, dominant $CD8^+$ T cells recognizing a small subset of pathogen-derived peptides are sufficient for adaptive immune control of infection. In comparison, protozoan parasites are more complex antigenically due to their larger genomes and proteomes as well as lifecycles involving distinct extracellular and intracellular stages occurring within a host. Though dominant $CD8^+$ T cells have recently been described for several intracellular parasites (6-10), the role that these populations play in immune resistance to infection is not fully understood.

In addition to being larger, many parasite genomes also contain greatly expanded sets of variant gene families encoding surface expressed and secreted proteins (11, 12). Our group has recently identified immunodominant $CD8^+$ T cells responding to acute and chronic *Trypanosoma cruzi* infection that are specific for the (H-$2K^b$-binding) TSKB20 (ANYKFTLV) and TSKB18 (ANYDFTLV) epitopes encoded by *trans*-sialidase (TS) gene family members (8). The dominant TSKB20-specific response (which also recognizes the cross-reactive TSKB21 peptide) represents approximately 20-30% of the total $CD8^+$ T cell compartment and the sub-dominant TSKB18-specific population (which recognizes the cross-reactive TSKB74 peptide) represents 4-10% of effector $CD8^+$ T cells at the peak of acute infection with Brazil strain *T. cruzi* in C57BL/6 (B6) mice. This degree of immunodominance is remarkable considering there are >1,400 annotated TS family gene members in the CL Brenner reference genome (of >12,000 annotated genes) (12). Moreover, distinct strains of *T. cruzi* appear to have unique sets of TS genes (8, 13), suggesting that this gene family has evolved at a population level under considerable immune pressure. Unlike persistent viral infections (14), the $CD8^+$ T cells recognizing TS-derived epitopes remain highly competent throughout this chronic infection, despite persistent antigen exposure (8, 15-18). However there is as yet no evidence that these sustained effector responses select for TS epitope-loss mutants of the parasite (17).

Although some TS gene products have been experimentally confirmed as capable of performing the critical enzymatic function of transferring sialic acid residues from host glycoproteins to molecules on the parasite's surface, the vast majority of TS gene family members lack evidence of this activity (19). Thus, it is unclear what the selective

advantage for expansion of the TS gene family is if they provide numerous targets for adaptive immunity (20, 21). Some have proposed that TS genes participate in immune evasion, promoting the chronic nature of *T. cruzi* infection (12, 17, 19, 22-26). The strong immunodomination by TS-derived epitopes predictably results in the out-competition of other epitope-specific $CD8^+$ T cell populations. However, the significance of the tight focusing of the $CD8^+$ T cell response on only a few of the vast array of possible parasite-derived epitopes is not known. Herein, we explore the role of immunodominant $CD8^+$ T cells in immune resistance to *T. cruzi* infection by inducing immunological tolerance to TS-derived epitopes during the course of acute infection. Though infection with this parasite elicits one of the strongest immunodominant $CD8^+$ T cells responses documented, we find the focus of the adaptive immune response to be remarkably plastic.

2.3 Materials and Methods

2.3.1 Mice and parasites

C57BL/6 and BALB/c mice were obtained from the National Cancer Institute at Frederick (Frederick, MD) and kept under specific pathogen-free conditions at the Coverdell Center animal facility (University of Georgia, Athens, GA). For *T. cruzi* infections, 8 week-old female mice were infected intraperitoneally (ip) with 1×10^3 trypomastigotes of the Brazil strain. Trypomastigotes were maintained in tissue culture by serial passage through Vero cells. Mice were euthanized by CO_2 inhalation. The University of Georgia Institutional Animal Care and Use Committee approved all animal use protocols.

2.3.2 Peptide treatments

Peptides were synthesized by SigmaGenosys (Saint Louis, Missouri) or GenScript (Piscataway, New Jersey). Peptides used were the H-2K^b-restricted TSKB20 (ANYKFTLV), TSKB74 (VNYDFTLV), and $OVA_{257-264}$ (SIINFEKL) peptides, or the H-2K^d-restricted peptides TSKD14 (IYNVGQVSI) and $LL0_{91-99}$ (GYKDGNEYI). Lyophilized peptide was suspended in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml and stored at -20° C. Stock peptide was diluted to the desired concentration in sterile saline (PBS) for intravenous (iv) injection (each mouse received 100 μl per injection). Peptide-treated mice initially received 300 μg peptide on day -7 and 100 μg on days -4 and -1. Mice were infected on day 0 and injected with 100 μg peptide weekly until the end of the experiment. An equal quantity of peptide was injected whether mice received one or two peptides simultaneously. Tolerized mice were sacrificed 7 days after final peptide treatment.

2.3.3 T cell phenotyping

For *ex vivo* lymphocyte phenotyping, spleens were removed and dissociated by rubbing between two glass slides in a medium of hypotonic ammonium chloride to lyse red blood cells. Cell numbers were determined on a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA). 5 x 10^6 washed splenocytes were suspended for staining in PBS with 1% bovine serum albumin and 0.05% sodium azide (PAB) (both from Sigma). TSKB20/K^b, TSKB74/K^b, and TSKD14/K^d tetramers were synthesized at the Tetramer Core Facility (Emory University, Atlanta, Georgia) and were labeled with PE (Molecular Probes, Carlsbad, CA). Antibodies used were CD8 Pacific Blue, CD4 PE-Cy5, CD127 PE-Cy7, FoxP3 PE (ebioscience, San Diego, CA), CD11b PE-Cy5, B220

PE-Cy5, CD25 APC (CALTAG), CD44 APC, CD11a FITC, and CD62L FITC (BD bioscience, San Jose, CA). Cells were stained at 4° C for 30 minutes, washed with PAB and fixed in 2% formaldehyde. The ebioscience intracellular staining kit was used for FoxP3 staining. At least 500,000 cells were collected for each sample on a Cyan ADP using Summit version 4.3 (Beckman Coulter). FlowJo Flow Cytometry Analysis Software Version 7 (Tree Star, Ashland, OR) was used for analyses.

2.3.4 T cell stimulation and intracellular cytokine staining

1.5 x 10^6 splenocytes were stimulated in 96-well round-bottom tissue culture plates (Costar, Corning, NY) at 37° C for 5 hr in the presence 1 μM peptide and brefeldin A (Golgi Plug, BD biosciences). For polyclonal activation, wells were pulsed with 30 μg anti-mouse CD3ε (ebioscience) for 1 hr at 37° C and excess antibody was removed prior to the addition of cells. Cells were stained with CD8 Pacific Blue and CD4 FITC (ebioscience) followed by intracellular staining with IFNγ APC (BD biosciences) and IL-10 PE (ebioscience) according to the cytofix/cytoperm kit (BD biosciences). At least 150,000 cells were collected for analysis.

2.3.5 In vivo cytotoxicity assay

Spleen cells from naïve mice were incubated for 1 hour at 37° C with 10 μM peptide or media alone, and then labeled with different concentrations of carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) as described (8) to produce CFSE high, medium, and low populations. Equal numbers of CFSE-labeled cells were transferred ip into recipients, and after 16 hours, splenocytes were isolated and CFSE-labeled cells were detected by flow cytometry. Percentage of specific killing was determined using the

formula 1-[(% $CFSE^{lo}$ naïve / % $CFSE^{med/hi}$ naïve) / (% $CFSE^{lo}$ infected / $CFSE^{med/hi}$ infected)] X 100%.

2.3.6 Real-time PCR

Mouse hind leg muscles were collected and popliteal lymph nodes were removed as well as extraneous adipose tissue prior to DNA extraction as described (27). Extracted DNA was analyzed by real-time PCR essentially as described (27). PCR reactions consisted of iQ SYBR Green Supermix (BioRad) and primers specific for *T. cruzi* or mouse genomic DNA (27). An iQ5 Multi-Color Real-Time PCR Detection System was used with iQ5 Standard Edition Optical System Software Version 2 (both BioRad). *T. cruzi* equivalents were calculated as the quantity of *T. cruzi* satellite DNA divided by the quantity of mouse TNFα DNA in each sample.

2.3.7 Statistical analysis

Statistical significance was calculated using a two-tailed students *t* test.

2.4 Results

2.4.1 Repetitive intravenous administration of peptide results in depletion of epitope-specific $CD8^+$ T cells during acute T. cruzi infection

In order to determine the importance of immunodominant TS-peptide-specific $CD8^+$ T cell responses in *T. cruzi* infection, we first developed a system in which immunodominant TS-specific $CD8^+$ T cell responses were ablated. Because the TSKB20 and TSKB74 epitopes (and cross-reactive epitopes) are encoded by numerous TS genes (more than 200 in the case of TSKB20/21; (8)), it was not feasible to generate gene knock-out parasites which do not express either antigen. We instead induced immune tolerance to these epitopes by administration of high doses of soluble peptide

intravenously (iv) (Fig. 2.1 A), a method previously used to induce and maintain epitope-specific tolerance in models of viral infection (28-32).

At the peak of the T cell response (~3 weeks post-infection; (8) and data not shown), TSKB20- and TSKB74-specific $CD8^+$ T cells in the spleens of mice injected with the respective peptides were nearly undetectable (Fig. 2.1 B). Infected control mice injected with the irrelevant $OVA_{257-264}$ peptide had normal proportions of TSKB20 and TSKB74 $tetramer^+$ $CD8^+$ T cells compared with infected mice treated with PBS + DMSO or non-treated mice (data not shown). Importantly, TSKB20-tolerized mice had a normal complement of TSKB74 $tetramer^+$ $CD8^+$ T cells and TSKB74-injected mice had TSKB20 $tetramer^+$ $CD8^+$ T cells (Fig. 2.1 B), demonstrating that depletion via TS-derived peptides was epitope-specific and did not prevent priming of other $CD8^+$ T cells specific for homologous TS epitopes. Peptide treatments effectively prevented expansion of peptide-specific T cells throughout the course of acute infection (Fig. 2.1 C-D). Interestingly, spleens of TSKB20-treated mice had significantly more TSKB74 $tetramer^+$ $CD8^+$ T cells at the peak of expansion compared to $OVA_{257-264}$-treated mice (Fig. 2.1 D). A similar compensation in immunodominance hierarchies has been noted in virus infection with epitope-loss variants and deletion mutants (33-36), and suggests that *T. cruzi*-specific $CD8^+$ T cells with alternative specificities expand in the absence of competition by the normally dominant TSKB20-specific $CD8^+$ T cell population.

2.4.2 Peptide tolerized mice lack epitope-specific $CD8^+$ T cell effector functions

Depletion of $tetramer^+$ $CD8^+$ T cells by repetitive peptide treatment also resulted in epitope-specific immune tolerance as assessed by the failure to produce IFNγ in response to stimulation with peptide *ex vivo* (Fig. 2.2 A-C). Additionally, both TSKB20

and TSKB74 tolerized mice were deficient in peptide-specific cytotoxicity *in vivo* at 28 days post-infection (Fig. 2.2 D-E). Compared to $OVA_{257-264}$-treated mice, TSKB20-treated mice killed the majority of TSKB74-pulsed target cells but not TSKB20-pulsed targets, and TSKB74-treated mice efficiently killed most TSKB20-loaded targets but not TSKB74-loaded targets (Fig. 2.2 D-E). Though peptide-treated mice exhibited essentially background levels of tolerizing epitope-specific $CD8^+$ T cells (Fig. 2.1 and 2.2 A-C), low levels of tolerizing epitope-specific cytotoxicity were apparently maintained *in vivo* (Fig. 2.2 D-E). Nevertheless, this residual killing was less than that previously observed for very sub-dominant *T. cruzi*-specific $CD8^+$ T cells (8, 37). Notably, $CD8^+$ T cells from $OVA_{257-264}$-treated mice produced IFNγ after stimulation with TSKB20 or TSKB74 peptide but did not produce IFNγ after stimulation with $OVA_{257-264}$ peptide (Fig. 2.2 A), indicating that the peptide treatment does not stimulate T cell priming in the face of infection-induced inflammation. From these experiments we conclude that peptide treatments prevented the normally robust expansion of functional $CD8^+$ T cells specific for the tolerizing peptide.

2.4.3 Peptide induced tolerance does not lead to enhanced regulatory T cell populations

Although the evidence above indicates that the decreased $tetramer^+$ populations and functions were epitope-specific, we considered the possibility that the high-dose of peptide administered could induce enhanced regulatory T cell (Treg) populations capable of general immune suppression. Approximately 15% of splenic $CD4^+$ T cells in naive mice expressed the FoxP3 transcription factor (two-thirds co-expressed the IL-2Rα chain (CD25)) (Fig. 2.3 A), whereas the proportion of Tregs decreased in spleens after *T. cruzi* infection (Fig. 2.3 A-B). Importantly, the proportion of Tregs in spleens of all peptide

treated groups were similar over the course of acute infection (Fig. 2.3 A-B), indicating that tolerization with *T. cruzi*-derived epitopes did not lead to an atypical expansion of Tregs.

Production of IL-10 by $CD4^+$ T cells has also been shown to suppress $CD8^+$ T cell function during infections (38). Polyclonally stimulated $CD4^+$ T cells from infected peptide-treated mice exhibited robust IFNγ production whereas few IL-10-producing $CD4^+$ T cells were detectable (Fig. 2.3 C). Both $OVA_{257-264}$ and TS peptide-treated mice maintained similar populations of cytokine producing $CD4^+$ T cells during acute infection (Fig. 2.3 C and data not shown). Furthermore, stimulation of splenocytes with TSKB20 or TSKB74 peptides *in vitro* failed to elicit IL-10 production irrespective of the *in vivo* peptide treatment (data not shown). Thus, we found no evidence that peptide-induced T cell tolerance was due to extrinsic T cell regulation, and conclude that it likely was the result of deletion of peptide-specific $CD8^+$ T cells.

2.4.4 Mice tolerized against immunodominant T. cruzi epitopes control acute infection

T. cruzi Brazil strain-infected mice genetically deficient for, or depleted of, $CD8^+$ T cells exhibit uncontrolled parasitemia and mortality by approximately one month post-infection (39-41). However, neither TSKB20- nor TSKB74-tolerized mice deficient in the respective immunodominant T cell population succumbed to acute infection of up to 35 days. Since skeletal muscle is a site of *T. cruzi* persistence in this model, we measured parasite load in muscle to determine the quality of immune control of *T. cruzi* infection in tolerized mice. The level of parasites, as measured by real-time PCR, was similar between peptide-treated groups of mice through-out infection (Fig. 2.4), with the exception that TSKB74-treated mice had an increased number of parasites at day 21 post-

infection compared with control $OVA_{257-264}$-treated mice (p=0.03) (Fig. 2.4). Although several TSKB20-tolerized mice had elevated numbers of parasites at 21 days post-infection, the group average was not statistically different (p=0.4) when compared to $OVA_{257-264}$ -treated mice. We observed slightly greater cellular infiltration as well as parasitized host cells in muscle sections of individual tolerized mice exhibiting increased parasite loads (data not shown), further suggesting that depleting TSKB20- or TSKB74-specific $CD8^+$ T cells can have a negative, though minor, impact on control of infection. Ultimately, both TS-peptide tolerized groups controlled parasite loads similar to $OVA_{257-264}$-treated mice (Fig. 2.4), demonstrating that immune control of *T. cruzi* infection occurs despite the absence of the normal immunodominant $CD8^+$ T cell population.

2.4.5 Tolerized mice generate protective effector $CD8^+$ T cell responses despite the absence of immunodominant $CD8^+$ T cells

Since the TSKB20- and TSKB74-specific $CD8^+$ T cells can represent as much as 40% of the total *T. cruzi*-specific $CD8^+$ T cell population in infected mice (8), we next assessed the effect of depleting the immunodominant T cells on the overall size of the responding $CD8^+$ T cell population bearing an activated phenotype. Most $CD8^+$ T cells in a naïve spleen express the lymph node homing receptor, CD62L, and the IL-7Rα chain, CD127, but very few of these naïve T cells have an antigen-experienced phenotype ($CD44^{hi}$ $CD11a^{hi}$) (Fig. 2.5 A and C). However, after *T. cruzi* infection, a large proportion of $CD8^+$ T cells from the control $OVA_{257-264}$-treated mice had up-regulated surface expression of both CD44 and CD11a (Fig. 2.5 A) and down-regulated CD62L and CD127 (Fig. 2.5 C). Spleens of TSKB20- and TSKB74-tolerized mice contained similar proportions of antigen-experienced ($CD44^{hi}$ $CD11a^{hi}$, $CD62L^{lo}$ $CD127^{lo}$) $CD8^+$ T

cells (Fig. 2.5 A-C) indicating that *T. cruzi*-specific T cells recognizing alternative parasite epitopes expand during *T. cruzi* infection when the normally immunodominant T cell populations are absent. The effector function of these T cells was confirmed by demonstrating their production of IFNγ in response to αCD3 stimulation (Fig. 2.5 D).

B6 mice tolerized simultaneously against both TSKB20 and TSK74 (labeled as TS tx) showed a predictable decrease in both TSKB20- and TSKB74-specific $CD8^+$ T cells (Fig. 2.6 A and B) but interestingly had increased numbers of activated ($CD44^{hi}$ $CD11a^{hi}$) $CD8^+$ T cells (Fig. 2.6 C) and a greater percentage of $CD8^+$ T cells capable of producing IFNγ in response to αCD3 stimulation (Fig. 2.6 B). Few activated $CD8^+$ T cells had decreased expression of CD3 or TCR β-chain in either control or TS-peptide tolerized mice (data not shown), excluding the possibility that the $CD44^{hi}$ $CD11a^{hi}$ $CD8^+$ T cell population consisted of expanded TSKB20- or TSKB74-specific cells that were undetected by tetramer staining due to downregulation of the TCR. Furthermore, the TS-tolerized mice had an increased number of tissue parasites at the peak of infection ($p=0.018$ at 21 days post-infection), although they had effectively controlled their parasite load by 28 days post-infection (Fig. 2.6 D). Thus, TSKB20- and TSKB74-specific $CD8^+$ T cells are required for optimal control of *T. cruzi* at the peak of the infection, but other $CD8^+$ T cells of unknown specificity can substitute to eventually contain the acute infection.

2.4.6 BALB/c mice tolerized against a dominant TS-derived epitope do not exhibit enhanced susceptibility to T. cruzi infection

BALB/c mice infected with Brazil strain *T. cruzi* also generate immunodominant $CD8^+$ T cells specific for a TS-derived epitope (13, 42), although the response to the H-

$2K^d$-restricted TSKD14 (IYNVGQVSI) is at a substantially lower frequency than observed for TSKB20 and TSKB74 in B6 mice (Fig. 2.7 A-D) (13, 42). However, similar to tolerized B6 mice, BALB/c mice tolerized with TSKD14 peptide showed the expected reduction in the TSKD14-specific response (Fig. 2.7 A-E) and also effectively controlled acute *T. cruzi* infection with parasite loads similar to control $LL0_{91\text{-}99}$-tolerized mice throughout the acute infection (Fig. 2.7 F). Furthermore, TSKD14-tolerization did not enhance regulatory T cell populations (data not shown) and a normal expansion of activated $CD8^+$ T cells was observed (data not shown) in mice depleted of TSKD14-specific $CD8^+$ T cells.

2.5 Discussion

Adaptive immunity to intracellular pathogens depends on $CD8^+$ T cell recognition of host cells presenting foreign antigen. The consequences of antigen-specific $CD8^+$ T cell responses focusing on a restricted versus broader set of pathogen-derived epitopes is not fully understood. The issue of immunodominance is particularly complicated for understanding immune control of protozoan parasites potentially presenting an expansive set of antigenic determinates, especially in comparison to viral pathogens where immunodominance has been extensively investigated. In this study, we addressed the role that immunodominant $CD8^+$ T cells play in host resistance to *T. cruzi* infection by ablating epitope-specific T cells via administrating high-doses of peptide. We achieved significant epitope-specific tolerance against the dominant TSKB20 and sub-dominant TSKB74 peptides in H-$2K^b$-restricted B6 mice, and the dominant TSKD14 peptide in H-$2K^d$-restricted BALB/c mice during acute *T. cruzi* Brazil strain infection. B6 mice tolerized to TSKB20, TSKB74, or both epitopes simultaneously, exhibited modest and

transitory increases in parasite load, suggesting that these greatly expanded T cell populations contribute to control of *T. cruzi*. Although these immunodominant TS-specific $CD8^+$ T cells represent a significant portion of the parasite-specific response, deleting them during infection ultimately had minor consequences for the outcome of infection; thus, they are not required for the acute resistance provided by the adaptive immune response to *T. cruzi*.

Immunodominant $CD8^+$ T cells are implicated as important for control of intracellular pathogens since they represent a majority of the responding T cell pool in circulation and at sites of infection. Attempts to determine the necessity of immunodominant T cells have often relied upon experimental infections with natural mutants or engineered viruses and bacteria that lack the epitope of interest (34-36, 43-49). Tolerance induction has also been used as a means of depleting mice of epitope-specific $CD8^+$ T cells during viral infection (30, 31, 50-55). In some cases, loss of the immunodominant $CD8^+$ T cell population impaired viral control (31, 34, 36, 44, 47, 50-54) or enhanced disease without affecting viral load (34), while in other situations, pathogen load was not affected (30, 43, 46, 49) or disease manifestations were ameliorated (30, 47, 52, 55). Compensation in the dominance hierarchy in the absence of immunodominant T cells occurred in many of these infection models (34-36, 43, 46-48), though often T cells recognizing minor epitopes emerge instead of enhanced sub-dominant responses (35, 36, 46, 47). Thus, elimination of immunodominant $CD8^+$ T cell responses has variable results depending on the infection model employed and is not readily predictable.

The observation that TS-peptide tolerized mice are resistant to acute *T. cruzi* infection directed us to question which parasite-derived peptides protective $CD8^+$ T cells respond to in the absence of the normally dominant responses. In our studies, both B6 and BALB/c mice depleted of the previously identified immunodominant TS-specific T cells expanded effector $CD8^+$ T cell populations to a similar level as control mice. These responding effector $CD8^+$ T cells had an antigen-experienced $CD44^{hi}$ $CD11a^{hi}$ phenotype and rapidly produced IFNγ in response to stimulation with anti-CD3 antibodies, whole *T. cruzi* lysate, or *T. cruzi*-infected dendritic cells (Rosenberg, unpublished results). However, we were unable to identify the antigen specificity of these compensating T cells in screens against previously predicted $CD8^+$ T cell targets (8) in either mouse strain (Rosenberg, unpublished results). A broader screen for epitope-specific responses will help identify the focus of compensating $CD8^+$ T cells in TS-peptide tolerized mice, though *T. cruzi*'s large proteome (>12,000 genes) may preclude a full description of all epitopes recognized in the mouse model.

Comparison of the reference genomes for the related trypanosomatids *T. cruzi*, *Trypanosoma brucei*, and *Leishmania major* revealed massive expansion in several gene families encoding surface proteins uniquely in *T. cruzi* (12, 56). Since surface expressed or secreted proteins are excellent sources of epitopes for both B cell and T cell recognition (57, 58), it is hypothesized that these large gene families have expanded because of immune selective pressure (26) and likely are involved in immune evasion (19, 59, 60). The TS gene-family has drastically expanded to represent upwards of 6% of the annotated *T. cruzi* CL Brener genome (12), and this may underestimate by half the true number of full and partial TS sequences (Weatherly, in preparation). Several

hundred TS genes encode epitopes recognized by TSKB20-specific $CD8^+$ T cells (8), and many of these gene products are represented in the proteome of the mammalian-dwelling stages of *T. cruzi* Brazil strain (22). Furthermore, distinct strains likely have distinct sets of TS genes (8), resulting in strain-variant immunodominance patterns (8, 13, 61). The benefit of carrying within an otherwise fairly compact genome a large number of genes encoding related but variable surface proteins is clear in cases where a pathogen expresses only one variant at a time, as with African trypanosomes (62). The relative benefit in terms of immune evasion of simultaneously expressing variants, some of which contain the same immunodominant epitope, is less evident. However, the fact that *T. cruzi* persists in hosts despite highly functional parasite-specific immune responses suggests that its strategy of immune evasion is successful – if not entirely obvious.

Rodrigues *et al.* (13, 23) have proposed that the strong immunodominance by TS-specific T cells restricts the generation of a broader, more protective immune response and allows *T. cruzi* to escape complete destruction. While immunodomination certainly restricts the focus of the immune response, it is probably not the primary reason why the majority of *T. cruzi*-infected hosts are unable to achieve sterile immunity. First, the documented immunodominant $CD8^+$ T cells of known specificity do not account for all of the T cells responding during infection; it is possible that these T cells of as yet unknown specificity are reactive to a broader set of *T. cruzi* epitopes. Second, vaccination to boost TS-specific $CD8^+$ T cells enhances protection in mice (17, 20, 63); if immunodominance prevented immune control then one would expect a stronger dominant response induced by prior vaccination to be deleterious for these hosts. Third, though humans and mice generate TS-specific $CD8^+$ T cells, the strong

immunodominance observed in B6 mice is somewhat anomalous compared to that observed for other mouse haplotypes (13, 42, 63) and humans (8, 64, 65). Therefore, either there are highly immunodominant $CD8^+$ T cell responses whose specificity has yet to be identified in these hosts, or immunodominance *per se* is not required for persistence. Finally, as shown in this study, tolerizing B6 mice against the immunodominant TS-epitopes had a transient negative impact on host control of *T. cruzi* replication, but little influence on the ultimate outcome of acute infection. It remains to be determined if diverting the focus of the $CD8^+$ T cell response away from these particular TS-encoded epitopes allows for the recognition of a broader set of epitopes (encoded by TS and other large gene-families or perhaps a more conserved set of genes), or otherwise alters the development of chronic disease due to the long-term persistence of *T. cruzi* in its host.

Since intracellular protozoan parasites do not rely on host cell machinery for gene expression, the pool of proteins readily introduced into the MHC I presentation pathway is controlled at the level of the parasite. Dominant antigens from numerous parasites are surface expressed and secreted proteins (6-8, 10, 66), therefore it seems likely that these pathogens have evolved to balance the necessary function of these proteins with the possibility of them serving as targets for immune recognition. Intriguingly, protective epitopes encoded in several secreted antigens of other parasites display significant variation both within (10) and between strains (6), similar to TS genes in *T. cruzi*. This variation has significant outcomes in terms of immunodominance (10) and cross-protection (6, 67). The issue of if and how variant T cell epitopes influence the outcome of these human diseases remains to be determined.

Figure 2.1. *Repetitive intravenous administration of peptide depletes epitope-specific $CD8^+$ T cells during acute T. cruzi infection.* (A) Protocol used to induce epitope-specific tolerance using high-dose peptide administration. Mice were injected with 100 μl peptide on indicated days before and after infection with 1,000 Brazil trypomastigotes. The primary injection was with 300 μg peptide and 100 μg was administered in the following treatments. Tolerized mice were allowed to rest for 7 days after final treatment before the experimental endpoint. Spleens of peptide-treated mice were assayed for the presence of epitope-specific $CD8^+$ T cells at 21 days post-infection with Brazil strain *T. cruzi*. (B) Splenocytes from $OVA_{257-264}$-, TSKB20-, and TSKB74-treated B6 mice were stained for CD44 and TSKB20/K^b or TSKB74/K^b tetramers. Histograms are gated on $CD8^+$ cells that were $CD4^-$ $CD11b^-$ $B220^-$. Numbers indicate percentage of $tetramer^+$ cells of total $CD8^+$ T cells. Data are from individual mice and are representative of five experiments. The total numbers of TSKB20/K^b (C) or TSKB74/K^b (D) $CD8^+$ T cells per spleen were calculated. Data are mean ± SEM and are cumulative from 3 separate experiments (n=4-11 per group). *, $p<0.05$ compared to OVA Tx group.

Figure 2.1

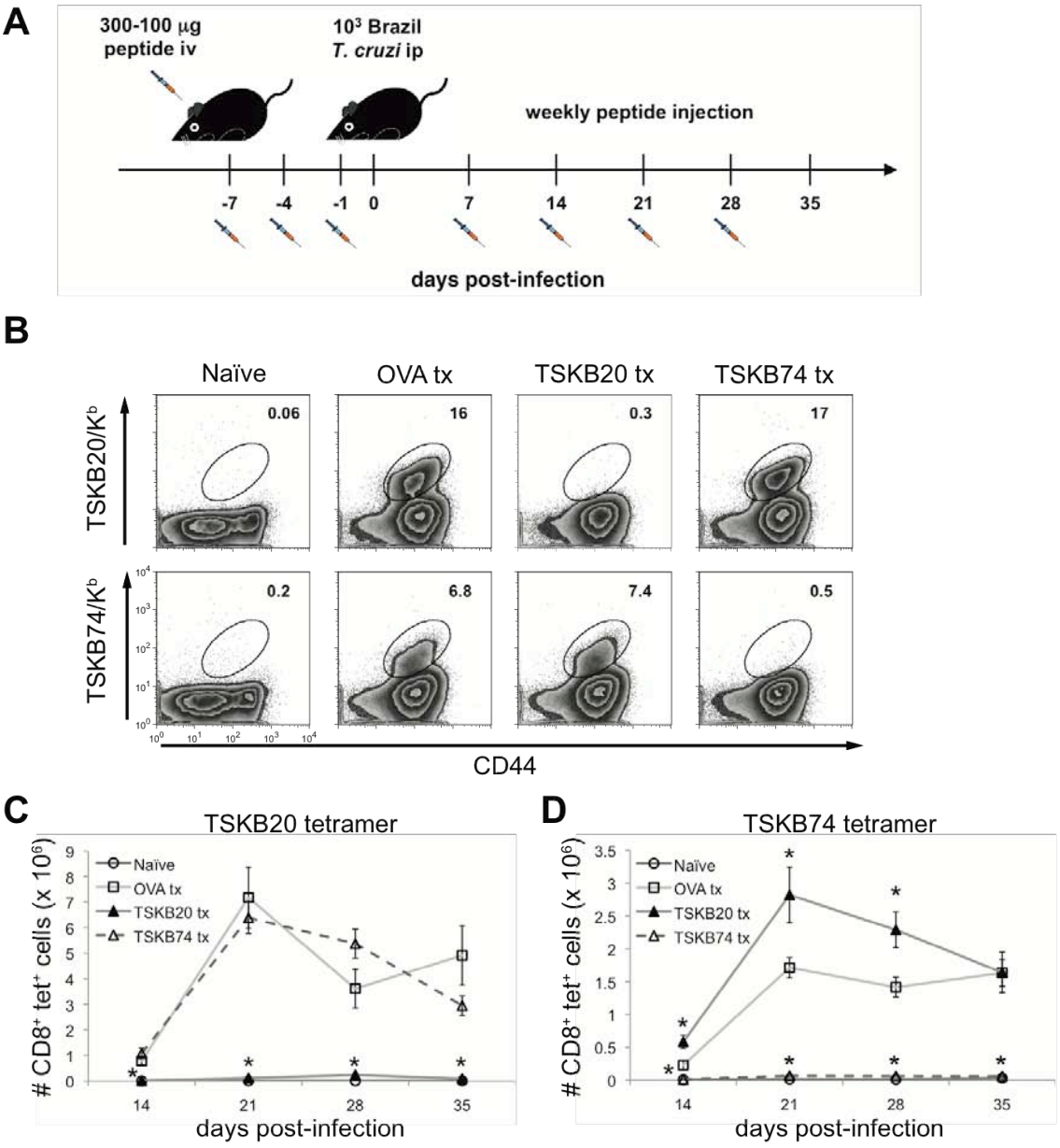
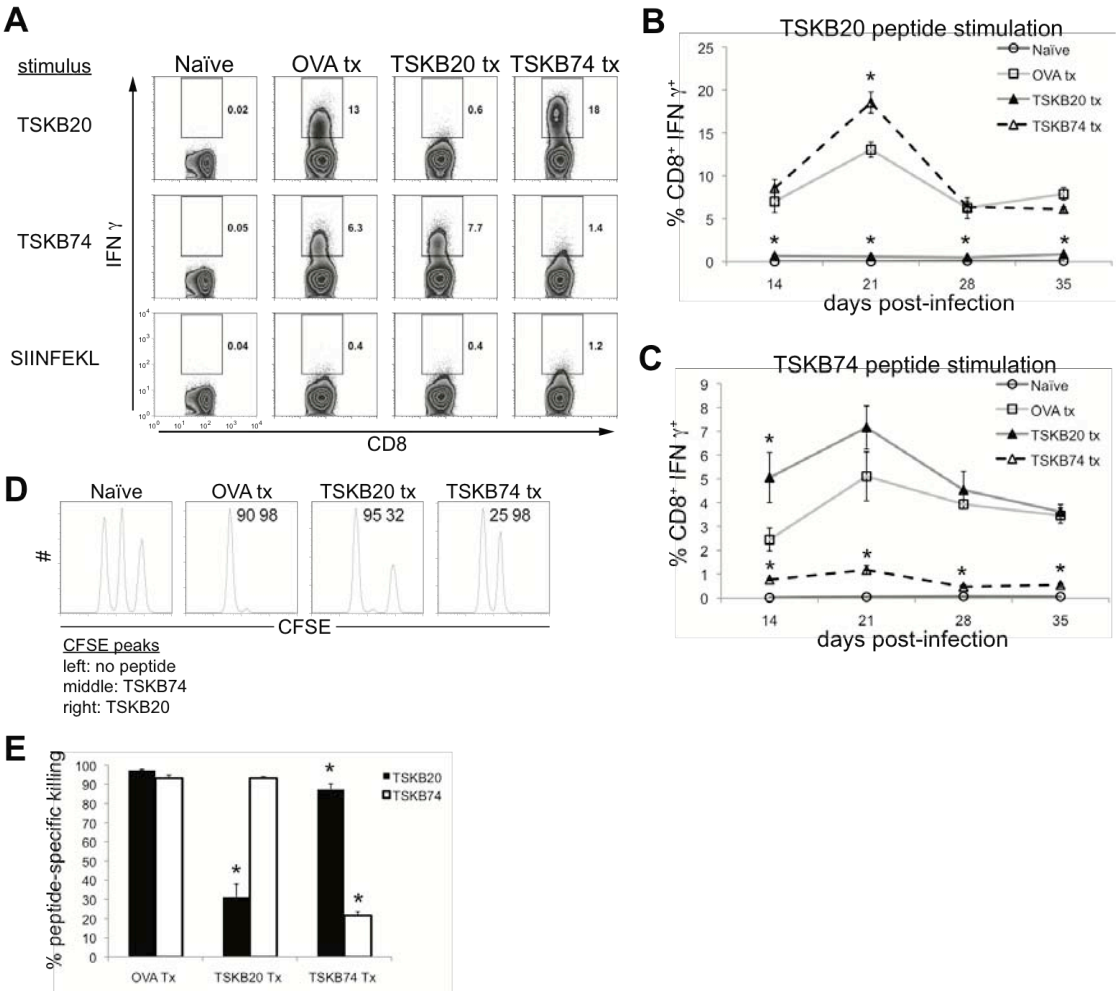

A
300-100 μg peptide iv
10³ Brazil T. cruzi ip
weekly peptide injection
-7
-4
-1
0
7
14
21
28
35
days post-infection
B
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
TSKB20/Kᵇ
TSKB74/Kᵇ
0.06
16
0.3
17
0.2
6.8
7.4
0.5
CD44
C
TSKB20 tetramer
CD8⁺ tet⁺ cells (x 10⁶)
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
days post-infection
D
TSKB74 tetramer
CD8⁺ tet⁺ cells (x 10⁶)
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
days post-infection

Figure 2.2. *Peptide tolerized mice lack epitope-specific CD8$^+$ T cell effector functions.* Peptide-treated mice were assayed for epitope-specific effector functions during acute *T. cruzi* infection. Splenocytes were incubated for 5 hours in the presence of 1 μM peptide and brefeldin A. (A) Representative intracellular IFNγ staining at 21 days post-infection. Histograms are gated on CD8$^+$ CD4$^-$ lymphocytes and numbers indicate percentage of cytokine-producing CD8$^+$ T cells. Data are from individual mice and are representative of five experiments. The percentage of CD8$^+$ T cells producing IFNγ in response to (B) TSKB20- or (C) TSKB74-peptide stimulation over the course of acute infection. Data are mean ± SEM from 1 experiment (n=4-5 per group) and are representative of five experiments. *, $p<0.05$ compared to OVA Tx group. Naïve splenocytes were pulsed with 1 μM TSKB20, 1 μM TSKB74, or no peptide and then labeled with high, medium, or low concentrations of CFSE, respectfully. At 28 days post-infection, equal numbers of each population were co-transferred iv into mice then detected in the spleens after 16 hours. Numbers indicate the percentage of specific lysis measured for individual mice. (E) Data are mean ± SEM and are cumulative of two *in vivo* CTL experiments (n=6-7 per group). *, $p<0.05$ compared to OVA Tx group.

Figure 2.2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stimulus
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
TSKB20
TSKB74
SIINFEKL
IFN γ
CD8
0.02
13
0.6
18
0.05
6.3
7.7
1.4
0.04
0.4
0.4
1.2
B
TSKB20 peptide stimulation
% CD8+ IFN γ+
days post-infection
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
C
TSKB74 peptide stimulation
% CD8+ IFN γ+
days post-infection
D
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
90 98
95 32
25 98
#
CFSE
CFSE peaks
left: no peptide
middle: TSKB74
right: TSKB20
E
% peptide-specific killing
TSKB20
TSKB74
OVA Tx
TSKB20 Tx
TSKB74 Tx

Figure 2.3. *Peptide induced tolerance does not enhance regulatory T cell populations.* Tolerized mice were monitored for regulatory T cell populations (A-B) as well as IL-10 production (C) during infection. (A) Representative intracellular staining for the FoxP3 transcription factor at 21 days post-infection. Histograms are gated on $CD4^+$, and were co-stained with CD25. Numbers indicate the percentage of cells in each quadrant. (B) The percentage of FoxP3 expressing $CD4^+$ T cells in spleens over the course of infection. Data are mean ± SEM from 2 experiments (n=3-12 per group). Splenocytes were incubated for 5 hours in media alone or with plate-bound mouse-CD3 monoclonal antibody in the presence of brefeldin A. Histograms are gated on $CD4^+$ $CD8^-$ cells, and numbers indicate the percentage of IL-10 and/or IFNγ positive cells per quadrant. Data are from individual mice and are representative of three experiments.

Figure 2.3

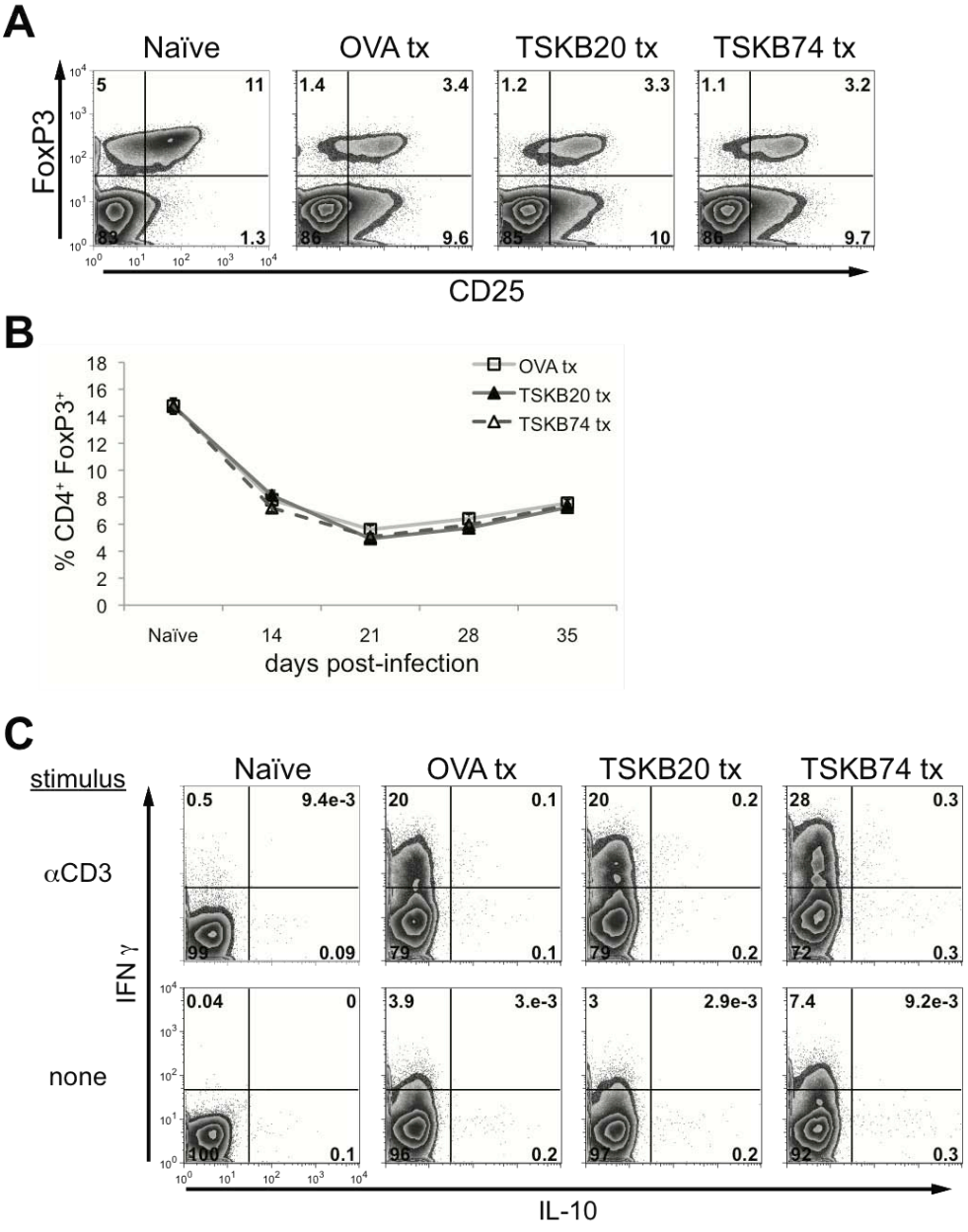

A
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
FoxP3
CD25
5
11
89
1.3
1.4
3.4
86
9.6
1.2
3.3
85
10
1.1
3.2
86
9.7
B
OVA tx
TSKB20 tx
TSKB74 tx
% CD4+ FoxP3+
Naïve
14
21
28
35
days post-infection
C
stimulus
αCD3
none
IFN γ
IL-10
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
0.5
9.4e-3
99
0.09
20
0.1
79
0.1
20
0.2
79
0.2
28
0.3
72
0.3
0.04
0
100
0.1
3.9
3.e-3
96
0.2
3
2.9e-3
97
0.2
7.4
9.2e-3
92
0.3

Figure 2.4. *Mice tolerized against immunodominant T. cruzi epitopes control acute infection.* The quantity of *T. cruzi* DNA in skeletal muscle of peptide-treated mice was detected at indicated time points by real-time PCR. Data points are for individual mice and bars are the mean from five experiments (n=11-17 per group). Several individuals were removed from the analysis because they passed the Grubbs' outlier test (GraphPad software). *, $p<0.05$.

Figure 2.4

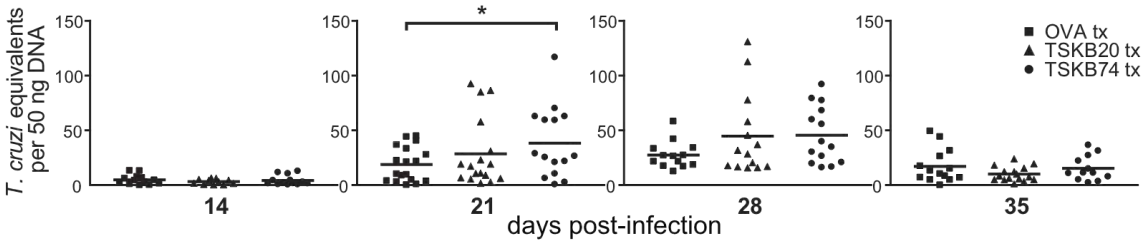

T. cruzi equivalents per 50 ng DNA
150
100
50
0
*
14
21
28
35
days post-infection
OVA tx
TSKB20 tx
TSKB74 tx

Figure 2.5. *Tolerized mice generate normal effector $CD8^+$ T cell populations despite the absence of immunodominant $CD8^+$ T cells.* Spleens of peptide-treated mice were assayed for the presence of antigen-experienced effector $CD8^+$ T cells. (A) Representative staining for CD44 and CD11a expression at 21 days post-infection. Histograms are gated on $CD8^+$ cells that were $CD4^-$ $CD11b^-$ $B220^-$. Similar results were obtained in three separate experiments. (B) The total number of $CD44^{hi}$ $CD11a^{hi}$ $CD8^+$ T cells per spleen was calculated. Data are mean ± SEM from one experiment (n=4-5 per group). *, $p<0.05$ compared to the OVA Tx group. (C) Representative staining for CD62L and CD127 at 21 days post-infection. Histograms are gated on $CD8^+$ cells that were $CD4^-$ $CD11b^-$ $B220^-$. Numbers indicate the percentage of $CD8^+$ T cells that have lost (left) or retained (right) expression of each marker (bold lines). Shaded lines are a fluorescence-minus-one control for the indicated marker. Similar results were obtained in three separate experiments. (D) Splenocytes were incubated for five hours in media alone or with plate-bound mouse-CD3 monoclonal antibody in the presence of brefeldin A at 35 days post-infection. Data are the mean (± SEM) percentage of $CD8^+$ T cells producing IFNγ for each condition (n=3-17 per group from three separate experiments). The percentage of $CD8^+$ T cells capable of producing IFNγ was also similar between groups at 14, 21, and 28 days post-infection (data not shown).

Figure 2.5

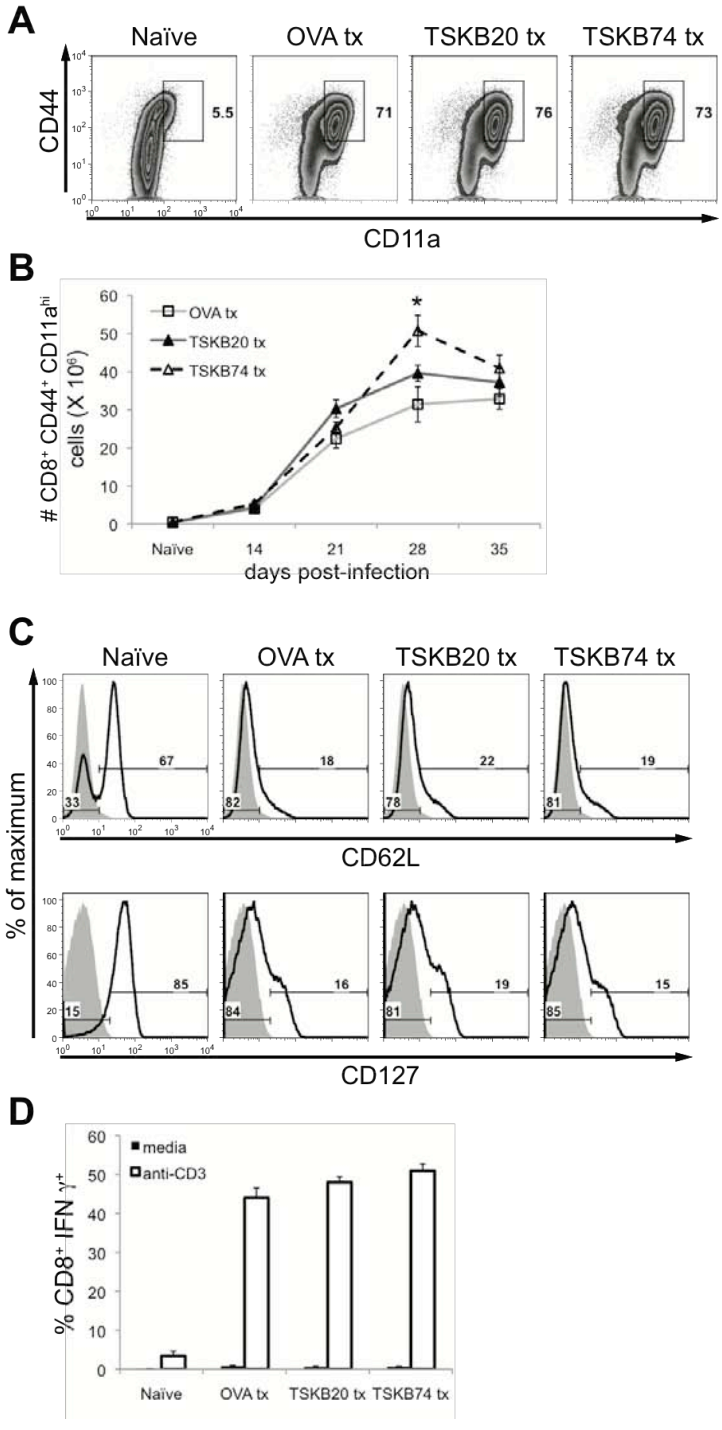

A
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
CD44
CD11a
5.5
71
76
73
B
CD8+ CD44+ CD11ahi cells (X 10^6)
OVA tx
TSKB20 tx
TSKB74 tx
*
Naïve
14
21
28
35
days post-infection
C
Naïve
OVA tx
TSKB20 tx
TSKB74 tx
% of maximum
CD62L
67
18
22
19
33
82
78
81
CD127
85
16
19
15
15
84
81
85
D
% CD8+ IFNγ+
media
anti-CD3
Naïve
OVA tx
TSKB20 tx
TSKB74 tx

Figure 2.6. *Mice tolerant to both TSKB20 and TSKB74 are ultimately resistant to T. cruzi infection.* B6 mice were injected with both TSKB20 and TSKB74 peptides (TS tx) similar to the description in Fig. 1A. TS Tx mice received the same total quantity of peptide as OVA Tx mice, i.e. half the effective dose of each individual peptide compared to experiments described in Fig. 1-5. (A) Representative tetramer staining at 21 days post-infection. Histograms are gated on $CD8^+$ cells that were $CD4^-$ $CD11b^-$ $B220^-$. Numbers indicate percentage of $tetramer^+$ cells out of $CD8^+$ T cells. Data are from individual mice and are representative of five experiments. (B) Representative intracellular staining for IFNγ produced in response to the indicated stimulus (see Materials and Methods). Histograms are gated on $CD8^+$ $CD4^-$ cells. Numbers indicate percentage of $IFN\gamma^+$ $CD8^+$ T cells. (C) The total number of $CD44^{hi}$ $CD11a^{hi}$ $CD8^+$ T cells per spleen was calculated. Data are mean ± SEM from one experiment (n=3-5 per group). *, $p<0.05$ compared to the OVA Tx group (same control individuals as in Fig 5B). (D) Quantity of *T. cruzi* DNA in skeletal muscle of peptide-treated mice detected by real-time PCR. Data points are individual mice and bars are the mean from two experiments (n=5-8 per group). One individual outlier was removed from the analysis. *, $p<0.05$.

Figure 2.6

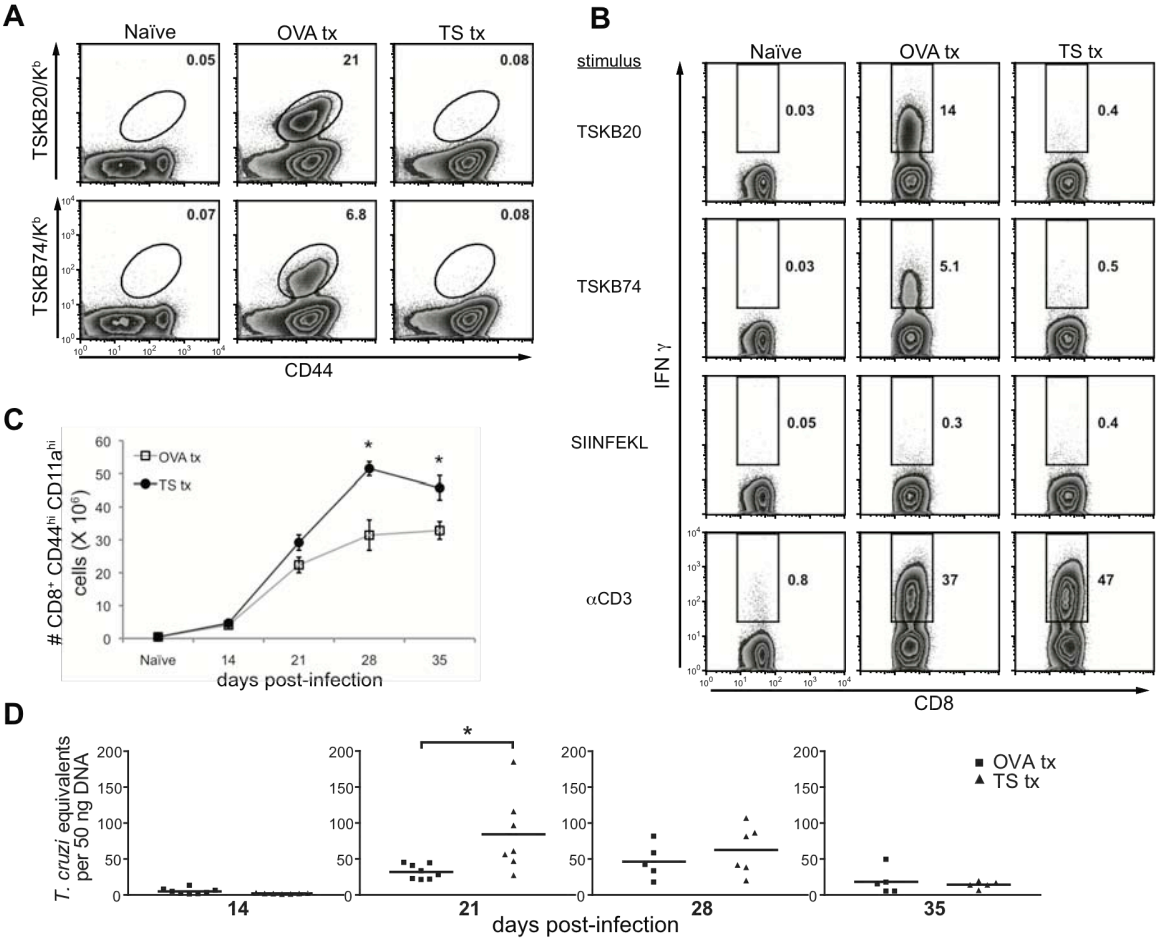
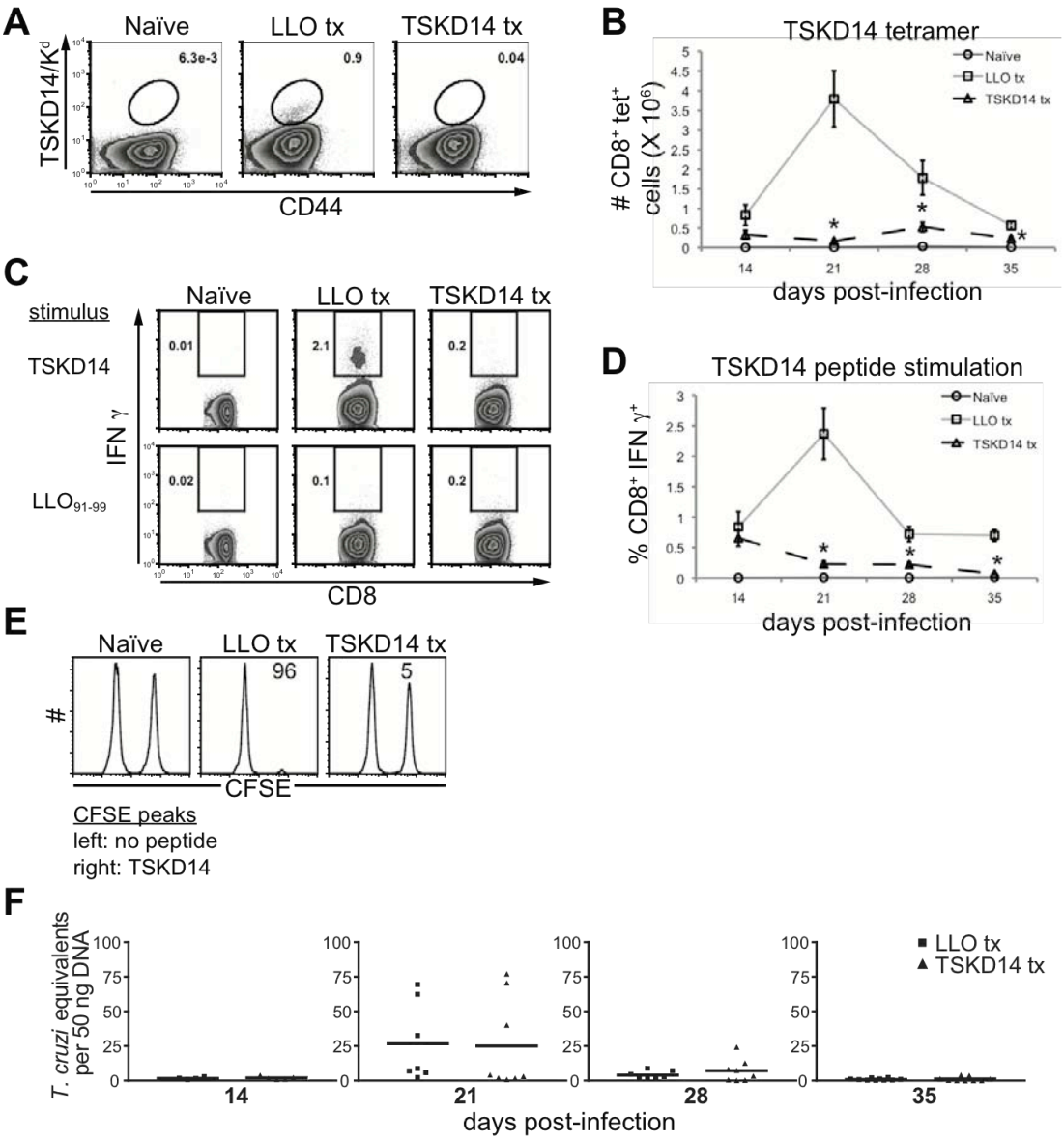

A
Naïve
OVA tx
TS tx
TSKB20/Kb
TSKB74/Kb
0.05
21
0.08
0.07
6.8
0.08
CD44
B
stimulus
Naïve
OVA tx
TS tx
TSKB20
TSKB74
SIINFEKL
αCD3
0.03
14
0.4
0.03
5.1
0.5
0.05
0.3
0.4
0.8
37
47
IFN γ
CD8
C
CD8+ CD44hi CD11ahi cells (X 10^6)
OVA tx
TS tx
Naïve
14
21
28
35
days post-infection
D
T. cruzi equivalents per 50 ng DNA
OVA tx
TS tx
14
21
28
35
days post-infection

Figure 2.7. *TSKD14 tolerized BALB/c mice remain resistant to T. cruzi infection.* BALB/c mice were treated with H-2K^{d}-restricted TSKD14 or LLO$_{91\text{-}99}$ peptide as described in Fig. 1 A. (A) Splenocytes from TSKD14- or LL0$_{91\text{-}99}$-treated BALB/c mice were stained for CD44 and TSKD14/K^{d}. Histograms are gated on CD8^{+} cells that were CD4^{-} CD11b^{-} B220^{-}. Numbers indicate percentage of tetramer^{+} cells of total CD8^{+} T cells. Data are from individual mice and are representative of three experiments. (B) The total number of TSKD14/K^{d} CD8^{+} T cells per spleen. Data are mean ± SEM and are cumulative from one experiment (n=4-5 per group). *, $p<0.05$ compared to LLO Tx group. (C) Representative intracellular staining for IFNγ produced in response to TSKD14 or LL0$_{91\text{-}99}$ peptide stimulation. Histograms are gated on CD8^{+} CD4^{-} cells. (D) The percentage of IFNγ producing CD8^{+} T cells specific for TSKD14 during acute infection. Data are mean ± SEM from two experiments (n=4-11 per group). *, $p<0.05$ compared to LLO Tx group. (E) TSKD14-specific *in vivo* cytotoxicity at 28 days post-infection. (F) Quantity of *T. cruzi* DNA in skeletal muscle of peptide-treated mice detected by real-time PCR. Data points are individual mice and bars are the mean from two experiments (n=4-10 per group). Several individuals were removed from the analysis because they passed the Grubbs' outlier test (GraphPad software).

Figure 2.7


A
Naïve
LLO tx
TSKD14 tx
TSKD14/K^d
6.3e-3
0.9
0.04
CD44
B
TSKD14 tetramer
CD8+ tet+ cells (X 10^6)
Naïve
LLO tx
TSKD14 tx
days post-infection
C
stimulus
Naïve
LLO tx
TSKD14 tx
TSKD14
LLO91-99
IFN γ
0.01
2.1
0.2
0.02
0.1
0.2
CD8
D
TSKD14 peptide stimulation
% CD8+ IFN γ+
Naïve
LLO tx
TSKD14 tx
days post-infection
E
Naïve
LLO tx
TSKD14 tx
#
96
5
CFSE
CFSE peaks
left: no peptide
right: TSKD14
F
T. cruzi equivalents per 50 ng DNA
LLO tx
TSKD14 tx
days post-infection

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CHAPTER 3

LONG-TERM IMMUNITY TO *TRYPANOSOMA CRUZI* IN THE ABSENCE OF IMMUNODOMINANT *TRANS*-SIALIDASE-SPECIFIC CD8+ T CELLS[1]

[1]Rosenberg, C.S. and Tarleton, R.L. 2011. Manuscript in preperation for submission to *The Journal of Experimental Medicine*.

3.1 Abstract

Trypanosoma cruzi infection drives the expansion of CD8+ T cells recognizing peptide epitopes encoded by the parasite's *trans*-sialidase (TS) gene family members. The immunodominance of TS is remarkable; up to 40% of all CD8+ T cells in C57BL/6 mice express T cell receptors capable of recognizing the dominant TSKB20 and sub-dominant TSKB18 epitopes, these responses are among the strongest documented in any infection. However, mice fail to completely clear *T. cruzi* and subsequently develop chronic disease despite generating such highly focused T cell responses, indicating these TS-focused responses are insufficient for complete immune clearance. Furthermore, mice tolerized to either the dominant or sub-dominant TS-derived epitope, or both simultaneously, control acute infection, likely due in part to the activities of CD8+ T cells specific for unidentified parasite-encoded epitopes. Here, we assessed the ability of these normally non-dominant CD8+ T cells to mediate long-term control of *T. cruzi* by generating transgenic mice expressing the TSKB20 or TSKB18 peptides as self-antigen to drive central tolerance. In agreement with our previous findings, mice expressing TSKB20, TSKB18 or both peptides, readily controlled *T. cruzi* infection and further developed functional effector CD8+ T cells that migrated into parasite-infected muscle and expressed signs of local activation there. Though mice deleted of the normally dominant CD8+ T cells controlled *T. cruzi* infection, they went on to develop chronic infection and similar disease as their infected wild-type littermates. Though immunodomination by TS-specific T cells interferes with the development of responses targeting other parasite-encoded epitopes, the absence of these described dominant CD8+ T cells did not appear to enhance protective immunity. Though these data do not support

a role for immunodominance as the main mechanism exploited by *T. cruzi* to promote its persistence in the immune host, they do indicate that strong responses against TS-derived epitopes are non-essential for long-term resistance. Furthermore, the relative position of an epitope within a CD8+ immunodominance hierarchy does not necessarily predict importance for parasite control.

3.2 Introduction

Though pathogens potentially express hundreds of thousands of antigenic peptides, a reproducible hierarchy of a limited set of dominant and sub-dominant T cells recognizing specific peptides expand in response to infection in a given host. Such immunodominance in CD8+ T cell responses is commonly observed in animal models of infection as well as humans infected with viral, bacterial, and protozoal pathogens (1-3). The generation of immunodominance hierarchies can be attributed to numerous factors (4-8), including competition for space and essential resources by dominant T cell clones (immunodomination) (9). Immunodominance is likely often beneficial to the host since energy and resources are invested in generating the most relevant antigen-specific T cells capable of pathogen clearance while eliciting minimal immunopathology. T cell recognition of epitopes located in conserved proteins may place evolutionary pressure on pathogens selecting for mutants that are less fit and more easily controlled. However, epitope-loss mutations may in turn evolve that benefit the pathogen by allowing escape of immune recognition. Immunodominance can also be detrimental to the host as overzealous CD8+ T cell responses can cause severe immunopathology as is the case for re-infections in hosts with highly focused pre-existing immunity or cross-reacting T cell populations (10). Persistently infecting pathogens also pose a problem since long-term antigen persistence drives chronic immunopathology that can be detrimental to the host (11, 12).

In contrast to viral and bacterial models in which immunodominance has been extensively studied (1, 2), less is known concerning immunodominant CD8+ T cells and their importance for control of intracellular protozoan parasites. Having relatively large

genomes and stage-regulated proteomes, these eukaryotic pathogens are more complex in terms of the array of antigens expressed by individual stages occurring within the same host. Furthermore, many parasites of medical importance to humans infect chronically or can re-infect immune individuals, suggesting immunity developed towards these pathogens is somewhat lacking (13). Recent studies have described CD8+ T cell immunodominance during infection with *Trypanosoma cruzi* (14, 15), an intracellular-replicating parasite that often persists for the lifetime of its mammalian host (16). Though *T. cruzi*'s genome contains several large gene families encoding surface proteins (20 to > 1000 annotated genes per family) (17, 18), many of which are likely shed and gain access to MHC-I presentation (19), the majority of the CD8+ T cells responding to infection in C57BL/6 (B6) mice target epitopes encoded by the strain-variant trans-*sialidase* (TS) family genes (14, 15). The dominant TSKB20-specific CD8+ T cells expand to represent 20-30% of the CD8+ population, and the sub-dominant TSK18-specific population represents 4-10% of CD8+ T cells during the acute peak of the response. These parasite-specific CD8+ T cells are maintained for the lifetime of mice at relatively high levels and exhibit robust effector functions even in the chronic phase of infection (20). Given that > 300 genes and pseudogenes encode the peptide epitopes recognized by these T cells (14), it is not surprising that these parasite-specific CD8+ T cells accumulate at such high numbers. Nevertheless, mice fail to completely clear *T. cruzi* despite developing such highly focused parasite-specific CD8+ T cell populations (20).

We previously analyzed the importance of immunodominant TS-specific CD8+ T cells during infection with *T. cruzi* (21). We found that mice tolerized against either

TSKB20 or TSKB74 (a cross-reactive peptide recognized by TSKB18-specific CD8+ T cells (14)) alone, or simultaneously, exhibited slight increases in parasite load during the peak of acute infection, though were ultimately resistant to infection and eliminated *T. cruzi* to a similar extent as control-treated mice. Since the TS genes evolved to expand uniquely in *T. cruzi* (22) and TS gene sequences exhibit considerable intra- and inter-strain variability (14, 17), it is hypothesized that this gene family is involved in immune evasion (21, 23-27). The observation that protective immunity is generated independent of CD8+ T cell recognition of the identified immunodominant TS-derived epitopes indicates that the described TS-focused CD8+ responses are not necessary and may block the generation of alternative CD8+ responses more capable of eliminating the parasite via immunodomination. To determine if diverting the focus of parasite-specific CD8+ T cells away from these TS-encoded epitopes alters the course of persistent *T. cruzi* infection, we have generated transgenic mouse lines expressing the TSKB20 and TSKB18 epitopes as self-antigens ensuring central tolerance to these epitopes in the context of infection. We find that TSKB-peptide transgenic mice generate effective CD8+ T cell responses that compensate in the absence of the known immunodominant TS-specific responses and mediate long-term control of persisting parasites. Identification of the targets recognized by these compensating CD8+ T cells will aid the development of novel immunotherapeutics against *T. cruzi* infection. Vaccines designed to boost sub-dominant responses might be a viable strategy for enhancing control of infections in which immunodominant responses have proven ineffective at establishing immunological cure, as is the case for parasites like *T. cruzi* that infect chronically.

3.3 Materials and Methods

3.3.1 Generation of TSKB20 and TSKB18 transgenic mice

For transgenic expression of the TSKB20 epitope (ANYKFTLV) or TSKB18 epitope (ANYDFTLV), a modified version of chicken ovalbumin (HA-OVA) was inserted into the eukaryotic expression vector pBroad3 (InvivoGen, San Diego, CA), which contains the mouse ROSA26 promoter and the human beta-globin gene 3' UTR. Briefly, HA-OVA was cloned from pTEX.HA-OVA (19) into pDONR-201 (Invitrogen, Carlsbad, CA). The sequence encoding the SIINFEKL epitope was replaced with a 54-bp oligonucleotide encoding TSKB20 or TSKB18 (including the endogenous residues flanking SIINFEKL) between StuI and AvaII. A 591-bp fragment of modified HA-OVA was amplified from pDONR-201-OVA/TSKB20 or pDONR-201-OVA/TSKB18 with PCR using primers designed to add 5' BglII followed by ATGGCC, and 3' TGA followed either by EcoRI or HindIII-PvuII-XbaI. These fragments were digested and ligated into the pBroad3 multiple cloning site between BglII and EcoRI to generate pBroad-TSKB20 or between BglII and XbaI to generate pBroad-TSKB18. The fidelity of transgenic TSKB20 and TSKB18 epitope expression in the context of the ovalbumin gene fragment was validated by generating transgenic EL4 tumor cells expressing either OVA/TSKB20 or OVA/TSKB18. C57BL/6 mice were injected intraperitoneally (i.p.) with the transgenic EL4 cell lines, and monitored for the development of transgene-specific CD8+ T cell responses. Mice challenged with EL4-OVA/TSKB20 generated TSKB20-specific CD8+ T cells, and mice challenged with EL4-OVA/TSKB18 only expanded TSKB18-specific CD8+ T cells. The 2,955-bp transgene expression cassette was liberated from the prokaryotic vector by double digestion with PacI and ApaLI and

the purified TSKB20 and TSKB18 transgene constructs were independently microinjected into fertilized C57BL/6 embryos by the MD Anderson Cancer Center Genetically Engineered Mouse Facility (Houston, Tx). Transgene positive founders were identified by PCR amplification of a 937-bp product using a forward primer located in the ROSA26 promoter (GGGAGAAGGGAGCGGAAAAG) and a reverse primer located in the human beta-globin 3'UTR (ATTAGGCAGAATCCAGATGC). Founder mice were mated with C57BL/6 mice obtained from the National Cancer Institute at Frederick (Frederick, MD) and kept under specific pathogen-free conditions at the Coverdell Center animal facility (University of Georgia, Athens, GA). Further identification of TSKB20- and/or TSKB18-specific transgenes was made using the described forward primer located in the ROSA26 promoter and the transgene-specific reverse primers EcoRI-MscI (TGGCCAGAATTCTCAATTGA) for TSKB20 or HindIII-PvuII (CAGCTGAAGCTTTCAATTGA) for TSKB18 transgene identification.

3.3.2 Parasites and mice

For *T. cruzi* infections, 8-12 week-old mice were infected ip with 1 x 10^3 trypomastigotes of either the Brazil strain or the Brazil-OVA strain (19). Trypomastigotes were maintained in tissue culture by serial passage through Vero cells (ATCC, Manassas, VA). C57BL/6 mice obtained from the National Cancer Institute at Frederick (Frederick, MD) and RAG1$^{-/-}$ OT-I TCR transgenic mice (CD45.1) were a kind gift of Dr. Kimberly Klonowski (University of Georgia, Athens, GA). All mice were kept under specific pathogen-free conditions at the Coverdell Center animal facility (University of Georgia, Athens, GA). Mice were euthanized by CO_2 inhalation. The University of Georgia Institutional Animal Care and Use Committee approved all animal use protocols.

3.3.3 T cell phenotyping

For *ex vivo* lymphocyte phenotyping, spleens were removed and dissociated by rubbing between two glass slides in a medium of hypotonic ammonium chloride to lyse red blood cells. Cell numbers were determined on a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA). For staining directly *ex vivo*, 5 x 10^6 washed splenocytes were incubated with antibodies in PBS with 1% bovine serum albumin and 0.05% sodium azide (PAB) (both from Sigma). Blood was collected from the retro-orbital sinus into a sodium citrate solution, washed in PAB and depleted of erythrocytes as described above. TSKB20/K^b and TSKB18/K^b tetramers were synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA) and were labeled with PE or APC (Molecular Probes, Carlsbad, CA). The SIINFEKL/K^b tetramer was prepared as described (28) and labeled with PE. Antibodies used were CD8 efluor450, CD4 PE-Cy5, CD44 FITC, KLRG-1 PE-Cy7, CD127 APC-efluor780, CD127 PE-Cy7, CD62L PerCP-Cy5.5 (eBioscience, San Diego, CA), CD11b PE-Cy5, B220 PE-Cy5 (CALTAG), CD44 APC, CD11a FITC, CD69 PerCP-Cy5.5, CD45.1 FITC (BD bioscience, San Jose, CA) and PD-1 PE-Cy7 (clone RMP1-30) (BioLegend, San Diego, CA). Cells were stained at 4° C for 30 minutes, washed with PAB and fixed in 2% formaldehyde. At least 500,000 cells were collected for each sample on a Cyan ADP using Summit version 4.3 (Beckman Coulter). FlowJo Flow Cytometry Analysis Software Version 9 (Tree Star, Ashland, OR) was used for analyses.

3.3.4 Lymphocyte isolation from peripheral tissues

Lymphocytes were isolated from lung and skeletal muscle essential as described (29). Mice were perfused prior to tissue removal by severing the hepatic vein and slowly

injecting 20 ml of a sodium citrate solution into the heart ventricles. Tissues were minced and incubated with stirring at 37°C for 30 min in Hanks' balanced salt solution (Sigma) with 1.25 mM EDTA, followed by treatment at 37°C for at least 1 hr with 260 mg/ml collagenase (250 units/mg) (Gibco) in RPMI with 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, and 5% FBS. The digested tissue slurry was filtered through 40 μM nylon cell strainers (BD Biosciences) and pelleted by centrifugation. Cell pellets were resuspended in 44% Percoll (GE Healthcare, Waukesha, WI) layered on 67% Percoll, followed by centrifugation at 850 X g to generate an interface. Cells were collected from the gradient interface and washed in RPMI with 10% FBS.

3.3.5 Synthetic peptides

Peptides were synthesized by GenScript (Piscataway, NJ) and solubalized in DMSO at 5 mM. Peptides were each diluted to 1 μM for stimulation assays. Published peptide sequences are as follows: TSKB20 (ANYKFTLV), TSKB18 (ANYDFTLV), TSKB38 (VNYNFTLV), TSKB60 (LSHSFTLV), TSKB81 (LSHSFTLV), TSKB92 (VGRPTTVV), TSKB388 (ANHRFTLV), ASP-1 (P14) (VNHDFTVV), ASP-2 (P8) (VNHRFTLV), TSA-1 (Pep77.2) (VDYNFTIV), Crz5 (PSVRSSVPL), Crz9 (VPLNKCNRL), Gft16 (SVPIRLLVL), Gft17 (LGFQERNVL), LYT-1p5 (ELTMYKQLL), PAR-1 (PFR-1) (YEIQYVDL), PAR-3 (PFR-3) (RVVSFTQM), and SIINFEKL.

3.3.6 T cell stimulation and intracellular staining

Splenocytes (1.5×10^6) were stimulated in 96-well round-bottom tissue culture plates (Costar, Corning, NY) at 37° C for 5 hr in the presence 1 μM peptide and brefeldin A (Golgi Plug, BD biosciences). For polyclonal activation, wells were pulsed with 30 μg

anti-mouse CD3e (eBioscience) for 1 hr at 37° C and excess antibody was removed prior to the addition of cells. For CD107a labeling, 2 μl CD107a FITC was added to the well during stimulation at 37° C. Cells were stained with CD8 efluor450 and CD4 on FITC (CALTAG) or PE (BD biosciences) followed by intracellular staining with IFNγ APC (BD biosciences) and TNFα PE-Cy7 (eBioscience) according to the cytofix/cytoperm kit (BD biosciences). At least 150,000 cells were collected for analysis. For intracellular granzyme B staining, cells were directly stained *ex vivo* without stimulation. Cells were surface stained followed by intracellular staining with anti-human granzyme B AF647 (clone GB11) (BD biosciences) using the cytofix/cytoperm kit.

3.3.7 In vivo cytotoxicity assay

Spleen cells from naïve mice were incubated for 1 hour at 37° C with 10 μM peptide or media alone, and then labeled with different concentrations of carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) as described (14) to produce CFSE high, medium, and low populations. Equal numbers of CFSE-labeled cells were transferred ip into recipients, and after 16 hours, splenocytes were isolated and CFSE-labeled cells were detected by flow cytometry. Percentage of specific killing was determined using the formula 1-[(% $CFSE^{lo}$ naïve / % $CFSE^{med/hi}$ naïve) / (% $CFSE^{lo}$ infected / $CFSE^{med/hi}$ infected)] X 100%.

3.3.8 Real-time PCR

Mouse hind leg muscles were collected and popliteal lymph nodes were removed as well as extraneous adipose tissue prior to DNA extraction as described (30). Extracted DNA was analyzed by real-time PCR essentially as described (30). PCR reactions consisted of iQ SYBR Green Supermix (BioRad) and primers specific for *T. cruzi* or mouse genomic

DNA (30). An iQ5 Multi-Color Real-Time PCR Detection System was used with iQ5 Standard Edition Optical System Software Version 2 (both BioRad). *T. cruzi* equivalents were calculated as the quantity of *T. cruzi* satellite DNA divided by the quantity of mouse TNFα DNA in each sample.

3.3.9 Statistical analysis

Statistical significance was calculated by 1-way ANOVA with Bonferroni's Multiple Comparison Test using Prism 4.0 software (GraphPad Software, San Diego, CA).

3.4 Results

3.4.1 TSKB20- and TSKB18-peptide transgenic mice exhibit central tolerance

To generate transgenic mice expressing the TSKB20 or TSKB18 epitope as neo-self-antigens, we constructed DNA sequences encoding the peptide fragments that allows for processing and H-2K^b-restricted presentation of TSKB20 or TSKB18 by mouse cells. We replaced the H-2K^b-restricted SIINFEKL epitope from the model antigen ovalbumin (OVA) with the sequences encoding TSKB20 or TSKB18 since the amino acid residues flanking minimal epitopes influence proteolytic cleavage and processing of antigenic peptides (31), and these sequences vary considerably at the TS genes encoding TSK20 and TSKB18 (17). We inserted the OVA-TSKB20 or OVA-TSKB18 fragments into a vector controlled by the mouse ROSA26 promoter to allow for ubiquitous expression (Fig. 3.1 A), and these ROSA.TSKB20 and ROSA.TSKB18 constructs were individually microinjected into fertilized B6 embryos to generate TSKB20 Tg and TSKB18 Tg mouse strains, respectively. Mice expressing both TSKB20 and TSKB18 were generated by crossing TSKB20 Tg and TSKB18 Tg mice to produce TSKB20/18 Tg mice that can be

identified using transgene-specific primers to PCR amplify either transgene sequence (Fig. 3.1 B and data not shown).

We confirmed that expression of TSKB20 and TSKB18 results in tolerance by infecting TSK20 Tg, TSKB18 Tg, TSKB20/18 Tg, and WT littermates with Brazil strain *T. cruzi* and assessed the level of TSKB20/Kb- or TSKB18/Kb-tetramer+ CD8+ T cells at the peak of acute infection (Fig. 3.1 C). Tetramer+ CD8+ T cells specific for the transgene-encoded epitope were not detected in spleens of TSKB20 Tg, TKSB18 Tg, or TSKB20/18 Tg mice, though TSKB18/Kb- or TSKB20/Kb-tetramer+ CD8+ T cells readily expanded in TSKB20 Tg and TSKB18 Tg mice, respectively, and activated CD44+ CD8+ T cells expanded normally in all *T. cruzi*-infected mice (Fig. 3.1 C and data not shown). Furthermore, *in vivo* cytotoxicity towards the respective transgene-encoded epitope remained undetectable during acute *T. cruzi* infection (Fig. 3.1 D). Thus, transgenic expression of TSKB20 and TSKB18 as neo-self antigens results in epitope-specific central tolerance, and does not prevent the activation and expansion of alternative parasite-specific CD8+ T cells in response to *T. cruzi* infection.

3.4.2 Long-term control of T. cruzi infection by TSKB20 and TSKB18 transgenic mice

Neither TSKB20- or TSKB18-specific CD8+ T cells are required for early/initial control of Brazil-strain *T. cruzi* infection in mice (21). Since *T. cruzi* infections can last for years in mice (and decades in humans), we asked if the TSKB20- or TSKB18-specific CD8+ T cells were essential for continued control of the persistent infection or contributed to disease development later in infection. We compared the long-term survival of TSK20 Tg, TSKB18 Tg, TSKB20/18 Tg, and WT littermates infected with *T. cruzi* and observed no differences in mortality for WT and TSKB-Tg groups up to a year

post-infection. To confirm that infected TSKB-Tg mice maintained *T. cruzi*-specific immunity, we monitored their peripheral blood for expansion of TSKB20/Kb- or TSKB18/Kb-tetramer+ CD8+ T cells as well as activated CD8+ T cells that had up-regulated surface expression of CD44 and KLRG-1, a marker of short-lived effector T cells (32), since we lacked suitable reagents to directly track *T. cruzi*-specific effector CD8+ T cells other than the TSKB20- and TSKB18-specific CD8+ T cells. Though infected TSKB-Tg groups never expanded tetramer+ CD8+ T cells recognizing their respective transgenic epitope (Fig. 3.2 A), they maintained expanded populations of CD8+ T cells recognizing the alternative non-tolerogenic TSKB-epitope (Fig. 3.2 A). Furthermore, all infected groups maintained frequencies of CD8+ T cells expressing CD44 and KLRG-1 comparable to that of wild type mice throughout the experiment (Fig. 3.2 B and C). During chronic *T. cruzi* infection, parasite persistence is required for the maintenance of elevated numbers of epitope-specific CD8+ T cells (20) as well as a KLRG-1+ phenotype (Bustamante and Rosenberg, unpublished), so it seemed likely that all groups of mice remained infected with *T. cruzi*. In contrast to mice that have fully resolved *T. cruzi* infection by drug-induced cure (20), the re-expression of surface CD127 on antigen-experienced CD8+ T cells occurred slowly in infected TSKB-Tg mice but at a similar rate as in chronically infected WT mice (Fig. 3.2 E and (20, 33)). The accumulation of CD127-expressing CD8+ T cells indicated that a subset of antigen-experienced CD8+ T cells acquired a resting memory phenotype in both WT and TSKB-Tg groups, reflecting decreased levels of parasites due to successful immune control, but not elimination.

If immunodomination by CD8+ T cells recognizing TS-derived epitopes facilitates immune escape by *T. cruzi*, then mice tolerized against the most dominant TS-specific responses might develop parasite-specific CD8+ T cell responses more capable of controlling infection and disease. To determine if parasite control was enhanced in the absence of the normally immunodominant TS-specific CD8+ T cells, we measured parasite loads by qPCR in skeletal muscle (a major site of Brazil strain *T. cruzi* persistence) of chronically infected (>8 months post-infection) TSKB-Tg and WT littermates. Similar low levels of parasite DNA were detectable in all groups (Fig. 3.2 F), and some individuals in each group maintained parasite levels below the limit of quantification by this assay. The difference between parasite levels in TSKB-Tg groups compared with infected WT littermates was not statistically different. Furthermore, pathology as a result of persisting parasites was similar as measured by H&E staining of skeletal muscle in all groups (Fig. 3.2 G-I). Thus, recognition of the immunodominant TSKB20 and TSKB18 epitopes is not required for long-term immunity to *T. cruzi*, and control of the parasite does not appear to be significantly enhanced in the absence of these responses.

3.4.3 Effector phenotype CD8+ T cells compensate in the absence of TSKB20 and TSKB18 immunodominance

The TSKB20 Tg mice consistently had a higher number of the normally sub-dominant TSKB18-specific CD8+ T cells in their spleens at the peak of infection (1.7-fold more than WT) (Fig. 3.3 A), yet the TSKB18-response rarely achieved the magnitude of the TSKB20-specific CD8+ T cells in the WT or TSKB18 Tg mice (3.1-fold more TSKB20/K^b+ than TSKB18/Kb+ CD8+ T cells in WT spleens) (Fig. 3.3 A).

The TSKB18-response was not significantly enhanced in TSKB20 Tg mice during the chronic phase of infection (Fig. 3.3 B). Therefore, normally non-dominant CD8+ T cells likely expand and perform critical effector functions that mediate parasite control in the absence of the described immunodominant CD8+ T cells, however, it does not appear that a single sub-dominant response alone completely replaces the dominant population.

We confirmed that *T. cruzi*-infected TSKB-Tg mice maintained effector CD8+ T cell populations during the acute and chronic phase. Compared with infected WT mice, the TSKB-Tg groups maintained similar proportions of CD8+ T cells expressing the markers of previous antigen encounter, CD44 and CD11a (Fig. 3.3 C), and the total number of antigen-experienced CD8+ T cells per spleen was not different between groups (Fig. 3.3 D). Most of the $CD44^{hi}$ CD8+ T cells co-expressed KLRG-1 during the acute phase (Fig. 3.2 B and data not shown), and numerous T cells expressing this short-lived effector phenotype were still detectable in the blood and spleens of chronically infected mice (Fig. 3.2 B and data not shown), albeit at a reduced level compared to the acute peak. Since the progressive contraction of KLRG-1-expressing CD8+ T cells occurs after antigen clearance (32) (Bustamante and Rosenberg, unpublished) and antigen-independent memory populations do develop during persistent *T. cruzi* infection (33), we considered that subtle differences in parasite load between WT and TSKB-Tg mice might be reflected in the accumulation of antigen-experienced CD8+ T cells expressing memory markers. Compared to acutely infected mice, more $CD44^{hi}$ $CD11a^{hi}$ CD8+ T cells expressed CD127 and CD62L during chronic infection (Fig. 3.3 E), however, similar proportions of these antigen-experienced memory cells were detected in spleens of chronically infected WT and TSKB-Tg groups (Fig. 3.3 F).

3.4.4 Functional effector CD8+ T cells migrate into non-lymphoid tissues and exhibit signs of recent activation in chronically infected skeletal muscle

During *T. cruzi* infection, parasite-specific CD8+ T cells migrate into peripheral non-lymphoid tissues including skeletal muscle, cardiac muscle, fat, lung and liver (Collins, manuscript in preparation) (34). Compared to lymphoid tissues, parasite-specific CD8+ T cells isolated from non-lymphoid tissues are loaded with higher amounts of the cytotoxic molecule, granzyme B (GrzB) (Bustamante and Rosenberg, unpublished). This promiscuous migration pattern of effector CD8+ T cells is similar to what has been observed during viral infection (35-37). In the chronic phase of Brazil-strain *T. cruzi* infection, parasite persistence is primarily restricted to skeletal muscle (38), cardiac muscle, and fat tissues, but not other organs such as the lung. Consistent with the importance of CD8+ T cells in controlling parasite load at sites of persistence, *T. cruzi*-specific CD8+ T cells exhibit an effector phenotype (34) and express CD69, a marker of recent activation, in muscle and fat tissues, but not in the lung where the parasite does not persist (Collins, manuscript in preparation).

In support of the idea that parasite-specific effector CD8+ T cells participate in control of *T. cruzi* in the absence of the TSKB20- and TSKB18-specific CD8+ T cells, we found a substantial fraction of $CD44^{hi}$ cells containing abundant GrzB in non-lymphoid tissues of chronically infected TSKB20/18 Tg mice (Fig. 3.4 A and B, top). Though GrzB-containing CD8+ T cells were also observed in the lungs, and to a lesser extent, the spleens of infected mice, only the skeletal muscle contained $CD44^{hi}$ CD8+ T cells that highly expressed CD69 (Fig 3.4 A and B, middle). Importantly, TSKB20/18 Tg mice had similar proportions of GrzB+ or CD69+ $CD44^{hi}$ CD8+ T cells compared to the

same tissues of infected WT-littermates (Fig. 3.4 B). We detected more CD44^{hi} CD8+ T cells expressing PD-1 in skeletal muscle in contrast to the spleen and lungs of infected mice (Fig. 3.4 A and B, bottom), however, TSKB20/18 Tg mice did not exhibit a greater frequency of PD-1+ CD8+ T cells in any tissue compared to infected WT tissues (Fig. 3.4 B bottom). PD-1 is expressed by activated CD8+ T cells and is up regulated on persistently activated T cells that undergo functional exhaustion (39, 40). To differentiate these two functional states in *T. cruzi*-infected muscle, we assessed the phenotype of PD-1+ and PD-1- CD44^{hi} CD8+ T cells to determine if T cells lacking effector function segregated with PD-1 expression. Unsurprisingly, infected muscle was the only tissue that had numerous GrzB+ effector CD8+ T cells that expressed CD69 (Fig. 3.4 C, middle), and importantly, the PD-1+ subset of CD8+ T cells contained the same proportion of GrzB+ effector CD8+ T cells as the PD-1- subset did in each tissue (Fig 3.4 C). Furthermore, the muscle-derived PD-1+ and PD-1- subsets contained a similar proportion of CD69+ cells, supporting the hypothesis that PD-1 expression is an indicator of recent activation in *T. cruzi*-infected muscle and not localized exhaustion.

In confirmation that effector CD8+ T cells do not suffer increased levels of exhaustion in the absence of control by the TSKB20- and TSKB18-responses, spleen-derived CD8+ T cells from chronically infected TSKB20/18 Tg mice responded to TCR-stimulation by rapid production of the cytokines IFNγ and TNFα (Fig. 3.4 D, top), and release of cytotoxic granule contents as measured by surface CD107a staining (Fig. 3.4 D, bottom). Importantly, chronically infected WT and TSKB-Tg mice maintained similar proportions of multi-functional CD8+ T cells (Fig. 3.4 E). Collectively, these data support a role for continued effector CD8+ T cell control of persistent *T. cruzi* in non-

lymphoid tissues of TSKB20/18 Tg mice and further demonstrate that the compensating effector CD8+ T cells remain highly functional in the absence of the immunodominant TSKB20- and TSKB18-specifc CD8+ T cells.

3.4.5 Sub-dominant parasite-derived epitopes are infrequently recognized by few CD8+ T cells in infected WT and TSKB20/18 Tg mice

To determine if the compensating effector CD8+ T cells that develop in infected TSKB20/18 Tg mice recognize previously identified parasite-derived epitopes, we incubated spleen cells from acutely or chronically infected WT and TSKB20/18 Tg mice with H-2K^b-restricted peptides and assessed specific responsiveness by intra-cellular staining for IFNγ. The irrelevant epitope, SIINFEKL, from chicken albumin, was used as a negative control, and we stimulated with TSKB20 and TSKB18 peptides as positive controls for WT CD8+ T cell responsiveness and also to confirm non-responsiveness by the TSKB20/18 Tg CD8+ T cells. We first measured responsiveness to TS-encoded epitopes that are homologues of TSKB20 and TSKB18 (14), and found few CD8+ T cells in WT or TSKB20/18 Tg mice that responded to the epitopes encoded by the TS vaccine candidates TSA-1 (Pep77.2) (41, 42), ASP-1 (P14) and ASP-2 (P8) (43, 44) (Fig. 3.5 A and B). Previously identified variant TS-peptides encoded at homologous (TSKB60, TSKB81, TSKB38, TSKB388) and non-homologous (TSKB92) positions of TS-genes (14) were also not recognized by many effector CD8+ T cells in the infected WT and TSKB20/18 Tg (Fig. 3.5 A and B). Though the pooled TSKB38 and TSKB388 peptides did consistently stimulate the WT CD8+ T cells to produce IFNγ, CD8+ T cells from TSKB20/18 Tg mice rarely responded to the same extent at acute or chronic stages of infection. Sub-dominant epitopes encoded by cruzipain (Crz5 and Crz9) and β-

galactofuranosyl transferase (Gft16 and Gft17) gene family members (14, 33, 45) were also not significant targets of effector CD8+ T cells in infected TSKB20/18 Tg mice (Fig. 3.5 A and B). Several non-TS vaccine candidates have been identified from *T. cruzi*'s paraflagellar rod proteins (PAR-1-derived PFR-1 and PAR-3-derived PFR-3) (46) as well as LYT-1 (LYT-1p5) (47), however we found few CD8+ T cells responding to the described H-2K^{b}-restricted peptides encoded by these proteins (Fig. 3.5 A and B) as well. Thus, in the absence of the immunodominant TSKB20- and TSKB18-responses, the main focus of the responding CD8+ T cells cannot be attributed to these known parasite-derived epitopes.

3.4.6 Parasite-specific CD8+ T cells recognizing an engineered dominant epitope partially compensate in the absence of the TSKB20- and TSKB18-response

Though our observations supported the hypothesis that effector CD8+ T cells control *T. cruzi* in the absence of the described immunodominant responses (Fig. 3.2-3.4), we lacked conclusive evidence of their specificity for *bona fide* parasite-derived epitopes. To confirm that the effector CD8+ T cells generated by infected TSKB20/18 Tg mice are in fact specific for parasite antigen, we infected WT and TSKB-Tg mice with Brazil-strain *T. cruzi* parasites stably transfected with the model antigen ovalbumin (Brazil-OVA) (48). Both WT and TSKB-Tg groups expanded SIINFEKL/K^{b}-tetramer+ CD44^{hi} CD8+ T cells (Fig. 3.6 A) and we observed similar numbers of SIINFEKL-specific CD8+ T cells in spleens of all groups infected with Brazil-OVA (Fig. 3.6 B). Since the T cells that survive thymic selection differ between WT and mice transgenically expressing TSKB20 and TSKB18, we considered that this altered TCR-repertoire might impact the ability of the endogenous SIINFEKL-specific CD8+ T cells

to fully compensate in the absence of TSKB-peptide dominance. However, transferred TCR transgenic OT-I cells specific for SIINFEKL expanded to the same extent in the Brazil-OVA infected WT and TSKB-Tg mice (Fig. 3.6 C). The majority of responding OT-I cells expressed surface KLRG-1 and many contained GrzB (Fig. 3.6 D and E), supporting these effector phenotypes as surrogate markers for parasite-responsive CD8+ T cells. Though slightly more OT-I cells stained for GrzB in Brazil-OVA infected WT spleens compared to the TSKB20/18 Tg group (Fig. 3.6 E), a similar proportion of OT-I cells produced cytokines and released cytotoxic granules when stimulated with SIINFEKL (Fig. 3.6 F). These data show that alternative parasite-derived epitopes are targeted by effector CD8+ T cells, however, these neo-immunodominant T cells do not necessarily expand more or function better in the absence of TSKB20- and TSKB18-immunodomination.

3.5 Discussion

How critical for control of infection are CD8+ T cells specific for only a few of the thousands of potential pathogen-derived epitopes? Despite numerous studies on immunodominant CD8+ T cells, relatively few studies have attempted to elucidate the relationship between immunodominance and protective immunity (1, 4, 49). This is especially true for parasitic diseases where few studies have been conducted to determine the mechanisms accounting for immunodominance (3, 13-15, 50, 51), and the roles of these parasite-specific CD8+ T cells in controlling infection (21, 52). Some suggest that narrow responses favor establishment of chronic infections (4, 53), whereas broadly focused responses are more capable of long-term control (27, 53).

We have addressed the importance of CD8+ T cells recognizing the described immunodominant epitopes encoded by *T. cruzi*'s TS-gene family ((21) and this study). Control of acute infection was observed in *T. cruzi*-infected mice that were peptide-tolerized against the TSKB20 or TSKB18 (cross-reactive) epitopes, as well as both epitopes simultaneously (21). Here, we confirm and extend this finding to long-term control of chronic *T. cruzi* infection using transgenic mice expressing TSKB20 and TSKB18 as self-antigen to ensure permanent deletion of peptide-specific CD8+ T cells. We found that immunity to *T. cruzi* is generated independent of recognition of the dominant TSKB20 and TSKB18 epitopes since TSKB20 and TSKB18 transgenic mice, as well as TSKB20/18 double transgenic mice, survive up to a year after *T. cruzi* infection. These findings are important since *T. cruzi* infection drives disease over long periods of chronic infection, and subtle differences in acute control may amplify over time to exacerbate disease. Furthermore, dominant CD8+ T cell responses may play a part in causing pathology during chronic infection when antigen persists. CD8+ T cells recognizing TS-derived epitopes likely contribute to control of *T. cruzi* (21, 44, 54, 55), although to a limited extent, as mouse strains generating highly focused TS-specific responses contain infection but cannot fully eliminate parasites (20, 56) and parasitological cure has not been demonstrated by TS immunizations alone (57).

We have shown previously (21), and here, that mice lacking TSKB20 immunodomination generate increased numbers of the normally sub-dominant TSKB18-specific CD8+ T cells, however, this increased response was isolated to the acute phase and was relatively subtle compared to the WT immunodominance exhibited by TSKB20-specific CD8+ T cells. Similar to their WT counterparts, chronically infected

TSKB20/18 Tg mice maintained effector and effector-memory phenotype CD8+ T cell populations that expressed a resting phenotype in lymphoid tissues and a highly activated phenotype within *T. cruzi*-infected muscle, supporting their specificity for parasite antigens presented locally in inflamed tissue. Furthermore, WT and TSKB20/18 Tg mice generate comparable SIINFEKL-specific responses during acute infection with Brazil-OVA parasites, and these engineered parasite-specific CD8+ T cells expressed a similar effector phenotype as CD8+ T cells observed in lymphoid organs of infected TSKB-Tg mice. In the absence of control by the normally immunodominant CD8+ T cells, compensating effector CD8+ T cells remained highly functional and did not exhibit signs of exhaustion due to chronic antigen stimulation, so CD8+ T cell mediated immunity to the parasite is apparently maintained by non-dominant CD8+ T cells. Furthermore, though competition by immunodominant CD8+ T cells can affect the expansion of sub-dominant CD8+ T cells, parasite-specific CD8+ T cells of unknown specificity expand and compensate to provide TSKB20/18 Tg with a highly functional effector T cell pool.

Observing that normally non-dominant CD8+ T cells generated by TSKB-Tg mice mediate long-term protection indicates it is a worthwhile endeavor to identify the alternative sources of protective antigens beyond TS for development of a *T. cruzi* vaccine. Using infected TSKB20/18 Tg mice should facilitate this search, and we attempted to address this here by screening for enhanced recognition of published *T. cruzi*-derived epitopes. Though we were unable to identify the epitope-specificity of compensating CD8+ T cells, we have ruled out several sources that include several epitopes encoded by TS (TSA-1, ASP-1 and ASP-2) (41, 43) and non-TS vaccine candidates (LYT-1, PAR-1, and PAR-3) (46, 47), as well as several reported sub-

dominant epitopes also encoded by TS or cruzipain and b-galactofuranosyl transferase (GFT) gene family members (14, 33, 45). Though immunizations with the previously mentioned vaccine candidates do elicit significant epitope-specific CD8+ responses that protect mice from *T. cruzi* challenge (42, 44, 46, 47), CD8+ T cells recognizing these epitopes are rarely detectable during normal Brazil-strain *T. cruzi* infection, even in the absence of competition by the described immunodominant CD8+ T cells. Many of these previously characterized responses are greatly reduced or absent in the infected TSKB20/18 Tg mice, suggesting these epitope-specific responses are in fact attributable to cross-reactive recognition by the promiscuous TCRs of TSKB20- and TSKB18-specific CD8+ T cells (14). These underwhelming responses observed indicate that a broader set of target genes must be assessed for recognition by the normally non-dominant CD8+ T cells. These target genes might include genes that are not members of variant surface antigens and are predicted to be released by *T. cruzi*, thus making them available for recognition during infection (19). Since the majority of these candidate target genes are uncharacterized and of low copy number in the genome, a high-throughput screening approach would likely facilitate these efforts.

T. cruzi's TS family exhibits considerable intra- and inter-strain variability in sequence (17, 58-60) and expression patterns (25) which impacts generation of CD8+ immunodominance hierarchies (14, 15). We and others (21, 23-27) hypothesize that variant TS genes function as a means of immune evasion, and it has been suggested that immunodominance by TS-epitope specific CD8+ T cells blocks the generation of parasite-focused responses better capable of parasite eradication (21, 27). If immunodomination by TSKB20- and TSKB18-specific CD8+ T cells inhibits

development of more protective CD8+ responses, one would predict that TSKB20/TSKB18 Tg mice should control *T. cruzi* infection better than WT counterparts, which is not the case as shown in this study. TSKB20/18 Tg mice develop effector CD8+ responses that mediate control of persisting parasites and chronic disease similar to those responses generated by infected WT mice (likely focusing on TSKB20, TSKB18, as well as the unidentified CD8+ targets that await discovery). However, finding that the normally immunodominant TS-specific CD8+ responses do not appear to inhibit optimal immunity does not disprove the hypothesis that TS functions as an immune evasion mechanism. *T. cruzi*'s genome contains thousands of variant TS genes (17, 18), as well as other gene families of surface expressed proteins that encode variant MHC-I-restricted epitopes as well (14). Since our manipulations have deleted CD8+ T cells recognizing only a few of the thousands of TS-encoded epitopes, we cannot exclude immunodomination by CD8+ T cells responding to other TS-encoded epitopes, or other large gene family member-encoded epitopes, as being responsible for preventing the generation of CD8+ response capable of sterile immune control. As we cannot genetically ablate all TS or other large gene family members, nor is it likely that we can tolerize mice against every recognized epitope encoded by variant surface antigens, we currently cannot fully define the role that immunodominance of *T. cruzi*'s variant surface antigens contributes towards immune evasion. However, we can conclude here that immunodominance by a given parasite-specific CD8+ T cell population does not define importance for parasite control, as mice lacking the described immunodominant CD8+ responses are clearly able to control *T. cruzi* infection as well as WT mice.

Development of T cell-based vaccines has focused on enhancing immunodominant T cells prior to pathogen encounter, though boosting sub-dominant or cryptic responses has also been suggested as an important goal for vaccines (61). Induction of broadly focused T cell responses might be particularly important for therapeutic vaccines that enhance control of chronically infecting pathogens where the immunodominant response has already proven ineffective at establishing immunological cure. In agreement with recent studies (62), our data strongly suggest that TS-based vaccination strategies might fail if applied in the field where *T. cruzi* strains express unique versions of TS genes (14) since even under controlled experimental conditions the recognition of immunodominant TS-derived epitopes is not required for protection. Using the TSKB20/18 Tg mice as an antigen discovery platform should facilitate identification of alternative sources of antigen that currently exist below the limit of assay detection.

Figure 3.1. *Transgenic expression of TS-derived peptides results in epitope-specific tolerance.*

(A) Map of TSKB20 and TSKB18 transgene constructs. Relevant restriction sites used for cloning are indicated. (B) PCR products of the transgene construct amplified using TSKB20- or TSKB18-construct-specific primers from gDNA of transgenic founder mice but not from wild-type C57BL/6 gDNA. Primers used were located in the ROSA26 promoter sequence and the unique OVA/TSKB20 or OVA/TSKB18 insert's 3' restriction sites. (C) Splenocytes from Naïve or acute phase *T. cruzi* infected WT, TSKB20 Tg, TSKB18 Tg or TSKB20/18 Tg mice were stained for CD44 and TSKB20/K^b or TSKB18/K^b tetramers. Histograms are gated on CD8+ CD4- and numbers indicate percentage of $CD44^{hi}$ tetramer positive CD8+ cells. Data are from infection-matched individuals and are representative of 2-4 experiments per acutely infected group. (D) Naïve splenocytes were pulsed with 1 μM TSKB20, 1 μM TSKB18, or no peptide and then labeled with high, medium, or low concentrations of CFSE, respectively. At 27 days post-infection, equal numbers of each population were co-transferred i.v. into mice and detected in the spleens after 16 h. Numbers indicate the percentage of specific lysis measured for representative individuals compared with Naïve. Data are from one experiment with 3-5 mice per infected transgenic group.

Figure 3.1

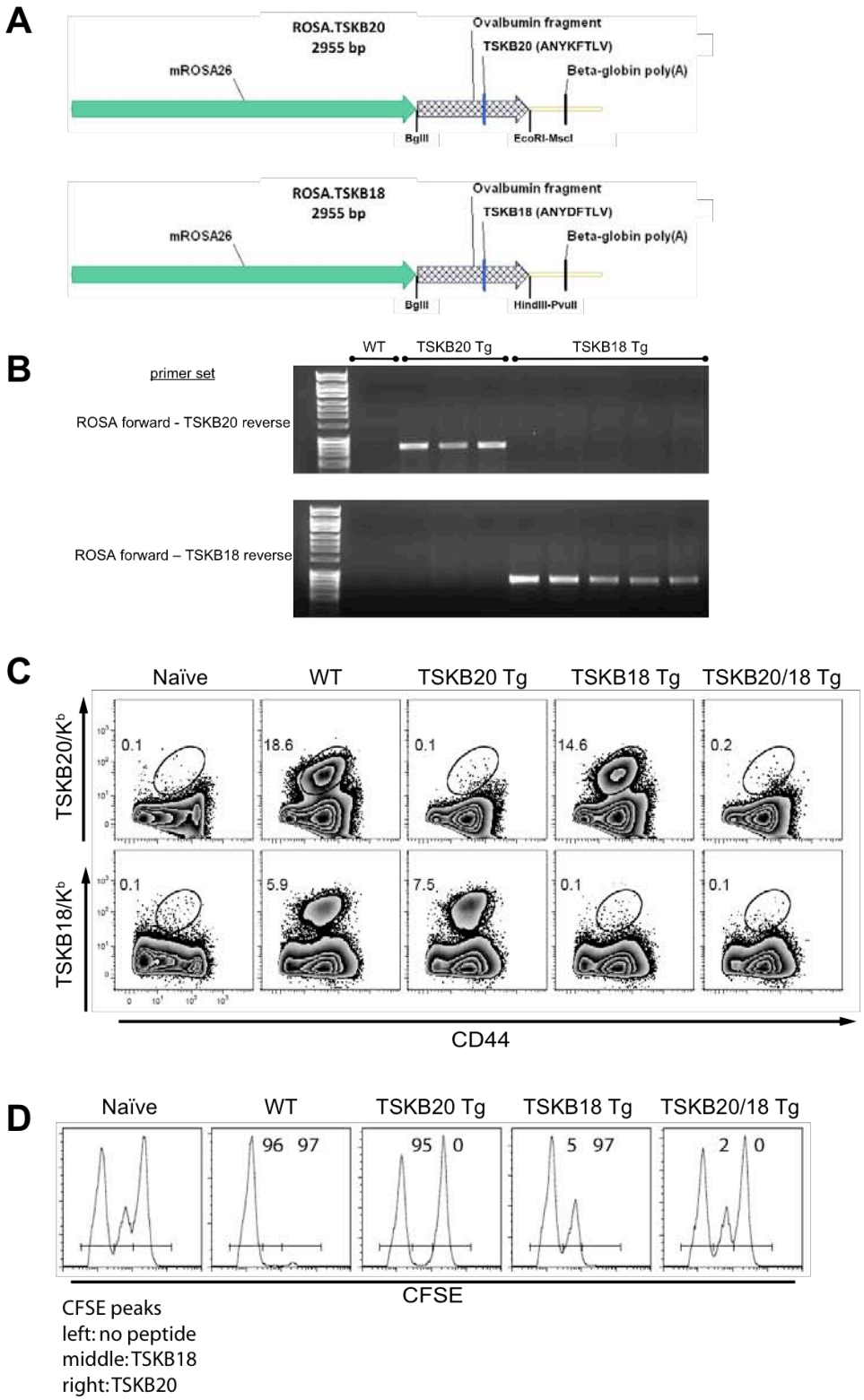

A
ROSA.TSKB20
2955 bp
Ovalbumin fragment
TSKB20 (ANYKFTLV)
mROSA26
Beta-globin poly(A)
BglII
EcoRI-MscI
ROSA.TSKB18
2955 bp
Ovalbumin fragment
TSKB18 (ANYDFTLV)
mROSA26
Beta-globin poly(A)
BglII
HindIII-PvuII
B
WT
TSKB20 Tg
TSKB18 Tg
primer set
ROSA forward - TSKB20 reverse
ROSA forward – TSKB18 reverse
C
Naïve
WT
TSKB20 Tg
TSKB18 Tg
TSKB20/18 Tg
TSKB20/Kb
TSKB18/Kb
0.1
18.6
0.1
14.6
0.2
0.1
5.9
7.5
0.1
0.1
CD44
D
Naïve
WT
TSKB20 Tg
TSKB18 Tg
TSKB20/18 Tg
96 97
95 0
5 97
2 0
CFSE
CFSE peaks
left: no peptide
middle: TSKB18
right: TSKB20

Figure 3.2. *Long-term control of T. cruzi by TSKB-peptide Tg mice.*

(A) TSKB20/K^b+ or TSKB18/K^b+ CD8+ T cells were measured longitudinally in peripheral blood of Naïve or *T. cruzi* infected WT, TSKB20 Tg, TSKB18 Tg or TSKB20/18 Tg mice as described in (Fig. 1 C). (B) $CD44^{hi}$ KLRG-1+ CD8+ T cells detected in blood of Naïve or *T. cruzi* infected TSKB20/18 Tg and WT littermates at 21 days post-infection. Histograms are gated on CD8+ CD4- lymphocytes and numbers indicate proportion of cells within gates. (C) $CD44^{hi}$ KLRG-1+ CD8+ T cells measured longitudinally in blood of mice analyzed as in *B*. (D) CD127 staining on peripheral blood $CD44^{hi}$-gated CD8+ T cells (black line) compared with CD127 staining on total CD8+ T cells from a naïve individual (red line). Filled grey histograms are $CD44^{hi}$-gated CD8+ T cells not stained for CD127. Numbers indicate proportion of $CD44^{hi}$-gated CD8+ T gated cells expressing low (left) or high (right) levels of CD127 at 220 days post-infection. Data in *B* and *D* are representative of 2 identical experiments (n=4-8 per infected group). (E) Proportion of $CD44^{hi}$-gated CD8+ T cells stained positive for CD127 measured longitudinally in blood of mice analyzed as in *D*. Data in *A*, *C*, and *E* are mean ± SEM (n=3-11 per infected group) from one experiment and are representative of two similar longitudinal experiments. (F) The quantity of *T. cruzi* DNA in skeletal muscle was detected by real-time PCR in mice during the chronic phase (238-337 days post-infection). Data points are individual mice and bars are means from 3 cumulative experiments (n=4-13 per infected group). LOD refers to the limit of detectable quantification based on serially diluted *T. cruzi*-spiked tissue DNA standards. (G-I) Representative H&E stained skeletal muscle sections from *G* Naïve or infected *H* WT and *I* TSKB20/18 Tg mice at 337 days post-infection. Scale bar is 200 μM.

Figure 3.2

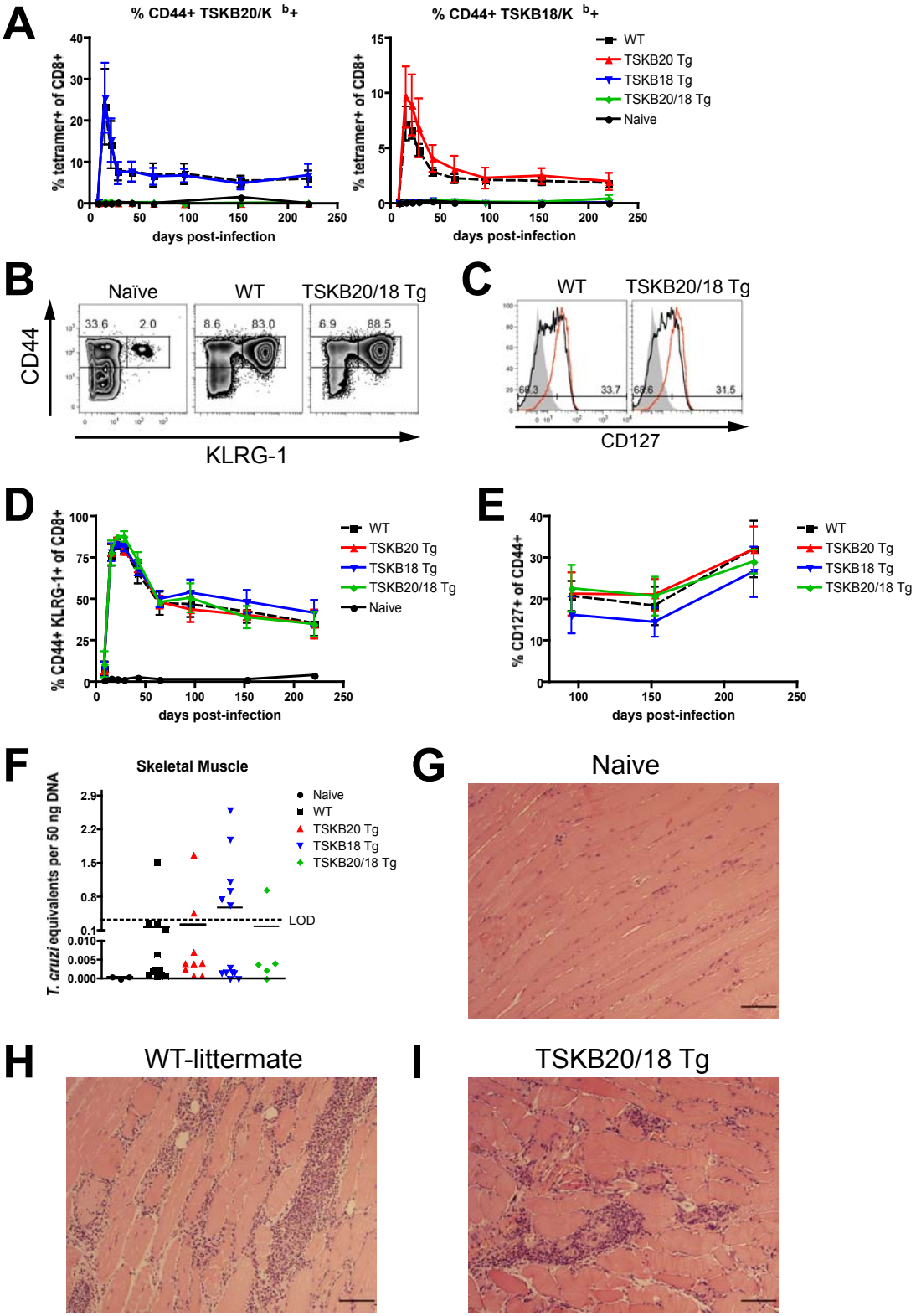

A
% CD44+ TSKB20/K b+
% CD44+ TSKB18/K b+
% tetramer+ of CD8+
days post-infection
WT
TSKB20 Tg
TSKB18 Tg
TSKB20/18 Tg
Naive
B
Naïve
WT
TSKB20/18 Tg
CD44
KLRG-1
33.6
2.0
8.6
83.0
6.9
88.5
C
WT
TSKB20/18 Tg
CD127
33.7
31.5
D
% CD44+ KLRG-1+ of CD8+
days post-infection
E
% CD127+ of CD44+
days post-infection
F
Skeletal Muscle
T. cruzi equivalents per 50 ng DNA
LOD
G
Naive
H
WT-littermate
I
TSKB20/18 Tg

Figure 3.3. *Effector CD8+ T cell populations expand and exhibit an effector-memory phenotype in the absence of TSKB20 and TSKB18 immunodominance*

(A-B) The total number of TSKB20/K^b+ or TSKB18/K^b+ CD8+ T cells per spleen of Naïve or *T. cruzi* infected WT, TSKB20 Tg, TSKB18 Tg or TSKB20/18 Tg mice at *A* 18-21 days post-infection or *B* 238-337 days post-infection. (C) Representative staining for CD44 and CD11a expression during acute (day 18) and chronic (day 238) phases of infection. Histograms are gated on CD8+ CD4- events, and numbers represent percentage of gated events. (D) The total number of $CD44^{hi}$ $CD11a^{hi}$ CD8+ T cells per spleen of age-matched Naïve or *T. cruzi* infected WT, TSKB20 Tg, TSKB18 Tg or TSKB20/18 Tg mice. (E) Representative staining for CD127 and CD62L expression during acute (day 18) and chronic (day 238) phases of infection. Histograms are gated on CD8+ CD4- for age-matched Naïve, and further gated on $CD44^{hi}$ $CD11a^{hi}$ antigen-experienced cells for infected WT and TSKB20/18 Tg mice. (F) Proportion of $CD44^{hi}$ $CD11a^{hi}$-gated CD8+ splenocytes that expressed the indicated marker during the chronic phase (238-337 days post-infection). Data points in *A-B*, *D* and *F* are individual mice and bars are means from 3 cumulative experiments in acute (n=3-8 per infected group) and 2 cumulative experiments in the chronic phase (n=4-13 per infected group). $*p<0.05$

Figure 3.3

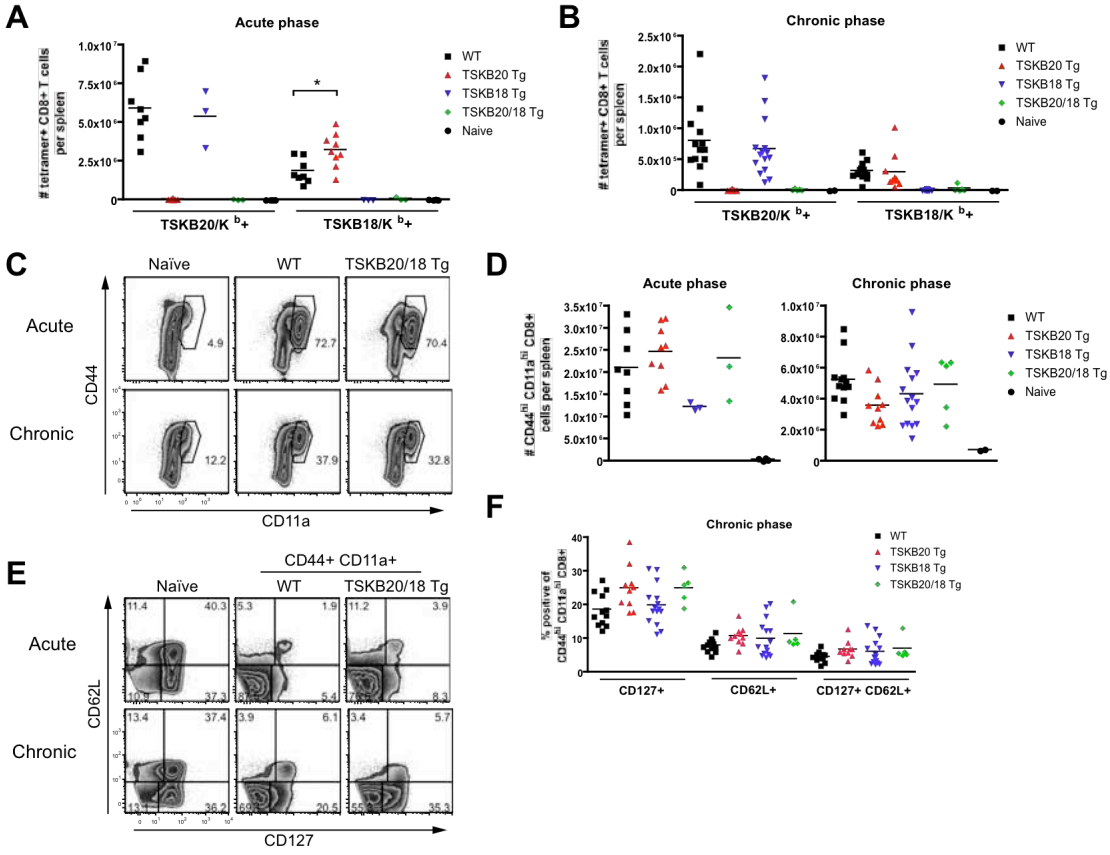

A
Acute phase
tetramer+ CD8+ T cells per spleen
WT
TSKB20 Tg
TSKB18 Tg
TSKB20/18 Tg
Naive
TSKB20/K b+
TSKB18/K b+
B
Chronic phase
C
Naïve
WT
TSKB20/18 Tg
Acute
Chronic
CD44
CD11a
D
Acute phase
Chronic phase
E
CD44+ CD11a+
CD62L
CD127
F
Chronic phase
CD127+
CD62L+
CD127+ CD62L+

Figure 3.4. *Phenotype of effector CD8+ T cells in non-lymphoid tissues*

(A) Representative staining of CD44 and intracellular GrzB (top), or surface expression of CD69 (middle) or PD-1 (bottom) on CD8+ CD4- gated lymphocytes isolated from the indicated tissues of chronically infected WT or TSKB20/18 Tg mice (338 days post-infection). Numbers indicate the proportion of events within gates. (B) Cumulative data from gates in panel *A* (n=5-13 mice per group). Data are mean +SEM. (C) Data are representative of 2 experiments. Histograms are gated on the indicated PD-1 postive or negative stained population (as gated in *A*) from chronically infected mice (338 days post-infection). (D) Representative intracellular staining of IFNγ and TNFα (top) or surface accumulation of CD107a (bottom) after 5 hr stimulation with plate bound anti-CD3. Histograms are gated on CD8+ CD4- events and numbers indicate proportion of events within the gated quadrant. Data are from 238 days post-infection. (E) Cumulative data from data represented in *D*. Data are 2 cumulative experiments in the chronic phase (238-337 days post-infection) (n=4-13 per infected group). Data are mean + SEM.

Figure 3.4

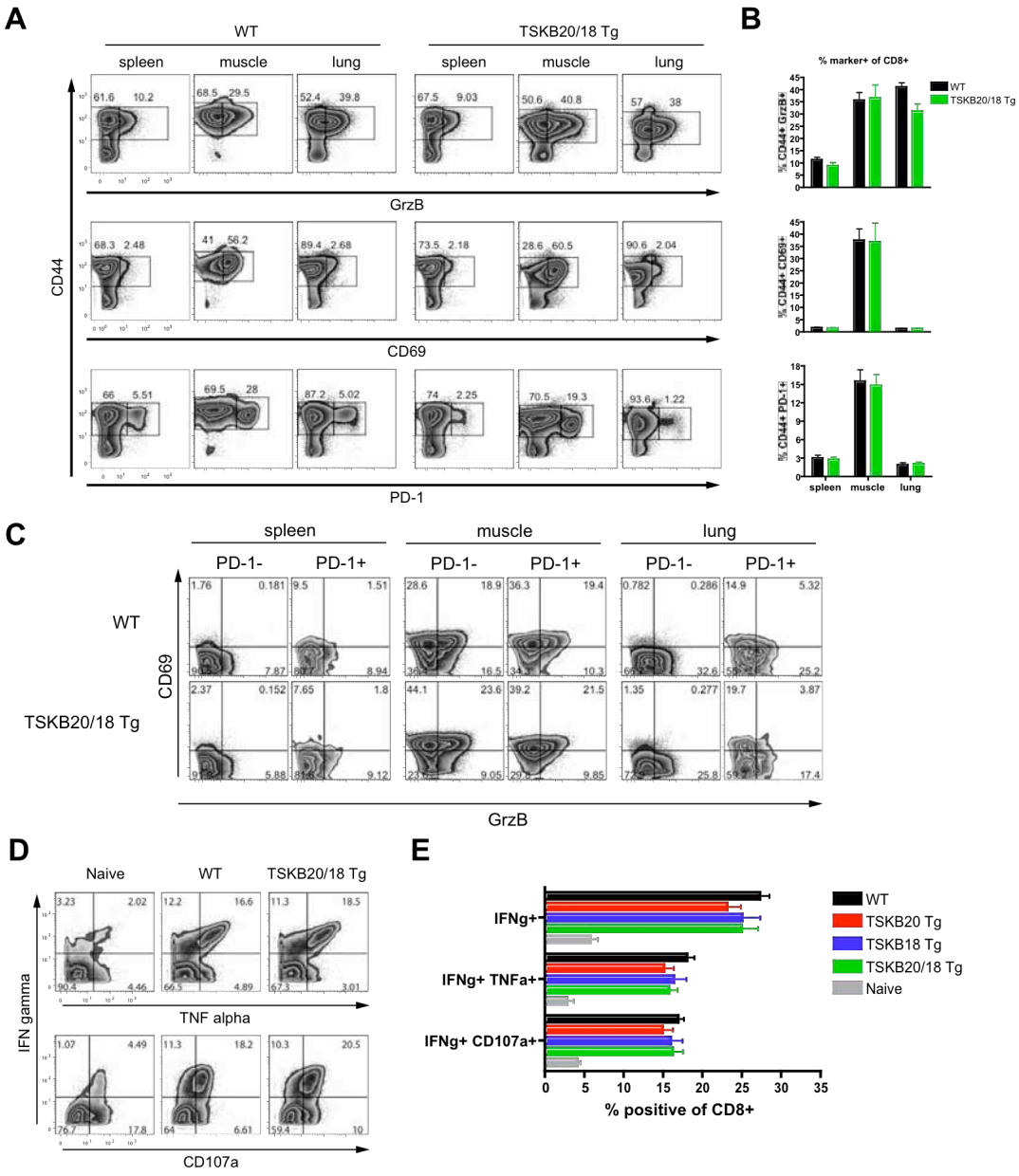

A
WT
TSKB20/18 Tg
spleen
muscle
lung
GrzB
CD44
CD69
PD-1
B
% marker+ of CD8+
WT
TSKB20/18 Tg
% CD44+ GrzB+
% CD44+ CD69+
% CD44+ PD-1+
C
PD-1-
PD-1+
CD69
GrzB
D
Naive
IFN gamma
TNF alpha
CD107a
E
IFNg+
IFNg+ TNFa+
IFNg+ CD107a+
TSKB20 Tg
TSKB18 Tg
Naive
% positive of CD8+

Figure 3.5. *Sub-dominant parasite-derived epitopes are recognized by relatively few CD8+ T cells in infected WT and TSKB20/18 Tg mice.*

(A-B) Cumulative data from peptide stimulated splenocytes from Naïve and *A* acutely or *B* chronically infected WT or TSKB20/18 Tg mice. CD8+ CD4- gated events were assessed for IFNγ staining after 5 hr incubation with 1 μM per indicated peptide alone or per indicated pool. Data are representative of 3 acute phase experiments at 18-21 days post-infection (3-8 mice per infected group) and 2 chronic phase experiments at 238-338 days post-infection (5-6 mice per infected group). Data are mean +SEM.

Figure 3.5

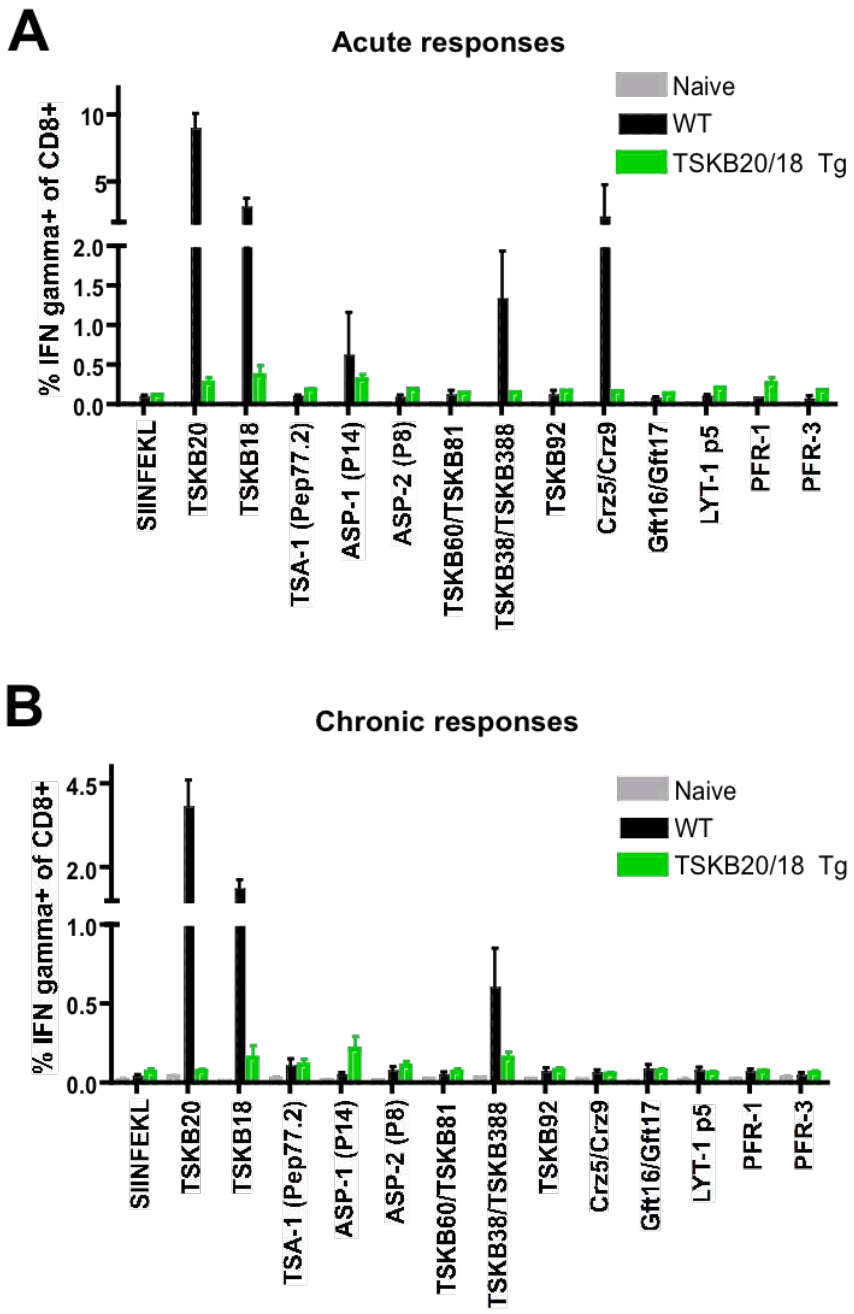
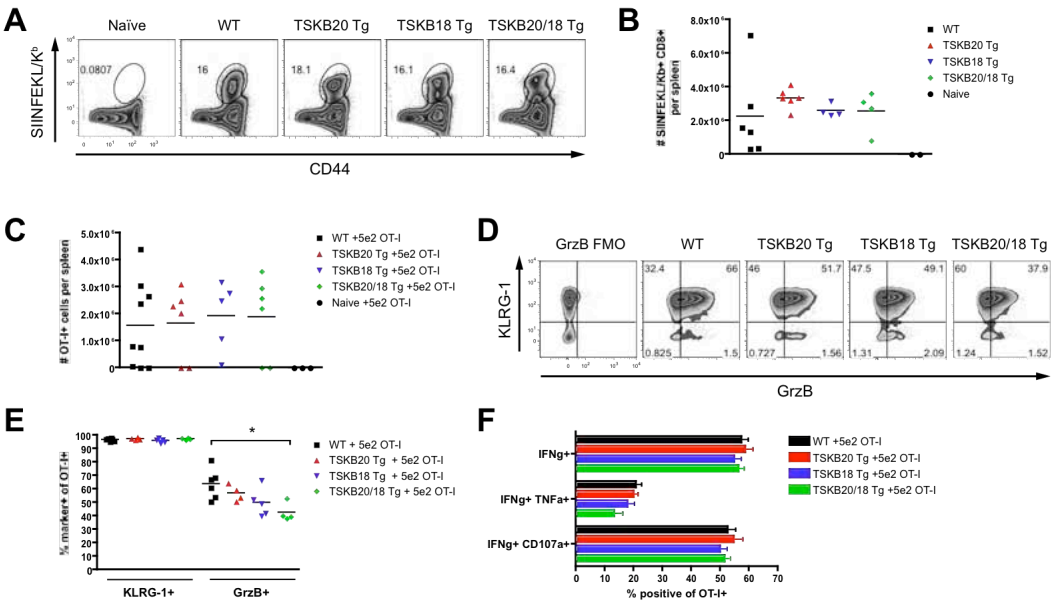

A
Acute responses
Naive
WT
TSKB20/18 Tg
% IFN gamma+ of CD8+
10
5
2.0
1.5
1.0
0.5
0.0
SIINFEKL
TSKB20
TSKB18
TSA-1 (Pep77.2)
ASP-1 (P14)
ASP-2 (P8)
TSKB60/TSKB81
TSKB38/TSKB388
TSKB92
Crz5/Crz9
Gft16/Gft17
LYT-1 p5
PFR-1
PFR-3
B
Chronic responses
Naive
WT
TSKB20/18 Tg
% IFN gamma+ of CD8+
4.5
2.0
1.0
0.5
0.0
SIINFEKL
TSKB20
TSKB18
TSA-1 (Pep77.2)
ASP-1 (P14)
ASP-2 (P8)
TSKB60/TSKB81
TSKB38/TSKB388
TSKB92
Crz5/Crz9
Gft16/Gft17
LYT-1 p5
PFR-1
PFR-3

Figure 3.6. *Partial compensation by an engineered dominant SIINFEKL-specific response in the absence of TSKB20 and TSKB18 immunodominance.*

(A) Representative SIINFEKL/K^b-tetramer staining of splenocytes from Naïve or WT, TSKB20 Tg, TSKB18 Tg, or TSKB20/18 Tg mice infected with *T. cruzi* Brazil-OVA at 22 days post-infection. Histograms are gated on CD8+ CD4- and numbers indicate percentage of $CD44^{hi}$ SIINFEKL/K^b+ of CD8+ cells. (B) The total number of SIINFEKL/K^b+ CD8+ T cells from spleens of Naïve or Brazil-OVA infected WT, TSKB20 Tg, TSKB18 Tg, and TSKB20/18 Tg mice at 21-22 days post-infection. Data are cumulative of 2 experiments (n=4-6 per infected group). (C) The total number of CD45.1+ OT-I cells from spleens of Naïve or Brazil-OVA infected WT, TSKB20 Tg, TSKB18 Tg, TSKB20/18 Tg mice at 21 days post-infection (n=6-9 per infected group). All mice received 5e2 OT-I cells i.v. prior to infection. Several individuals had no detectable OT-I cells despite exhibiting an immune phenotype consistent with infection. These data are included in this panel, and omitted in the following panels. (D) Representative KLRG-1 and intracellular GrzB staining of CD45.1-gated OT-I splenocytes from mice with expanded OT-I population in *C*. Numbers indicate percentage of events in the gate. (E) Cumulative of data represented in panel *D* (n=4-6 per infected group). Data points in *B*-*C* and *E* are individual mice and bars are means. (F) Proportion of OT-I (CD45.1+) CD8+ T cells stained positive for indicated marker in response to 5 hr SIINFEKL peptide stimulation from spleens of Brazil-OVA infected mice at 21 days post-infection (n=4-6 per infected group). $*p<0.05$

Figure 3.6


A
Naïve
WT
TSKB20 Tg
TSKB18 Tg
TSKB20/18 Tg
SIINFEKL/K^b
0.0807
16
18.1
16.1
16.4
CD44
B
SIINFEKL/Kb+ CD8+ per spleen
WT
TSKB20 Tg
TSKB18 Tg
TSKB20/18 Tg
Naive
C
OT-I+ cells per spleen
WT +5e2 OT-I
TSKB20 Tg +5e2 OT-I
TSKB18 Tg +5e2 OT-I
TSKB20/18 Tg +5e2 OT-I
Naive +5e2 OT-I
D
GrzB FMO
WT
TSKB20 Tg
TSKB18 Tg
TSKB20/18 Tg
KLRG-1
GrzB
32.4
66
0.825
1.5
46
51.7
0.727
1.56
47.5
49.1
1.31
2.09
60
37.9
1.24
1.52
E
% marker+ of OT-I+
KLRG-1+
GrzB+
WT + 5e2 OT-I
TSKB20 Tg + 5e2 OT-I
TSKB18 Tg + 5e2 OT-I
TSKB20/18 Tg + 5e2 OT-I
F
IFNg+
IFNg+ TNFa+
IFNg+ CD107a+
% positive of OT-I+
WT +5e2 OT-I
TSKB20 Tg +5e2 OT-I
TSKB18 Tg +5e2 OT-I
TSKB20/18 Tg +5e2 OT-I

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusion

Herein, I described studies I initiated to understand the role of CD8+ immunodominance during infection with the protozoan parasite, *Trypanosoma cruzi*. The *trans*-sialidase (TS)-derived TSKB20- and TSKB18-specific CD8+ T cells represent almost half of the responding CD8+ T cells during acute infection, and remain at heightened frequencies through the chronic phase of infection. Though *T. cruzi* infected C57BL/6 mice generate some of the largest documented pathogen-specific CD8+ T cell responses (1), these mice fail to completely clear *T. cruzi* infection and suffer chronic disease after years of persistent infection (2). To determine if the known immunodominant CD8+ T cells were required for control of *T. cruzi*, I deleted the epitope-specific CD8+ T cells by inducing immune tolerance to TSKB20 and TSKB18. Epitope-specific tolerance was maintained during acute and chronic phases of infection by repetitive injections of peptide, or by transgenic expression of the peptide as self-antigen. Mice tolerant to TSKB20, TSKB18, or both epitopes simultaneously, resolved acute *T. cruzi* infection comparably to control infected mice. Furthermore, deletion of the normally immunodominant CD8+ T cells resulted in similar levels of chronic infection and disease in WT and TSKB-peptide tolerant mice. Therefore, C57BL/6 mice generate immunity to *T. cruzi* independent of recognizing the dominant TSKB20 and TSKB18 epitopes. As antigen-specific T cell responses are inherently associated with

immunodominance (3), we cannot conclude the immunodominant CD8+ responses are not required for parasite control *per se*. However, these studies do clearly demonstrate that immunodominance by a given *T. cruzi*-specific CD8+ T cell population does not define importance for parasite control. Furthermore, these studies further question the wisdom of pursuing a *trans*-sialidase (TS)-based vaccine against *T. cruzi* (1, 4), as well as vaccines targeting immunodominant antigens encoded by variant gene families of other parasites. In contrast to some viruses and bacteria, optimal protection against parasitic infections likely needs to focus on more than a few immunodominant epitopes.

We questioned if the variant *trans*-sialidase (TS) proteins expressed by *T. cruzi* play a role in immune evasion and promote parasite persistence. Immunodominance by TS-derived epitopes may mask alternative parasite-derived epitopes (5). If recognition of the TSKB20 and TSKB18 epitopes diverts the focus of the CD8+ response away from more optimal targets, one would expect better control in mice tolerized to TSKB20 and TSKB18. Though TSKB20 and TSKB18 double transgenic mice control persisting parasites to very low levels, we did not see enhanced immunity in comparison to WT infected mice. Therefore, immunodominance by these TS-derived epitopes does not appear to subvert the generation of protective immunity in mice. This finding does not disprove an immune evasion hypothesis of TS evolution; as we cannot directly test immune control in the absence all TS genes by genetic ablation (~3,000 TS full and partial sequences) or tolerize mice to every TS-derived epitope, we currently cannot definitively demonstrate or refute the immune evasion role by TS. If the variant TS family facilitates immune evasion, the mechanism currently remains elusive. Future studies might focus more on the phenomenon of altered peptide ligand antagonism (6) by

cross-reacting TS-derived epitopes as modulating the function of parasite-specific T cells (7, 8) and further study the interaction of effector CD8+ T cells at the infected-host cell interface. Another avenue of research would be to define the timing of the parasite's expression of TS genes that encode TSKB20 and TSKB18, and investigate a role for stage-specific expression of these genes as an explanation for why these CD8+ responses are not essential for parasite clearance from infected tissues.

The fact that the strongest known TS-specific responses are not required for control indicates protective CD8+ T cells may target other sources of antigen. The likely alternative sources of antigen are large gene family members, such as TS, and may very well include TS genes encoding TSKB20 and TSKB18 peptides. Here, we conducted preliminary screens for enhanced responses to previously identified epitopes encoded by TS, other large gene family members, and several proteins shown to be good vaccine candidates, however, we found TSKB20/18 Tg mice lacked significant numbers of CD8+ T cells specific for the epitopes tested. A more thorough response screen should be carried out to determine the focus of responding CD8+ T cells in TSKB20/18 Tg mice. One place to start will be to screen for responsiveness to the >100 peptides previously tested for recognition in WT mice (1). Epitopes encoded outside of the TSKB20/TSKB18-homologous position of TS genes are also likely targets, and testing of responsiveness to these epitopes will either require production of many more synthetic peptides, or stimulation using whole protein preparations of TS gene products. A similar approach can be used to test recognition of other large gene family members, however, a cautionary finding should be recognized in my data concerning the lack of observed TSKB20/18 Tg CD8+ T cell recognition of similar sources of epitopes. It may prove

likely that many of the previously identified sub-dominant epitopes are in fact recognized by the cross-reactive TSKB20- and TSK18-specific TCRs.

The goal of searching for CD8+ T cell targets beyond TS should be to identify novel sources of protective antigen that will inform us how to design better immunoprophylactics or immunotherapeutics. Since *T. cruzi*'s TS and other large gene family members are strain-variant, characterized by numerous pseudogenes and partial sequences, and encoded at regions of the chromosome that allow for further sequence evolution, they are likely not the best targets for vaccination. Vaccination against individual TSs would only place further immune selective pressure on those genes to evolve and change (9); assuming that particular TS gene is encoded by the *T. cruzi* strain encountered after vaccination. Instead, induction of broadly focused responses against the parasite might be successful in field settings. Immunizations using avirulant *T. cruzi* strains, or attenuated strains generated by genetic manipulations, will likely be a useful vaccine platform to induce the B and T cell responses necessary for protective immunity in the field. Further manipulation of these vaccine strains to over-express CD8+ T cell targets normally out-competed by the immunodominant TS should be a useful strategy for inducing protective immunity against numerous *T. cruzi* strains, not just strains sharing a handful of TS sequences. Using TSKB20/18 Tg mice as an antigen discovery platform will facilitate the identification of these potentially non-variant targets.

Though *in silico* predictions and screening for responsiveness to synthesized MHC-I-restricted peptides uncovered the two immunodominant CD8+ responses recognized in C57BL/6 mice, this approach will be cumbersome to apply to the rest of *T. cruzi*'s genes, even those that are predicted to be surface expressed or secreted by the

parasite's vertebrate stages. Several studies have completely identified the targets of CD8+ T cells responding to several virus infections by tetramer staining or stimulating *ex vivo* with overlapping synthetic peptides that span the viral genome (10-13), though the size of *T. cruzi*'s genome precludes a similar screening method (by currently available technology). Instead, a high throughput screening approach will have a greater chance of successfully identifying the novel targets recognized by infected TSKB20/18 Tg mice. The novel targets identified in TSKB20/18 Tg mice would then be tested for their recognition in infected WT mice (in the face of TSKB20/TSKB18 immunodominance), or in WT mice infected or immunized with *T. cruzi* parasites manipulated to over express the target of interest. Protection afforded by immunizations that boost immunity to these novel CD8+ T cell targets should be tested to determine if diverting the focus of CD8+ T cells overcomes TS immunodominance during parasite challenge and otherwise benefits the host.

Since this proposed approach will require testing of many targets, any step of the process that can be carried out in a high throughput fashion should increase the likelihood of identifying useful vaccine candidates. Screening for responses to as many targets as possible will be facilitated by testing for recognition of whole *T. cruzi* gene products, so an approach similar to *Cooley et al* (14) could be used to uncover novel T cell responsiveness. Screening for CD8+ T cell responsiveness to the >1,000 cloned *T. cruzi* genes available (14) would become unwieldy if recombinant proteins are to be expressed and purified from bacteria and used to stimulate the CD8+ T cells *in vitro*. Since CD8+ T cells recognize specific peptide-MHC-I complexes produced endogenously by APC, we can theoretically express the cloned *T. cruzi* genes in cell lines that express (or over-

express) mouse MHC-I alleles, and use these transgenic cells to test for responsiveness to entire *T. cruzi* genes. A similar approach has been used to identify the gene-products targeted by the total CD8+ T cell response generated by viral infection (15, 16). Since generating drug-selected cell lines takes weeks to months, and several hundred potential genes will likely need to be screened, transient transfections using a 96-well plate based transfection system will increase the ability to screen numerous genes. A reporter system for specific CD8+ T cell recognition of epitopes presented on the surface of transiently transfected APC would also facilitate experimental readout during such initial screenings. One such reporter system would entail crossing IFNγ-biscistronic reporter mice (17, 18) with TSKB20/18 Tg mice to produce TSKB-peptide tolerized mice whose antigen-activated CD8+ T cells express YFP. Thus, YFP intensity measured by flow cytometry could be used to detect antigen-specific responses without the need for cell staining steps required to directly detect IFNγ production.

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