ENHANCING IMMUNITY TO *TRYPANOSOMA CRUZI* WITH TRANSGENIC EXPRESSION OF EXOGENOUS PAMPS AND ENDOGENOUS PARASITE PROTEINS

By

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(Under the direction of Rick L. Tarleton)

ABSTRACT

Trypanosoma cruzi establishes an indefinitely persistent infection in mammalian hosts and causes Chagas disease in humans. Adaptive cell mediated immune responses- including $CD8^+$ T cell responses- are critical in controlling this infection, making it a priority in the field to understand the dynamics of this response and ways to manipulate immune responses to potentially clear the infection. Compared to most other pathogens, *T. cruzi* has a relative deficiency in the expression of immunologically relevant pathogen associated molecular patterns (PAMPs), the molecules that allow the innate immune system to detect and initiate responses to invading pathogens. This relative absence of PAMPs in *T. cruzi* may aid in its ability to establish and persist in hosts. Heterologous expression of bacterial PAMPs in *T. cruzi* led to a more rapid and consistently stronger adaptive immune responses. Although co-inoculating with, or temporarily anchoring exogenous PAMPs on *T. cruzi* also resulted in initially stronger parasite-specific $CD8^+$ T cell responses, only the continuous expression of heterologous PAMPs sustained these responses and ultimately resulted in clearance of the infection.

However, the $CD8^+$ T cell responses in *T. cruzi* infection are often directed at members of large gene families, including trans-sialidase-like proteins that are highly diverse and vary widely among *T. cruzi* strains. We hypothesized that $CD8^+$ T cell responses directed against sub-dominant, invariant, non gene-family proteins may impart better cross-strain protection from *T. cruzi* infection. On observing that *T. cruzi* sacrifices its flagellum during entry of host cells, transgenic *T. cruzi* over-expressing the abundant flagellar protein PAR4 were generated, that induced stronger and more effective PAR4-specific $CD8^+$ T cell response, conferring better protection from subsequent *T. cruzi* challenges. These findings demonstrate that, the efficiency and strength of specific immune responses may be affected at and beyond their initiation by PAMPs, as well as by the over-expression of chosen target epitopes, to effect the overall improved immune control of a pathogen.

INDEX WORDS: *T. cruzi*, PAMPs, flagella, Chagas disease, paraflagellar

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DEDICATION

I dedicate this work to my wife, Tanushree for her countless sacrifices and unconditional support, as well as to my parents for their love and faith in me.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Chagas Disease and *Trypanosoma cruzi*

Chagas disease caused by the protozoan parasite *Trypanosoma cruzi* affects millions of people and animals, especially in the Americas. Though over 300,000 people are infected in the United States alone, Chagas disease has its highest impact in Latin America with around 8-10 million people infected, especially belonging to the low income populations. It is a progressive and debilitating parasitic disease that develops over decades in individuals chronically infected with *T. cruzi*. Parasite persistence in patients is thought to impair smooth muscle, peripheral nerve and cardiac functions leading to the development of digestive diseases and dilated cardiomyopathy, which often end fatally (Tanowitz et al., 2009). It is considered the leading cause of myocarditis worldwide, with 30,000-40,000 cases of chagasic cardiomyopathy reported in the US alone, every year (Bern and Montgomery, 2009).

The transmission of Chagas disease in nature is primarily by deposition of parasite-laden feces by the hematophagous insect vector- *Triatoma* on wounded skin after a blood meal, the mucous membrane or even orally. Transmission to humans is promoted by poor housing conditions that provide a habitat for the triatomine bugs (Massad, 2008) as well as by the presence of reservoir animals such as dogs and cats in human dwellings (Cohen and Gurtler, 2001). Safe drugs or vaccines for Chagas disease do

not exist and given the public health impact of the disease, it is imperative that research is directed towards strategies to control the infection.

1.2 *T. cruzi* in the mammalian host

T. cruzi is a protozoan pathogen belonging to the order Kinetoplastida. Epimastigotes are the replicating form of *T. cruzi* found within the gut of the triatomine vector. Parasites transform into infective metacyclic trypomastigotes as they pass into the hindgut, where they are excreted with the feces, soon after the vector feeds on the mammalian host. Upon entry into the mammalian host through the insect bite wound, *T. cruzi* can infect virtually any nucleated cell in the body, with the vertebrate life cycle stages of *T. cruzi* being obligately intracellular. *T. cruzi* enters the host cells through a lysosome dependent (Burleigh and Woolsey, 2002) or independent (Andrade and Andrews, 2004) pathway and within a few hours, escapes the vacuole, converts into a morphologically distinct amastigote, and begins to divide in the cytoplasm. After approximately 4-5 days, the host cell is destroyed and the infective, recirculating trypomastigotes are released to invade other cells or to be taken up by the triatomine vector during a blood meal to continue its invertebrate-host life cycle. The trypomastigotes may be detectable in the blood and amastigotes in tissue sections of infected organs in the mammalian host. The immune response generated in the vertebrate host often results in clearance of the majority of *T. cruzi* from the tissues, though incompletely resulting in an indefinite persistence of the parasite in the mammalian host. *T. cruzi* persistence within myocytes, adipocytes and other cells, and may be a key cause for disease progression to the chronic stage of Chagas' disease.

This aspect of the biology of the parasite makes targeting the intracellular life stage of *T. cruzi* critical to the scheme of developing vaccines or drugs to control the infection.

1.3 Immunity to *T. cruzi* infection

T. cruzi has a complex life-cycle with various (possibly antigenically distinct) life-stages in the host. Hence it is not surprising to have diverse sets of innate and adaptive immune responses that control this parasite (Martin and Tarleton, 2004; Tarleton, 2007).

Initial recognition and control of invading pathogens is usually accomplished by the innate immune system, which senses pathogen associated molecular patterns (PAMP) via pattern recognition receptors (PRR) such as Toll-like receptors (TLR) (Coffman et al., 2010; Iwasaki and Medzhitov, 2004, 2010). Though there has been considerable research examining the involvement of PRRs in generating immunity to *T. cruzi;* with multiple PAMPs (e.g. GPI anchors, *T. cruzi* DNA, GIPL-ceramide) being attributed to it (Almeida and Gazzinelli, 2001; Bafica et al., 2006; Campos et al., 2001; Ouaissi et al., 2002), they seem to be relatively insignificant to the downstream immune responses generated. Comparable adaptive immune responses were generated in mice responsive or (genetically) unresponsive to these putative PAMPs described in *T. cruzi* (Oliveira et al., 2010), leading to the speculation that these were “hidden” from their respective PRRs in live, intact, infective stages of the parasite. PAMPs, in general are highly conserved molecules that are extremely difficult for pathogens to alter or sacrifice given their consistent association with their pathogenicity (eg. Lipopolysaccharides in bacteria) or core biology (eg. CpG DNA). However, there appears to be some plasticity

in PAMP display in pathogens. For example, host detection of LPS in *Porphyromonas gingivalis and Escherichia coli* is modulated by differential acylation of lipid-A (Coats et al., 2007; Reife et al., 2006), while *Yersinia pestis* synthesizes LPS-lipid A that is a poor TLR4 ligand (Montminy et al., 2006). *Helicobacter pylori* produces a flagellin variant that is non-stimulatory to TLR5 (Andersen-Nissen et al., 2005) and *Pseudomonas aeruginosa* down-regulates its flagellin expression in airway passages (Wolfgang et al., 2004). Although it is unlikely that any pathogen will be able to make all of its PAMPs entirely invisible to the immune system, we believe that potential PAMPs discovered in *T. cruzi* may be rendered immunologically inconsequential by concealing or modifying them, without significantly impacting its biology. This is of immense significance evolutionarily, as the lack of adequate, relevant PAMPs may have driven the immunological privilege *T. cruzi* enjoys, by remaining relatively undetected initially during an infection, until it establishes in the host. The relative absence of effective PAMPs in *T. cruzi* also presents the unique opportunity to study the relevance of PAMPs in a pathogen's immunology, from an unprecedented view point, since almost all of the studies that examined this subject have relied on compromising PAMP recognition at the host side (for eg, using MyD88 Knockout mice), rather than modifying PAMPs in the pathogen.

Various aspects of adaptive immunity are critical to control of *T. cruzi* infection as well. $CD4^+$ T cells coordinate the immune-effector mechanism through secretion of cytokines, primarily by a type-1-biased T-helper (Th1) cell response involving interferon-gamma (IFN-γ) (Kumar and Tarleton, 2001; Martins et al., 1999). $CD4^+$ T cells may also be critical for the regulation of antibody class switching, activation of macrophages to kill

the intracellular parasite, and also for adequate $CD8^+$ T cell immunity. Similarly, humoral immunity has also been shown to contribute to the control of *T. cruzi* infection (Gupta and Garg, 2010; Kumar and Tarleton, 1998). However, a large body of evidence suggests that despite the contributions of $CD4^+$ T cells or the humoral immunity, the role of $CD8^+$ T cells is critical to the control of *T. cruzi* infection. When mice that lack $CD8^+$ T cells succumb to the infection very quickly (Tarleton, 1990; Tarleton et al., 1992), the antibody deficient mice survived longer(Kumar and Tarleton, 1998). Similarly, a robust *T. cruz*i-specific $CD8^+$ T cell response develops in the absence of $CD4^+$ T cell help, and displays similar effector functions as in $CD4^+$ T cell sufficient mice (Padilla et al., 2007). Thus, in the adaptive immunity arm, $CD8^+$ T cells are thought to be the most important subset that controls *T. cruzi* infection.

1.4 $CD8^+$ T cell response to *T. cruzi* infection

Though it has been well known that $CD8^+$ T cells are critical to the control of many viral and bacterial infections, it is rarely emphasized that these are critical to the control of many intracellular protozoans as well. Infection by *T. cruzi* may be one of the best studied among protozoans, for the significance of $CD8^+$ T cells in immunity (Tarleton et al., 1992). In its cytoplasmic niche, *T. cruzi* releases various proteins that are processed and presented on class I MHC molecules (Garg et al., 1997), which are detected by specific $CD8^+$ T cells. In addition to humans, *T. cruzi* naturally infects a wide range of mammals, including mice. Thus, we study the role of $CD8^+$ T cells in immunity to *T. cruzi* infection, in both the acute and chronic stages- in mice. Further, *T. cruzi* can also be manipulated genetically, to knockout individual parasite genes or to transgenically

introduce exogenous or native genes, to study its immunology (Garg et al., 1997). This makes *T. cruzi* a good model to study the generation and maintenance of $CD8^+$ T cell responses in a persistent infection, and learn how manipulating the strength or targets of these responses would impact *T. cruzi* immunology and its persistence in the host.

Any pathogen-specific $CD8^+$ T cell population generated in an infection recognizes the foreign peptide epitopes presented in the context of surface-bound class I MHCs using clonally diverse T cell receptors. Naïve $CD8^+$ T cells, undergo a relatively fixed program of expansion that allows the relatively fewer pathogen-specific $CD8^+$ T cells to amplify within a few days. Normally, this is followed by a contraction phase, as the pathogen is cleared or controlled. However, this general pattern and timing of T cell activation has been established based on acute viral or bacterial infections that are quickly controlled, to eventually establish a stable memory T cell population within a few weeks of infection. In *T. cruzi* infection though, the pattern of $CD8^+$ T cell induction and contraction are delayed in time, with the initial $CD8^+$ T cell activation not evident until 9-11 days after parasite introduction (Padilla et al., 2009a; Padilla et al., 2009b). As mentioned earlier, it is thought that the initial infection by *T. cruzi* is rather 'silent' (Costales et al., 2009) owing to the relative absence of PAMPs in *T. cruzi* (Oliveira et al., 2010) and the triggering of innate and hence the adaptive immune responses do not occur until the first round of parasite replication and reinvasion is complete (Padilla et al., 2009a). However, once the $CD8^+$ T cell response to *T. cruzi* is initiated, it displays the kinetics, similar to that in most viral and bacterial infections (Bixby and Tarleton, 2008; Bustamante et al., 2008), with a strong anti-*T. cruzi* $CD8^+$ T cell response that displays effective in vivo cytolytic activity (Bustamante et al., 2008).

In any $CD8^+$ T cell response generated in an infection, an intensive, reproducible hierarchy of epitope-specific $CD8^+$ T cells are generated, of which certain clones are represented in higher proportions (dominant) than others (subdominant) in a phenomenon called immunodominance (Yewdell and Bennink, 1999). Most of our understanding regarding the role of dominant and subdominant $CD8^+$ T cell responses in controlling an infection comes from bacterial (e.g.,*Listeria monocytogenes*) (Pamer, 2004) or viral (lymphocytic choriomeningitis, influenza, vaccinia viruses) (Yewdell, 2006) pathogens infecting mice. However, the antigenic repertoire in these simpler pathogens directing sufficiently protective dominant $CD8^+$ T cells are relatively few compared to complex pathogens like protozoans with intricate genetics and life stages, which may generate multiple layers of immunodominance in the $CD8^+$ T cell responses generated. Although dominant $CD8^+$ T cells have recently been described for several intracellular parasites (Blanchard et al., 2008; Martin et al., 2006), the role of these populations in resistance to these infections is not fully understood. In the C57Bl/6 mice that is used as a laboratory model for *T. cruzi* infection, a significant proportion (at least $\geq$ 50%) of the total $CD8^+$ T population at the peak of the infection is specific for a few peptides derived from *T. cruzi* trans-sialidase protein (Martin et al., 2006; Padilla et al., 2009a). Given that *T. cruzi* trans-sialidases are encoded by >3000 genes (Weatherly et al., unpublished) with most of them lacking any enzymatic activity (Frasch, 2000), and its other Kinetoplastid relatives possessing none (*Leishmania sp*.) to only a few (six in the case of *Trypanosoma brucei*) trans-sialidase genes, this possible gene amplification (Padilla et al., 2009a) and the perhaps associated, remarkable 'hijack' of immunodominance is thought to be of immunological advantage to the parasite.

Further, tolerizing mice to the most immunodominant trans-sialidase derived epitopes, TSKb20 (Rosenberg et al., 2010) and TSKb18 (Rosenberg et al., unpublished) appeared to have no impact on the control the infection in mice. This poses a concern from an immunoprophyllaxis point of view as well, making *T. cruzi* trans-sialidases unreliable candidates to direct vaccine efforts at, further worsened by the fact that *T. cruzi* trans-sialidases vary widely across different *T. cruzi* strains (Martin et al., 2006). This suggested that any attempt to improve protective $CD8^+$ T cell responses to *T. cruzi* may have to consider other invariant, though subdominant $CD8^+$ T cell epitopes as well. The crucial role of $CD8^+$ T cells in control of *T. cruzi* infection is evident from multiple studies demonstrating the inability of mice lacking $CD8^+$ T cell to survive an infection and the ability to better resist an infection by boosting $CD8^+$ T cell responses (Martin and Tarleton, 2004). In this work, we on one hand attempt to improve the overall immune response generated against *T. cruzi* by transgneically expressing strong PAMPs in the parasite, while on the other, try to at least partially redefine the immunodominance hierarchy led by trans-sialidase derived $CD8^+$ T cell epitopes by over-expression of other native *T. cruzi* proteins- all towards identifying ways to manipulate the immune responses to *T. cruzi* -to bring about its better control in the veterbrate host.

1.5. Summary and the major questions

Chagas disease is a very under-studied, neglected tropical disease with huge and growing global impact. Though millions of people and animals are infected with *T. cruzi*, we are still a long way from understanding the immunology of the infection, which

incidentally is a pre-requisite to devise schemes to successfully control or even eradicate the disease. The realization that $CD8^+$ T cell responses are critical to the control *T. cruzi* has convinced us to direct our efforts to tap into this very response to control the infection. In chapter 2, we attempt to drive a stronger and more rapidly mounted $CD8^+$ T cell response against *T. cruzi,* by transgenically expressing established, exogenous PAMPs in the parasite. We assess if this aids in hindering the establishment of, or induction of a better control- of *T. cruzi* infection. By doing this, we aim to answer the bigger question whether the relative absence of PAMPs provides an advantage to *T. cruzi*: to establish and persist indefinitely in its hosts. In chapter 3, we attempt to modify the immuno-dominance hierarchy of $CD8^+$ T cell response to *T. cruzi*, by transgenically over-expressing a native *T. cruzi* protein, in *T. cruzi*, to generate a more dominant $CD8^+$ T cell response against it. Based on the observation that *T. cruzi* sacrifices its flagella during intracellular amastigogensis, we chose to over-express a *T. cruzi* flagellar protein to generate a stronger $CD8^+$ T cell response directed at its constituent epitopes. We further assess if this now relatively stronger, dominant response can impart better control of a *T. cruzi* challenge.

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CHAPTER 2

PERPETUAL EXPRESSION OF PAMPS IS NECESSARY FOR OPTIMAL IMMUNE CONTROL AND CLEARANCE OF AN OTHERWISE PERSISTENT PATHOGEN

Samarchith P. Kurup and Rick L. Tarleton. Submitted to *Nature communications,* 02/22/2013

2.1 Abstract

Pathogen-associated molecular patterns (PAMPs) are known to be fundamental in instigating immune responses but their role in influencing these responses beyond their initiation is less well understood. Using the protozoan parasite *Trypanosoma cruzi,* which is deficient in strong PAMPs, we demonstrate a requirement for the continuous expression of PAMPs for optimal anti-pathogen immunity. Although co-inoculating with, temporarily anchoring of and transgenic expression of exogenous PAMPs all resulted in enhanced early adaptive immune responses, only the continuous expression of bacterial PAMPs on transgenic *T. cruzi* sustained these responses, resulting in enhanced pathogen clearance. These findings demonstrate that PAMPs function to potentiate adaptive immune responses well beyond their initiation and may determine the efficiency of control of pathogens capable of long-term persistence.

2.2 Introduction

Pathogen associated molecular patterns (PAMPs) are known to be critical in initiating innate immune responses and to inducing and directing subsequent adaptive immunity(Fearon and Locksley, 1996; Janeway and Medzhitov, 2002; Medzhitov et al., 1997; Schnare et al., 2001). PAMPs are effective indicators of the presence of particular pathogens in part because they are unique to classes of pathogens and because they are often required for pathogen survival and thus cannot be altered, suppressed or easily hidden by pathogens. Thus, most of the studies that established the role of PAMPs in innate and adaptive immune responses are based on negating the ability of the host to respond to these molecular patterns(Akira et al., 2001; Schnare et al., 2001) rather than by blocking PAMP expression. This however, has limited our ability to address the relevance of PAMPs to adaptive immunity after its onset.

In this work, we make use of the protozoan pathogen *Trypanosoma cruzi* to evaluate the role of PAMPs in influencing adaptive immunity at and beyond its initiation. *T. cruzi* is the etiological agent of Chagas disease, the highest impact human parasitic disease in the Americas. Previous studies have shown that *T. cruzi* trypomastigotes stimulate a very weak host cell response during invasion (Costales et al., 2009) and elicit significantly delayed adaptive immune responses (Padilla et al., 2009b), strongly suggesting the relative absence of potent PAMPs on live, invading parasites. Although several endogenous PAMPs have been identified in *T. cruzi* (Bafica et al., 2006; Campos et al., 2001), their failure to impact the strength of the adaptive immune responses (Oliveira et al., 2010) as well as the ability of certified PAMPs to potentiate

immune responses when co-inoculated with *T. cruzi* (Padilla et al., 2009b) have argued against the ready availability of these putative PAMPs on live *T. cruzi* (Tarleton, 2007) and their relevance to anti-*T. cruzi* immunity. This apparent absence of strong endogenous PAMPs, as well as the genetic pliability of *T. cruzi*, makes this pathogen an ideal template for expression of bona fide PAMPs from other pathogens, and thus for the study of role of PAMPs beyond the initial phases of immune recognition.

In this study, we generate transgenic *T. cruzi* expressing potent exogenous protein PAMPs and show that this expression not only induces superior innate immune responses, but also drives more rapid and persistently stronger adaptive immunity in mice. These studies demonstrate that constitutive expression of PAMPs are instrumental in maintaining strong adaptive immunity beyond its onset in an infection, leading to better pathogen control and in some cases, complete parasitological cure. Additionally, these findings provide new understanding of how the lack of molecular patterns on the part of some eukaryotic pathogens may fail to incite early immune responses and thus provide an opportunity for establishment of persistent infections.

2.3 Results

2.3.1 PAMP transgenesis in *T. cruzi* enhances innate immune responses

We chose to express protein PAMPs in *T. cruzi*, since PAMP expression can be stably generated by transgenesis of a single gene, in contrast to polysaccharides or nucleic acid PAMPs which would require transfer of entire biosynthetic pathways into *T. cruzi*. Genes encoding the *Salmonella typhimurium* flagellin (*fliC*), the ligand for both TLR5

(Hayashi et al., 2001) and Neuronal Apoptosis Inhibitory Protein (NAIP)5/ IL-1β-converting enzyme protease-activating factor (Ipaf) (Miao et al., 2006) and *Neisseria meningitidis* porin (*porB*), a ligand for TLR1/2 (Singleton et al., 2005) were amplified by PCR and cloned into the pTREX plasmid (Lorenzi et al., 2003) with a *T. cruzi* secretory signal peptide from gp72 (Garg et al., 1997) at their 5' end (Supplementary Fig. 2.1a). PAMP-transgenic *T. cruzi* expressing FliC (TcgFliC) or PorB (TcgPorB) were engineered by transfecting these constructs into wild-type (WT), Brazil strain *T. cruzi* (Tcwt). The signal peptide ensured secretion (Supplementary Fig. 2.1b) of the protein PAMPs expressed by the PAMP-transgenic *T. cruzi* in epimastigote, trypomastigote and amastigote life stages (Supplementary Fig. 2.1c).

Stimulation of TLRs 1/2 or 5 ultimately activates the transcription factors NFkB/ AP-1, to promote immunity, primarily by the production of inflammatory cytokines (Schnare et al., 2001; Takeda and Akira, 2004). NFkB/ AP-1 reporter cell lines exhibited substantially increased NFkB/ AP-1 activation by TcgFliC or TcgPorB live trypomastigotes (Fig. 2.1a) or epimastigote lysates (Supplementary Fig. 2.2a) relative to Tcwt parasites. FliC, is also an NAIP5/ipaf ligand that induces IL-1β production in antigen presenting cells (APCs)(Miao et al., 2006). FliC-expressing *T. cruzi* potentiated strong caspase1 activation (Fig. 2.1b) and production of IL1β in TLR5-deficient macrophages (Fig. 2.1c and 2.1d) demonstrating that *T. cruzi*-expressed FliC exhibits both of the PAMP properties of *Salmonella* flagellin.

The innate immune response-inducing activity of PAMP-transgenic *T. cruzi* trypomastigotes was also evident in IL-12*yet40* reporter mice in which cells expressing

IL12/23 p40 subunit also express yellow fluorescent protein (YFP) (Reinhardt et al., 2006). Peritoneal exudate macrophages exposed in vitro to PAMP-transgenic *T. cruzi*, produced IL-12 at an increased frequency relative to those exposed to WT *T. cruzi* (Fig. 2.2a). Additionally, TcgFliC and TcgPorB infections of IL-12*yet40* reporter mice resulted in a more rapid and increased infiltration of the IL-12 producing $CD11c^{+}$ $CD8\alpha^{+}$ classical dendritic cells (cDCs) into the draining lymph nodes (Fig. 2.2b and supplementary Fig. 2.2b). TcgFliC infection also altered the lineage bias of the inflammatory cells infiltrating or prevailing at the site of infection, with increased numbers of blood-derived monocytes ($CD45^{+}CD11b^{+}CD11c^{-}Gr\text{-}1^{int}$), macrophages ($CD45^{+}CD11b^{+}CD11c^{-}F4/80^{+}$), inflammatory DCs ($CD45^{+}CD11b^{+}CD11c^{hi}Gr\text{-}1^{int}$) and other non-classical ($CD45^{+}CD11b^{+}CD11c^{+}CD8\alpha^{-}$) DCs, as compared to WT *T. cruzi* infection (Fig. 2.3a and 2.3b). Classical ($CD45^{+}CD11b^{-}CD11c^{+}CD8\alpha^{+}$) DCs remained undetectable at the site of infection in either case (data not shown). TcgFliC infection was also associated with enhanced recruitment of IL-12 producing monocytes (Fig. 2.3a) and neutrophils ($CD45^{+}CD11b^{+}Gr\text{-}1^{hi}$) (data not shown) to the site of infection. The innate immune enhancing effect of PAMP transgenesis was also evident systemically with considerably higher serum levels of IL-12 and TNFα, as compared to Tcwt infection (Fig. 2.3c).

IFNγ produced by naïve $CD8^{+}$ T cells, in a TCR independent, IL-12 mediated manner early in the infection appears to serve vital functions in the initial immune responses to a number of pathogens (Berg et al., 2002; Cui et al., 2009). To measure the IFNγ induced early in response to *T. cruzi* infection, we used the IFNγ reporter (Yeti) mice, wherein cells expressing IFNγ concurrently express enhanced-yellow fluorescent protein

(eYFP)(Stetson et al., 2003). We observed significantly higher proportions of IFNγ$^+$CD8$^+$ T cells (but undetectable *T. cruzi* specific (TSKb20$^+$) CD8$^+$ T cells (not shown)) in the draining lymph nodes with TcgFliC infection, compared to that with Tcwt (Fig. 2.3d). Taken together, these results indicate that the expression of bacterial PAMPs in *T. cruzi* markedly enhances innate immune activation both in vitro and in vivo.

2.3.2 PAMP transgenesis in *T. cruzi* enhances adaptive immune responses

A robust *T. cruzi* specific CD8$^+$ T cell response is crucial for the control of *T. cruzi* infection in mice (Bustamante et al., 2008; Padilla et al., 2009a; Tarleton, 1990) and we have previously shown that the CD8$^+$ T cell response to *T. cruzi* in C57BL/6 mice is dominated by cells specific for peptides encoded by the trans-sialidase gene family (Martin et al., 2006). Hence, we can use TSKb20$^+$CD8$^+$ T cells as a surrogate for the total CD8$^+$ T cell response mounted against *T. cruzi*, and track this response using theTSKb20/K^b tetramers (Martin et al., 2006). Mice infected with PAMP- transgenic *T. cruzi* mounted a more rapid (Fig. 2.4a) and substantially stronger TSKb20$^+$ CD8$^+$T cell response that was also maintained at higher levels throughout the infection (Fig. 2.4b and 2.4c) relative to WT infected mice. This potentiation of T cell responses by PAMP-transgenic *T. cruzi* was also evident in the IFNγ and TNFα production by antigen-experienced CD8$^+$ (Fig. 2.4d) and CD4$^+$ (Fig. 2.4e) T cells. Infection with PAMP transgenic *T. cruzi* also elicited higher serum levels of *T. cruzi* specific antibodies compared to infection with Tcwt parasites (Fig. 2.4f).

To reaffirm that the enhanced adaptive immune responses observed with PAMP-transgenesis in *T. cruzi* were indeed dependent on signaling through pattern recognition receptors (PRRs) targeted by the transgenic PAMPs, we infected MyD88$^{-/-}$ mice, that are deficient in the primary adaptor for multiple TLRs and are unresponsive to TLR5, TLR1/2 (Schnare et al., 2001) or (IL-1β from) NAIP5/ipaf stimulation (Adachi et al., 1998). As expected, the *T. cruzi*-infected MyD88$^{-/-}$ mice showed a delayed generation of TSKb20-specific T cells relative to wild-type mice with the pattern of responses being similar irrespective of the expression or not of the bacterial PAMPs (Fig. 2.4g). This result indicates that the enhanced adaptive immune response to *T. cruzi* conferred by PAMP transgenesis is a result of increased triggering of host PRRs and the consequential effects downstream of MyD88 signaling.

Since many pathogens express multiple PAMPs that cooperate to potentiate adaptive immune responses(Napolitani et al., 2005; Querec et al., 2006), we attempted to co-express both FliC and PorB in *T. cruzi*. Although C57Bl/6 mice infected with TcgFliC-PorB generated a more rapid and stronger TSKb20^{+}CD8^{+} T cell response as compared to Tcwt, this response was not improved upon that generated by either PAMPs transgenically expressed alone (Supplementary Fig. 2.3a). This outcome may be a result of technical limitation of protein over-expression in this system since attempts to overexpress multiple other genes in *T. cruzi* also resulted in reduced TcgFliC potency and reduced expression of the second protein (Paraflagellar Rod Protein 4, PAR4) (Supplementary Fig. 2.3b and 2.3c). This result also suggests that the impact of PAMPs

on immune responses may be determined by their relative expression levels in a pathogen.

2.3.3 Continuous expression of FliC is required to sustain the enhanced adaptive immunity

A canonical concept in immunology is that strong innate immunity invokes more potent adaptive immune responses (Akira et al., 2001; Schnare et al., 2001). This concept is supported by many studies demonstrating that co-delivery of TLR-ligands with antigens or vaccines significantly boosts adaptive immune responses (Coffman et al., 2010). However, to our knowledge, no studies have directly investigated the impact on adaptive immunity- of a transient presence of PAMPS at the initiation of infection, to a continuous expression of PAMPs throughout the course of infection. Given that PAMP-transgenesis in *T. cruzi* not only initiated a more rapid TSKb20^{+}CD8^{+} T cell response in mice but also resulted in a response that was maintained at unusually high levels, we suspected that PAMPs may have a continuous instructive role in maintaining strong adaptive immune responses. To determine the consequences of transient versus continuous expression of PAMPs on adaptive immune responses to *T. cruzi*, we tethered various PAMPs to *T. cruzi* using GPI-anchors. Initial experiments showed that molecules linked in this fashion were readily incorporated into the surface of trypomastigotes of *T. cruzi* and had a half-life of ~ 12 hrs (Supplementary Fig. 2.4a). The signaling potency of FliC delivered by the GPI tether (Tc-GPI-FliC) or by endogenous expression (TcgFliC) was equivalent as indicated by their similar abilities to induce NFkb/AP-1 activation in reporter cells (Supplementary Fig. 2.4b), or IL-12

production in peritoneal exudate macrophages (Supplementary Fig. 2.4c). Additionally, Tc-GPI-FliC, TcgFliC or native FliC co-inoculated with *T. cruzi* all potentiated similar innate immune responses *to T.* cruzi in vivo (Fig. 2.5a), and resulted in nearly identical early TSKb20^{+}CD8^{+} T cells responses (Fig. 2.5b). However only in the infection with TcgFliC, was the TSKb20-specific response maintained above the level of the Tcwt infection through to the chronic stage (Fig. 2.5b). The delivery of other individual or combinations of PAMPs with *T. cruzi* infection by GPI-anchors or by co-inoculation enhanced innate (Supplementary Fig. 2.4d) immune responses in mice – some much more strongly than TcgFliC. But only infection with the PAMP-transgenic *T. cruzi* resulted in the long-term maintenance of enhanced adaptive responses (Supplementary Fig. 2.4e). Assuming that a general pro-inflammatory milieu(Gurunathan et al., 1998) maintained by the constantly expressed FliC may be driving the maintenance of higher *T. cruzi* specific immune response in TcgFliC, we attempted to simulate this in Tc-GPI-FliC or Tc-GPI infections by repeated inoculations(Salazar-Gonzalez et al., 2007) of native FliC. However, neither did we detect any (increased) IL-12 in the sera (data not shown) , nor an elevation or retention of the TSKB20^{+}CD8^{+} T cell levels in these mice (Supplementary Fig. 2.4f), possibly indicating an essential role for APC-PAMP interactions within the vicinity of infected cells. Thus, continued expression of PAMPs act to maintain stronger adaptive immune responses, exceeding those elicited by transient PRR engagement at the initiation of infection.

2.3.4 FliC transgenesis enhances control of *T. cruzi* infection in mice

To investigate the impact of the PAMP-induced enhancement of innate and adaptive immune responses on the parasite control during the course of *T. cruzi* infection, we first monitored the phenotype of *T. cruzi*-specific $CD8^+$ T cells in these mice. Drug-induced cure of *T. cruzi* infection results in a gradual shift in the $TSKb20^+CD8^+$ T cells from a predominant T-effector phenotype $CD127^{lo}$) to a majority T-central memory (T_{CM})-like ($CD127^{hi}$) phenotype (Bustamante et al., 2008), accompanied by a decrease in the frequency of $CD8^+$ T cells expressing KLRG1, a marker for repeated antigenic stimulation (Bustamante et al., 2008; Ibegbu et al., 2005). At 296 dpi, TcgFliC-infected mice exhibited higher proportions of T_{CM} among the $TSKb20^+CD8^+$ T cells and decreased numbers of $KLRG1^+(CD44^+)CD8^+$ T cells in comparison to mice infected with wild-type *T. cruzi* (Fig. 2.6a), suggesting more effective control of the infection with TcgFliC This supposition was confirmed using qPCR to measure *T. cruzi* DNA in muscle tissue from these mice (Supplementary Fig 2.5). At 400 dpi, *T. cruzi* DNA was undetectable in mice infected with TcgFliC, but was consistently detected in tissues from Tc-GPI-FliC, Tc-GPI+FliC and Tcwt infected mice (Fig. 2.6b).However, we did not observe T cell phenotype or tissue parasite loads indicative of a better control of TcgPorB infection relative to Tcwt infection (Supplementary Fig 2.6)

We have previously used immunosuppression to reveal otherwise undetectable infection and as a definitive measure of drug-induced cure in *T. cruzi* infection (Bustamante et al., 2008). One of three TcgFliC-infected mice immunosuppressed with cyclophosphamide exhibited no detectable parasites after immunosuppression,

indicating clearance of the infection (data not shown). The enhanced control of the TcgFliC infection relative to wild-type *T. cruzi* infection was neither due to a decrease in virulence of the FliC-transgenic parasites, as IFNγ$^{-/-}$ mice infected with TcgFliC or Tcwt showed similar peripheral blood parasite loads and mortality patterns (Supplementary Fig. 2.7), nor would it be due to immune responses directed at FliC presented as an antigen (Supplementary Fig. 2.8). Taken together, these data indicate that FliC transgenesis, but not co-inoculation or temporary surface-anchoring potentiates anti-*T. cruzi* adaptive immune responses that facilitates control and clearance of *T. cruzi* infection.

2.4 Discussion

One of the key paradigms in immunology is that innate immune mechanisms detect microbial infections through their characteristic PAMPs and trigger the specific antimicrobial host defense responses appropriate to that infection (Coffman et al., 2010; Iwasaki and Medzhitov, 2004, 2010). Once initiated, these pathogen-specific (adaptive) immune responses bring about control of the infection and often, a long-term specific immunological memory. However, we have very limited knowledge of the role of PAMPs in influencing the adaptive immunity beyond its initiation, in large part because, by their very nature, PAMPS are crucial for pathogen survival and thus cannot be turned off during an infection. In this study we provide unequivocal evidence that the expression of classical bacterial PAMPS in the protozoan pathogen *T. cruzi* results in substantially enhanced innate and adaptive immune responses and more efficient pathogen control. These data add to the wealth of information indicating that *T. cruzi* has an extremely

quiet entry into hosts (Costales et al., 2009) and a considerably delayed induction of anti-parasitic immune responses (Padilla et al., 2009b).

Though PAMPs are highly conserved structures that are extremely difficult for pathogens to alter or sacrifice, there is some plasticity in PAMP display. For example, host detection of LPS in *Porphyromonas gingivalis* and *Escherichia coli* is modulated by differential acylation of lipid-A (Coats et al., 2007; Reife et al., 2006), while *Yersinia pestis* synthesizes LPS-lipid A that is a poor TLR4 ligand (Montminy et al., 2006) and *Pseudomonas aeruginosa* down-regulates its flagellin expression when in airway passages(Wolfgang et al., 2004). Although it is unlikely that any pathogen will be able to make all its PAMPs entirely invisible to the immune system, we believe that potential PAMPs could be rendered immunologically inconsequential by concealing or modifying, without significantly impacting pathogen biology. Multiple PAMPs (e.g. GPI anchors, DNA, GIPL-ceramide), have been attributed to *T. cruzi* (Bafica et al., 2006; Campos et al., 2001) but these molecules seem to be relatively insignificant to the downstream immune responses generated, with comparable adaptive immunity in mice that are either responsive or (genetically) unresponsive to these ligands (Oliveira et al., 2010). Perhaps this is not surprising since these putative PAMPs would be "hidden" from their respective TLRs in live, intact *T. cruzi*. However, when strong bacterial PAMPs are transgenically expressed and released by *T. cruzi*, significantly improved innate and adaptive immune responses are generated. So we propose that the failure of *T. cruzi* to display potent PAMPs may indeed be another example of innate immune evasion employed by pathogens.

The observed evasion of innate immune responses may not only be important in delaying the adaptive immune response – thus allowing for firm establishment of the infection, but also may promote the persistence of *T. cruzi.* In the presence of a bacterial PAMP, *T. cruzi* infection is better controlled and even completely cleared in some cases. It has to be noted that complete clearance of *T. cruzi* infection is normally extremely rare. Given the increased level of *T. cruzi*-specific $CD8^+$ T cells with a Tcm phenotype and the nearly undetectable tissue parasite load in mice infected with PAMP transgenic *T. cruzi,* we predict that most of these mice would eventually cure these infections if allowed sufficient time. Importantly, this enhanced control of *T. cruzi* infection derived from expression of bacterial PAMPs is not associated with any evidence of increased immunopathology. .

The relative absence of PAMPs in *T. cruzi* provided a unique opportunity to study the importance of PAMP expression beyond the early induction of adaptive immune responses. Though there is a wealth of literature demonstrating how the strength of innate immunity determines the potency of adaptive immune responses(Iwasaki and Medzhitov, 2004, 2010), few studies have focused on the impact of innate immune responses on adaptive immunity once an infection is established. When potent bacterial PAMPs were either co-inoculated with, temporarily surface-anchored on, or constitutively expressed- by the invading *T. cruzi*, the resulting adaptive immune responses were not only accelerated, but also peaked early at levels that were above that seen in mice infected with WT *T.* cruzi. However, it was only when *T. cruzi* perpetually expressed the PAMPs that the stronger adaptive immune responses were

maintained throughout the course of the infection, eventually leading to a better control of the pathogen and sterile clearance in some cases. It is likely that the locally enhanced inflammatory milieu, coupled with the improved antigen processing and presentation by more highly activated APCs resulting from continuing PAMP exposure (Amigorena and Savina, 2010; Iwasaki and Medzhitov, 2010) potentiated the quality, quantity and longevity of T and B cells (Bocek et al., 2004; Kang et al., 2011; Mescher et al., 2006; Trinchieri, 2003). Although transgenic expression of FliC and PorB induced similar boosting of immune responses, it was only in the case of TcgFliC that this response corresponded with better parasite control. FliC is distinctive in its ability to induce IL-1β through the intracellular NAIP5/ipaf receptor stimulation and given that *T. cruzi* spends the majority of its time in vertebrates within the cytoplasm of host cells that are likely to express the NAIP5/ipaf receptor(Poyet et al., 2001), it is possible that NAIP5/ipaf-IL-1β activation contributes to enhanced recognition and control of TcgFliC infected cells. IL-1β levels have also been shown to correlate with $CD8^+$ T cell abundance in adipose tissue (Koenen et al., 2011) which incidentally is a major depot for *T. cruzi* persistence chronically. A possible confounder in the interpretation of our results is that FliC expressed by TcgFliC may act as a target antigen for adaptive immune responses, contributing to the control of FliC-expressing parasites. However the absence of detectable FliC specific T cells in TcgFliC-infected mice argues against this possibility. Additionally, *T. cruzi* expressing the highly immunogenic chicken ovalbumin (OVA) protein that induces very strong OVA-specific T cells does not appear to be controlled any better than WT *T. cruzi* (unpublished observation).

Heterologous expression of bacterial PAMPs in *T. cruzi,* despite prompting stronger adaptive immune responses, could only marginally (though significantly) accelerate the intial induction of these responses. Hence the deficiency of effective PAMPs in *T. cruzi* may be only one of the several factors that contribute to the marked delay in initiation of anti-*T. cruzi* immune responses. An additional important trigger for induction of adaptive responses is the exposure of damage associated molecular patterns (DAMPs) (Ahrens et al., 2012). Revelation of DAMPs from either host or *T. cruzi* would not be expected until 4-5 days post-infection, with the initial round of exit of *T. cruzi* from infected host cells.

This study provides significant new insights into the *T. cruzi*-host interface and identifies some of the contributing factors for the ability of *T. cruzi* to persist indefinitely in most hosts, despite the generation of potent immune responses. The contribution of inadequate PAMP expression in the persistence of other pathogens, in particular eukaryotic pathogens that lack many of the classical PAMPs, is worthy of further exploration. Our findings also advance the idea that innate immune responses may have an extended, instructive role on the adaptive immunity, thus playing an even more significant part in the effective control of pathogens than was previously appreciated. The inability of classical adjuvants to generate a long-lasting enhancement of T cell responses has been a hurdle in the development of T cell based vaccines (Coffman et al., 2010). Expression of heterologous PAMPs would be expected to enhance the effectiveness of live vaccines and periodic or continuous exposure to PAMPs might have therapeutic benefit in persistent infections wherein endogenous PAMPs are inadequate.

2.5 Methods

2.5.1 Mice, parasites, and infections

C57BL/6, B6.IFNγ–knockout (IFNγ$^{-/-}$), MyD88 Knockout (MyD88$^{-/-}$), B6.IL-12*yet40* reporter (IL-12*yet40*) and IFNγ reporter (Yeti) mice were purchased from The Jackson Laboratory or bred and maintained in our animal facility under specific pathogen-free conditions. *T. cruzi* epimastigotes were transfected as described previously (Garg et al., 1997) with pTREX plasmid (Lorenzi et al., 2003) containing the coding sequence of *Salmonella typhimurium* flagellin (FliC), *Neisseria meningitidis* (FAM18 strain) porin (PorB) or *T. cruzi* paraflagellar rod protein 4 (PAR4) genes, with or without fusion to an upstream N-terminal portion of the *T. cruzi* gp72 gene or influenza haemagglutinin (HA)-tag, to generate transgenic *T. cruzi*. All infections were initiated by inoculating vero cell culture passaged trypomastigote stage *T. cruzi,* intra-peritoneally (i.p) (10^4 parasites) or subcutaneously in the ear (s.c) ($5x10^4$ parasites) or the foot pad (f.p) (10^4 parasites). Native FliC was inoculated i.p in mice at 10µg/animal(Salazar-Gonzalez et al., 2007) at 10 days interval. All animal protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

2.5.2 Reporter cell assay for NFkB/ AP-1 activation and IL-12 production

The ability of various *T. cruzi* strains to induce NFkB/ AP-1 activation by TLR stimulation was assayed using THP1-Blue-CD14 reporter cells (Invivogen), following the manufacturer's protocol. 10^4 live *T. cruzi* trypomastigotes were incubated with $2x10^6$ reporter cells for 9 hours at 37°C/5% CO_2, and the nuclear translocation of activated

NFkB/ AP-1 was determined by colorimetrically quantifying the secreted embryonic alkaline phosphatase (SEAP). To determine the IL-12 production induced in cells by *T. cruzi*, 10^5 peritoneal exudate macrophages from IL-12yet40 reporter mice were incubated with 10^3 *T. cruzi* trypomastigotes for 18 hrs at 37°C/5% CO_2. The proportion of YFP^+ macrophages was determined by flow cytometry. LPS or media served as controls.

2.5.3 Determination of caspase 1 activity

Active caspases were detected with FLICA Apoptosis Detection kit (Immunochemistry Technologies) following the manufacturer's protocol. After 12 hours incubation of $2x10^4$ TcgFliC or Tcwt with $2x10^5$ RAW blue (TLR5-) mouse macrophages (Invivogen), the latter were incubated with a fluorescent inhibitor peptide specific to caspase 1 (FAM-YVAD-FMK) for 60 min at 37°C/5% CO_2. Inhibitors were removed by rinsing; the cells were fixed and then analyzed with a florescence plate reader

2.5.4 Western blot and ELISA

To determine the presence of FliC, TcgFliC lysate (Martin and Tarleton, 2005) or culture (12h) supernatant, were probed with anti-FliC Mab (Biolegend) by western blot. Sera collected from C57BL/6 mice infected with various *T. cruzi* strains, 30 dpi were assayed for anti-*T. cruzi* antibodies by ELISA. To determine the relative concentrations of haemagglutinin (HA) tagged protein (PAR4-HA) in the trypomastigote stage from various strains of transgenic *T. cruzi*, serial dilutions of whole cell lysates were assayed

with anti-HA antibody (Roche). A purified HA-tagged protein (*T. cruzi* PAR2) expressed in *E. coli* was used as the standard.

2.5.5 Intracellular cytokine staining for IL-1β, IFNγ and TNFα

To measure IL-1β production, $2x10^5$ RAW blue ($TLR5^-$) mouse macrophages (Invivogen) were incubated with $2x10^4$ TcgFliC trypomastigotes for 18hrs. *E. coli* lipopolysaccharide (LPS) +ATP or media served as controls. The induced IL-1β in macrophages were determined by staining using the Cytofix/Cytoperm intracellular staining kit (BD Pharmingen) following the manufacturer's protocol. Similarly, to determine intracellular IFNγ and TNFα production, $1.5x10^6$ spleen cells from TcgFliC, TcgPorB or Tcwt infected, or naïve mice were restimulated with *T. cruzi* peptide TSKb20 (5 μM), *T. cruzi* whole cell lysate (10 μg) or FliC and processed for intracellular cytokine staining (ICS). Recombinant Ovalbumin (ova) or recombinant Ovalbumin protein with TSKb20 peptide substituted for SIINFEKL(Ova-TS20) served as a controls. The splenocytes were washed in PAB (2% BSA, 0.02% azide in PBS) and stained for surface expression of CD4, CD44 and CD8 using anti-CD4 PE, CD44 FITC and anti-CD8 eFluor450 (BD Pharmingen). All cells for ICS were fixed and permeabilized using Cytofix/Cytoperm (BD Pharmingen) on ice for 15 min and washed in PermWash (BD Pharmingen). The cells were then stained with anti-IL-1β PE (R&D systems), anti-IFNγ APC or anti-TNFα PECy7 (both BD Pharmingen) for 30 min on ice. Cells were washed and fixed in 2% formaldehyde for 20 min at 4°C, then washed and resuspended in PAB for flow cytometric analysis.

2.5.6 Phenotyping cells by flow cytometry

T cell phenotypes were determined as described previously (Bustamante et al., 2008) and stained with tetramer-phycoerythrin (TSKb20-PE; ANYKFTLV peptide on $H2K^b$ NIH Tetramer Core Facility) and the following: anti-CD62L APC, anti-CD44 FITC, anti-CD8 efluor-450, anti-CD127 PEcy7 and anti-KLRG1 APCcy7 (all from eBioscience). Anti-CD4 PECy5 (Invitrogen) and anti-B220 PECy5 (Invitrogen) staining was used for a dump channel.

To determine the phenotypes of cells infiltrating the site of infection, the tissue (ear) was enzymatically digested to dissociate the cells as described before (Phythian-Adams et al., 2010). In the case of draining lymph nodes, the cells were dissociated by gently crushing between the ground edges of glass slides. After FcR (CD16/ 32) block, cell surface markers were used to differentiate several cell lineages as previously described (Phythian-Adams et al., 2010). DCs, infiltrating monocytes or resident macrophage subsets were differentiated using the following mAb conjugations: CD11c APC, CD8α efluor450, CD11b APC/eFluor780, Gr-1(Ly6C/Ly6G) PerCP/Cy5.5, F4/80 PECy7 and CD45 PE (BD Pharmingen, ebioscience, or BioLegend). DCs were defined with CD11c, with further differentiation into $CD11b^-CD8\alpha^+$ cDCs and $CD11b^+$ ($F4/80^-$) DCs. Monocytes and iDCs were defined as $CD11b^+$ $CD11c^-Gr\text{-}1^{int}$ and $CD11b^+$ $CD11c^+$ $Gr\text{-}1^{int}$ respectively (Turley et al., 2010). Macrophages were identified as $F4/80^+$ $CD11b^+$ $CD11c^-$. IL-12 producing DCs were defined as $CD11c^+$ $CD8\alpha^+$ ($CD11b^-$) YFP^+ in IL-12*yet40* reporter mice as described before(Reinhardt et al., 2006). Data is

represented as the percentage of each cell type over all the cells derived by enzymatic digestion, representing the total cellularity at the site.

IFNγ producing CD8 T cells in the draining lymph nodes of yeti mice were defined as $CD4^{-}B220^{-}CD8^{+}YFP^{+}$ as described before(Mayer et al., 2005). At least $5x10^{5}$ (blood) or $5x10^{6}$ (peripheral tissue/ lymph node) cells were acquired per sample using a CyAn flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

2.5.7 Serum cytokine assay

Blood collected from C57Bl/6 mice inoculated with TcgFliC or Tcwt and sera separated to assay for various cytokines using the Q-Plex™ Mouse Cytokine Screen ELISA (Quansys Biosciences) or for IL-12 alone using eBioscience mouse Il-12p70 flowCytomix following the manufacturer's protocol. Luminescence intensity of each sample was measured and the concentration of each cytokine was determined using the Q-View™ software (Quansys Biosciences) in the former or samples analyzed by flow-cytometry for the latter.

2.5.8 Temporary anchoring of PAMPs on *T. cruzi*

FSL-biotin GPI anchor with a single biotin F-moiety (FSL-CONJ(1Biotin)-SC2-L1, KODE Biotech Materials Ltd) was used to coat *T. cruzi* trypomastigotes as per the manufacturer's protocol. $1x10^{6}$ trypomastigotes were incubated with 2µg of FSL-biotin in 100 µl serum free RPMI 1640 media. After washing to remove the excess FSL-biotin, the parasites were incubated on ice with streptavidin (Sigma), at 5 times the molar concentration of FSL-biotin (to give Tc-GPI). Excess streptavidin was removed by

washing and various biotinylated ligands: FliC-biotin, Pam3CSK4-biotin (Pam3Cys-Ser-(Lys)4-biotin, invivogen), ODN-biotin (oligodeoxynucleotide- biotin, invivogen), all at 3x molar concentration of FSL-biotin or Pam3CSK4-biotin and ODN-biotin together, each at 1.5x molar concentration of FSL-biotin were incubated with the Tc-GPI for 30 min on ice to yield Tc-GPI-FliC, Tc-GPI-Pam3CSK4, Tc-GPI-ODN or Tc-GPI-ODN-Pam3CSK4 respectively. FliC was biotinylated using EZ-link sulfo-NHS-LC Biotinylation kit (Thermo scientific) following the manufacturer's protocol. The various PAMP anchored *T. cruzi* strains were washed twice with RPMI1640 to remove excess PAMPs, counted, re-suspended in complete RPMI1640 and used for in vitro or in vivo assays. Tc-GPI was used as the control. PAMPs when co-inoculated with *T. cruzi*, were used at approximately the same quantities (in w/v) as was used to label *T. cruzi* above.

2.5.9 Real-time PCR

The skeletal muscle tissue from mice were analyzed by real-time PCR for the presence of *T. cruzi* (DNA) as described before (Cummings and Tarleton, 2003)

2.5.10 Assessment of infectivity and clearance of *T. cruzi*

To assess the infectivity of different strains of *T. cruzi*, IFNγ$^{-/-}$ mice were inoculated with 10^4 trypomastigotes of TcgFliC or Tcwt strains. Blood was collected from the tail vein at 21dpi to quantify the number of parasites using a compound light microscope and expressed as the number of live trypomastigotes per 100 (40X) fields. Survival was monitored daily. Clearance of *T. cruzi* from infected mice were determined as previously described (Bustamante et al., 2008).

2.5.11 Statistical analysis

Data are presented as the mean plus/ minus the standard error of mean (s.e.m). Statistical analyses compared the groups with a two-tailed student t-test. Only p values of less than 0.05 were considered statistically significant.

Acknowledgements

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Figures

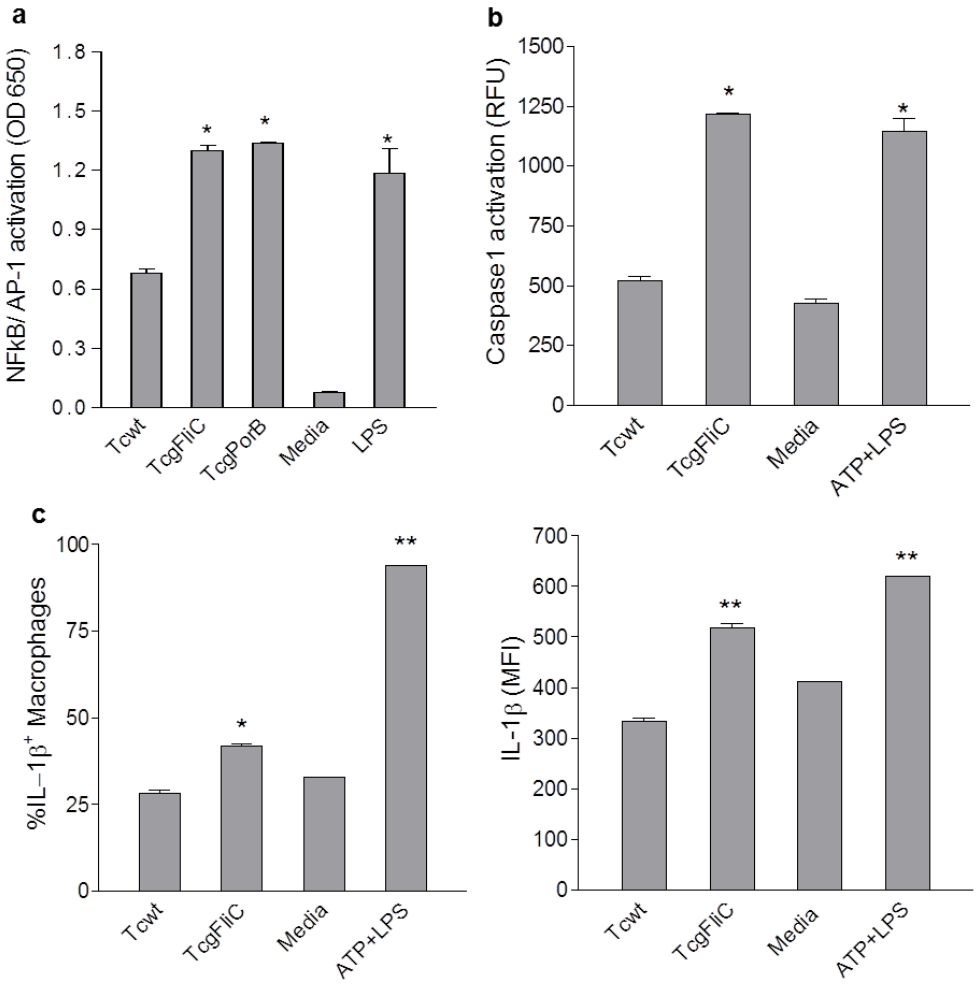


Figure 2.1. PAMP transgenesis in *T. cruzi* enhances cellular innate immune responses (a) NFkB/ AP-1 activation in reporter cells incubated with Tcwt, TcgFliC or TcgPorB trypomastigotes for 12 hrs. Media or *E. coli* derived LPS were used as negative and positive controls, respectively. (b) Caspase1 activation in (TLR5^{-}) macrophages incubated with Tcwt or TcgFliC trypomastigotes for 12 hrs. Media or ATP with *E. coli* derived LPS served as controls. (c) The proportion of (TLR5^{-}) macrophages producing IL-1β on incubation with Tcwt or TcgFliC trypomastigotes for 18 hrs. (d) IL-1β mean

fluorescence intensity (MFI) per cell by TLR5^{-} macrophages incubated with Tcwt or TcgFliC trypomastigotes for 18 hrs. Media or ATP with *E. coli* derived LPS served as controls. All data show mean $\pm$ s.e.m and are representative of at least 3 separate experiments. * or ** indicates $p \leq 0.05$ or ≤ 0.01 respectively, as determined by student t-test comparing the indicated groups to Tcwt.

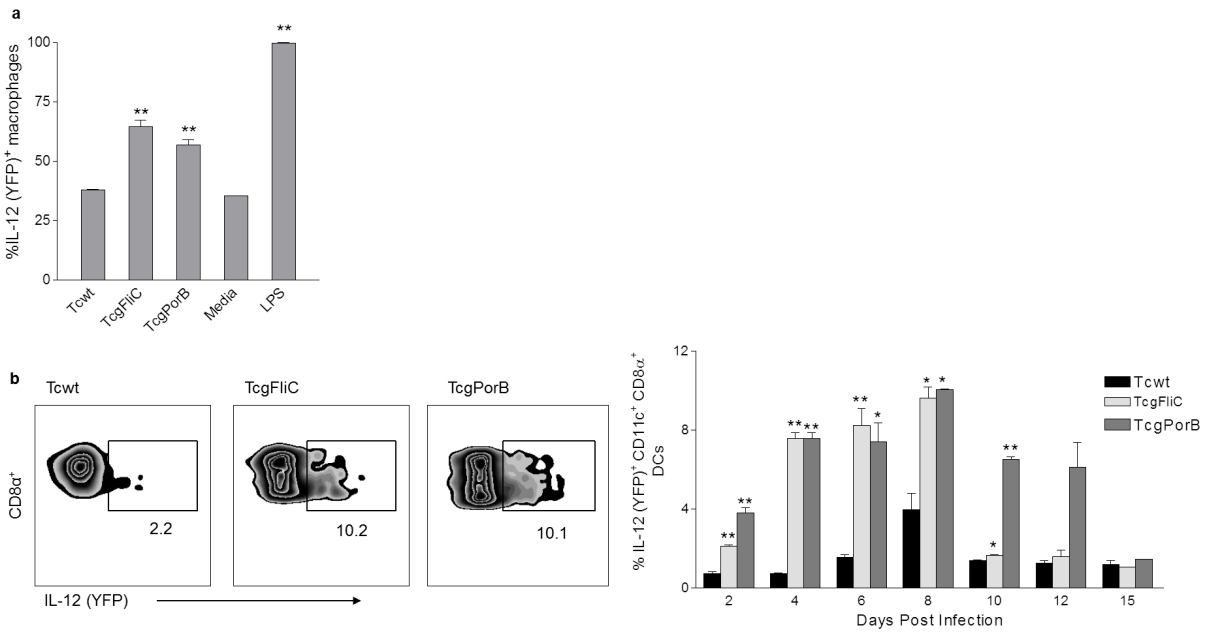


Figure 2.2. PAMP transgenic *T. cruzi* induces higher frequencies of IL-12 producing APCs (a) The proportion of peritoneal exudate macrophages with induced IL-12 (YFP) on incubation with Tcwt, TcgFliC or TcgPorB trypomastigotes for 18 hrs. Media or *E. coli* derived LPS served as controls. Data presented as mean $\pm$ s.e.m and are representative of 3 separate experiments. (b) IL-12 producing ($CD11c^+$ $CD8\alpha^+$) cDC recruitment into the draining lymph nodes on Tcwt, TcgFliC or TcgPorB infection of IL-12*yet40* mice. Flow plots show representative data from 6 dpi, with the numbers inset indicating the percentage of IL-12 producing cDCs. Right panel shows the kinetics of IL-12^+ ($CD11c^+$ $CD8\alpha^+$) cDC recruitment. Data are represented as mean$\pm$ s.e.m from one of 3 separate experiments, with at least 3 mice/ group. * or ** indicates $p \leq 0.05$ or $\leq$ 0.01 respectively, as determined by student t-test comparing the indicated groups to Tcwt (a), at the corresponding time points (b).

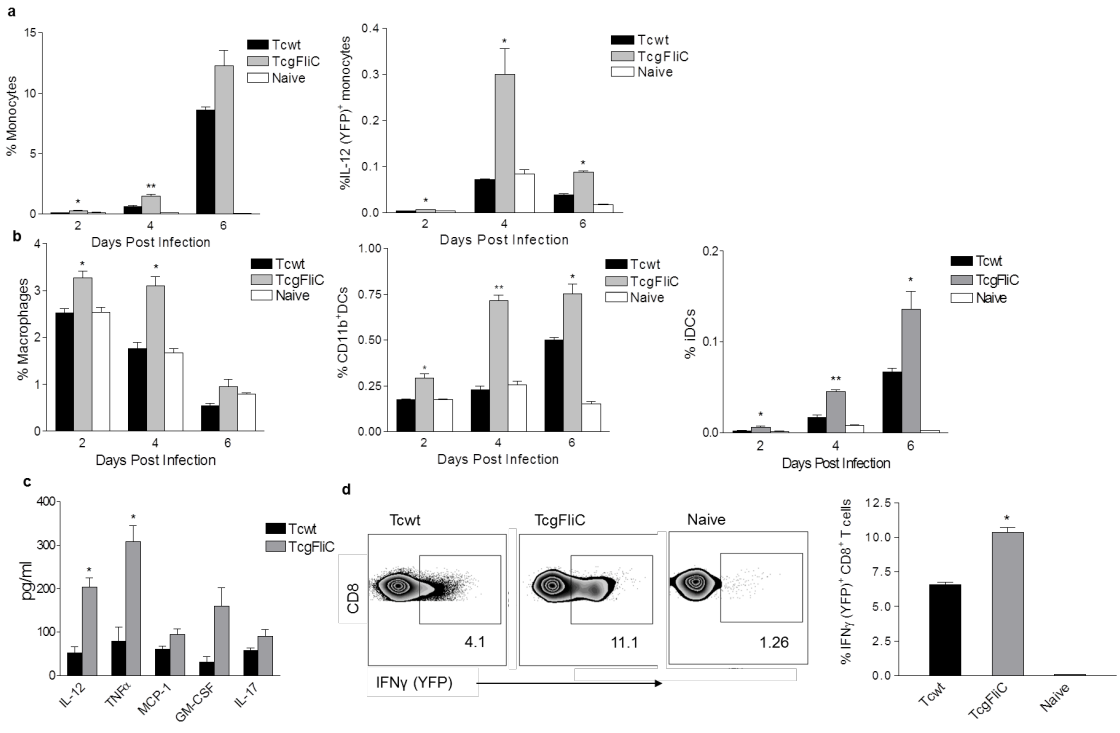


Figure 2.3. PAMP transgenesis in *T. cruzi* enhances the systemic innate immune responses. (a) The proportion of blood derived monocytes and their IL-12 producing subset at the site (s.c) of Tcwt or TcgFliC infection in IL-12*yet40* mice at various time points post infection. Naïve mice were inoculated with media alone. (b) The proportion of resident macrophages, $CD11b^+$ DCs or iDCs at the site of Tcwt or TcgFliC infection in IL-12*yet40* mice at various time points post infection. Naïve mice were inoculated with media alone. (c) Serum levels of various cytokines in C57Bl/6 mice infected with Tcwt or TcgFliC, 4 dpi. (d) Percentage of $CD8^+$ T cells producing IFNγ in the draining lymph nodes with Tcwt or TcgFliC infected (f.p) Yeti mice, 6dpi. Representative flow plots with the numbers inset indicating the percentage of IFNγ producing $CD8^+$ T cells. In the right panel, data represented as mean $\pm$ s.e.m from one of 3 separate experiments with 3-6 mice/group. * or ** indicates $p \leq 0.05$ or ≤ 0.01 respectively, as determined by student t-

test comparing the indicated groups to Tcwt (c and d), at the corresponding time points (a and b).

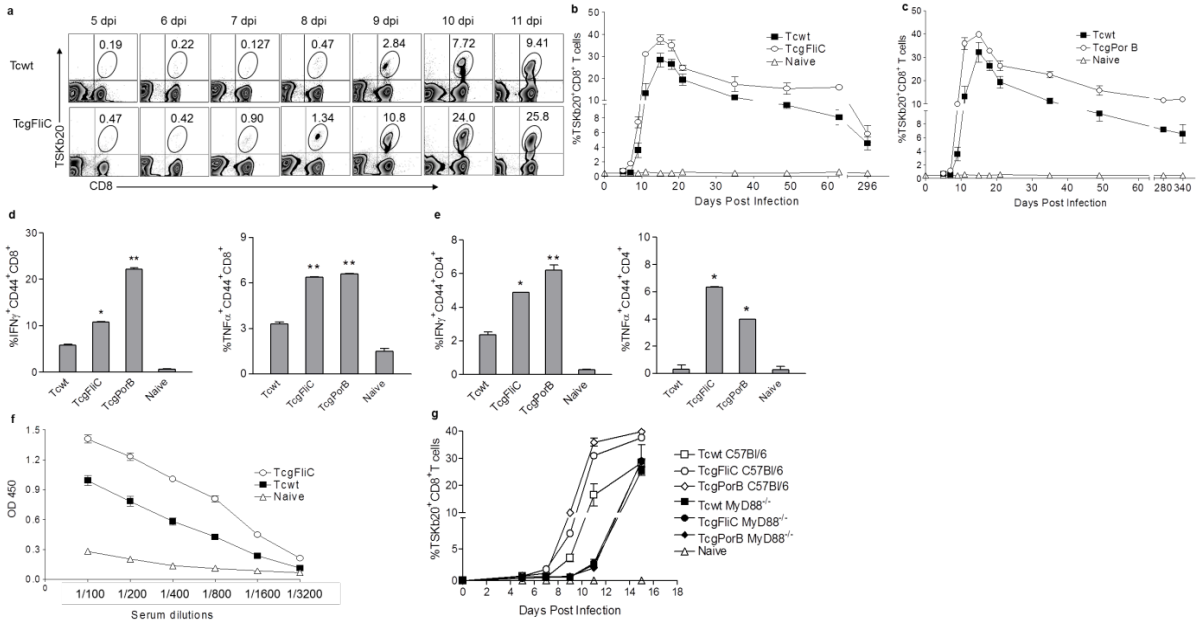


Figure 2.4. PAMP transgenesis in *T. cruzi* enhances the adaptive immune responses generated. (a) Flow plots show early time points post-infection in C57BL/6 mice with Tcwt or TcgFliC, with numbers inset indicating percentage of $TSKb20^{+}CD8^{+}$ T cells. (b and c) The kinetics of $TSKb20^{+}CD8^{+}$ T cell frequencies in circulation in TcgFliC (b) and TcgPorB (c) compared to Tcwt infection of C57BL/6 mice. Data represented as mean±s.e.m from one of 6 separate experiments, with at least 6 mice/ group. (d and e) The percentage of $CD8^{+}(CD44^{+})$ (d) or $CD4^{+}(CD44^{+})$ (e) T cells producing IFNγ (left) or TNFα (right) in response to TSKb20 peptide or *T. cruzi* whole cell lysate re-stimulation respectively in Tcwt, TcgFliC or TcgPorB infected C57BL/6 mice, 180dpi. Data represented as mean±s.e.m from one of 3 separate experiments, with 3 mice/ group. (f) Anti-*T. cruzi* antibody titers in sera of mice infected with Tcwt or TcgFliC in C57Bl/6 mice, 30dpi. Data shown as mean ± s.e.m, representing 2 separate experiments, with 3 mice/ group. (g) The kinetics of $TSKb20^{+}CD8^{+}$ T cell frequency in circulation on Tcwt, TcgFliC or TcgPorB infection of $MyD88^{-/-}$ or C57Bl/6 mice. Data represented as mean ±

s.e.m from one of 3 separate experiments, with 3-6 mice/ group. *p≤ 0.05 or **p≤ 0.01, as determined by student t-test comparing the indicated groups to Tcwt.

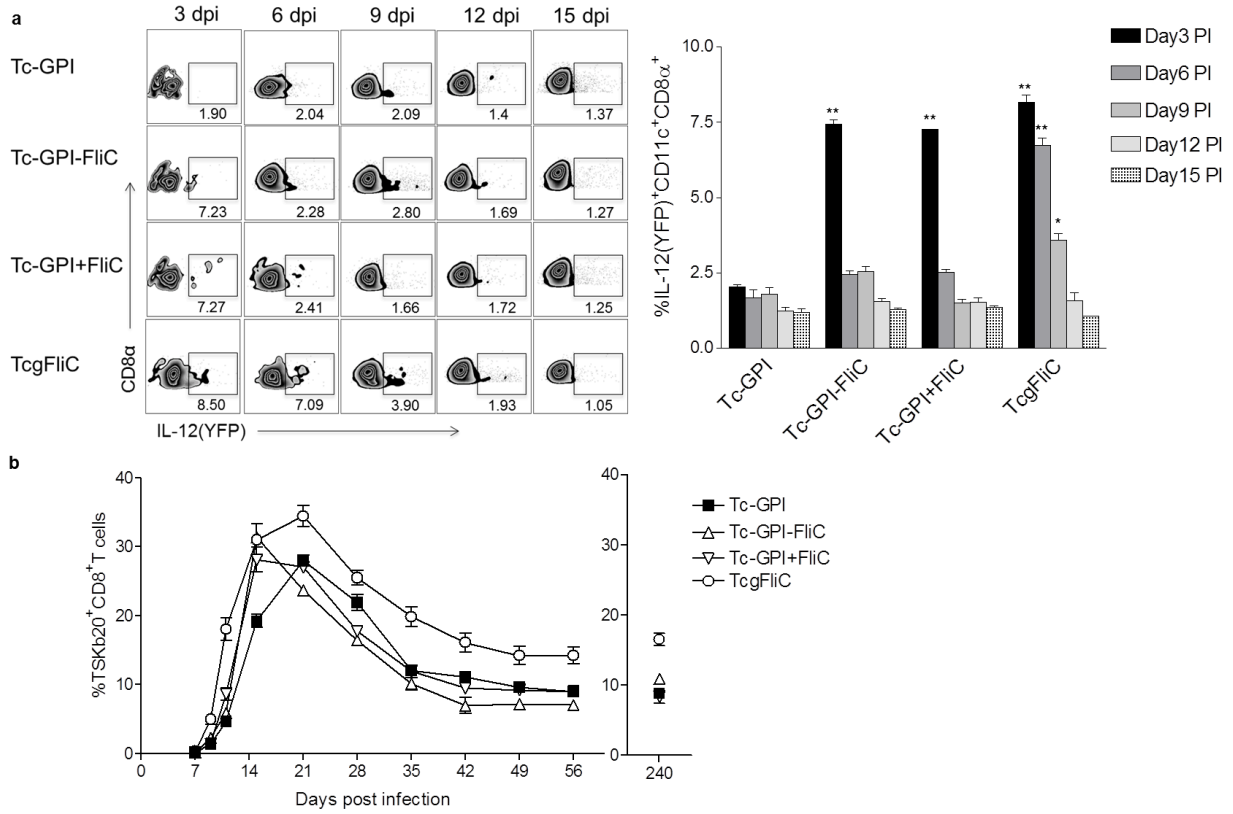


Figure 2.5. Continuous expression of FliC is required to sustain the enhanced adaptive immunity. (a) The proportions of IL-12 producing cDCs recruited into the draining lymph nodes on various days post infection with *T. cruzi* having FliC temporarily surface-anchored (Tc-GPI-FliC), co-inoculated with (Tc-GPI+FliC), or constitutively expressed in (TcgFliC), compared to the background strain (Tc-GPI) in IL-12*yet40* reporter mice. The flow panel shows representative flow plots, with the numbers inset indicating the %IL-12$^+$ cDCs. Data are represented as mean ± s.e.m from one of 3 separate experiments, with at least 3 mice/group/time point. (b) TSKb20$^+$CD8$^+$ T cell frequency in circulation in Tc-GPI, Tc-GPI-FliC, Tc-GPI+FliC or TcgFliC infection of C57BL/6 mice. Data represented as mean ± s.e.m combining two experiments, with 3-6 mice/ group.

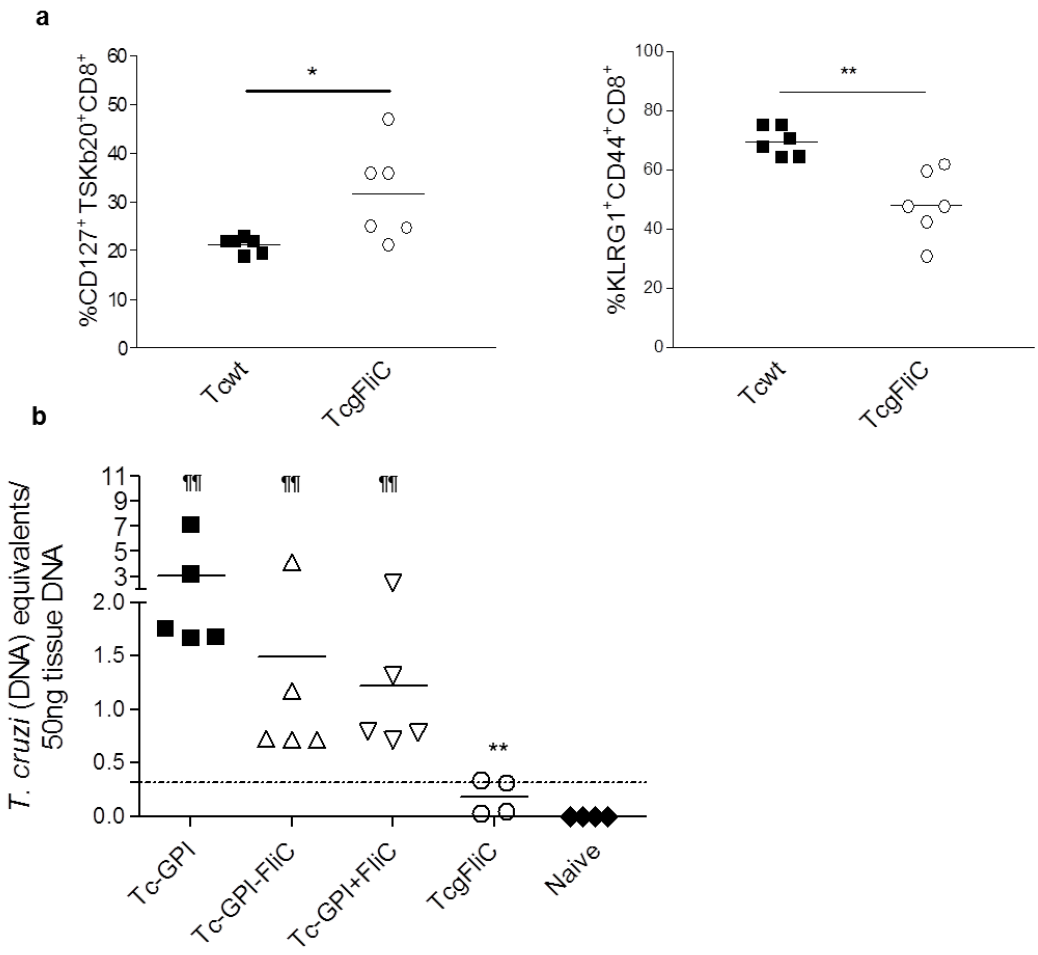
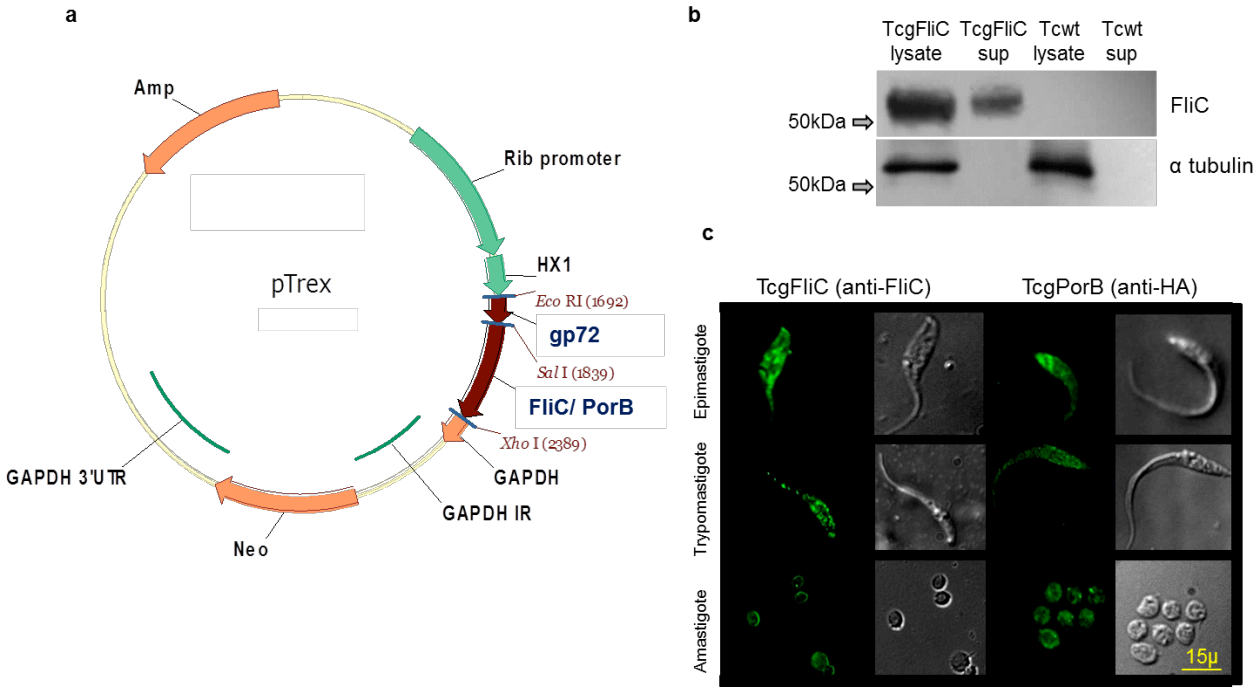
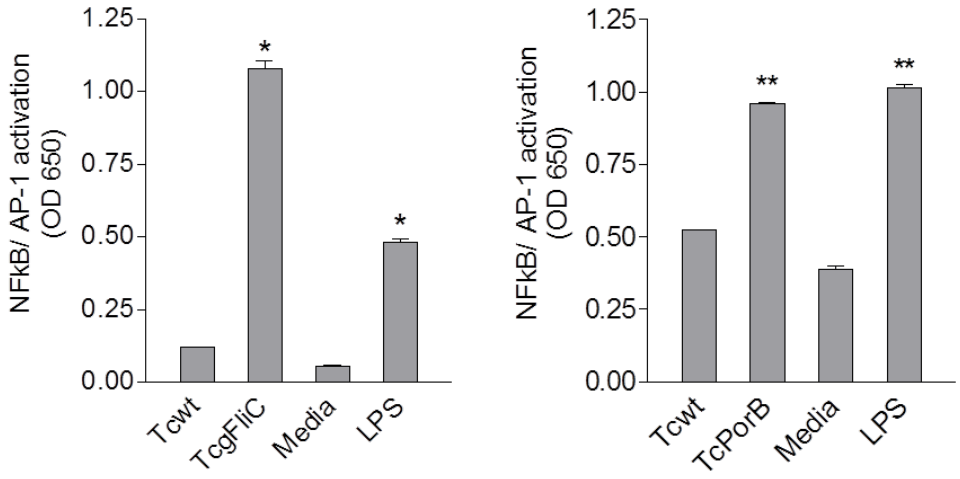


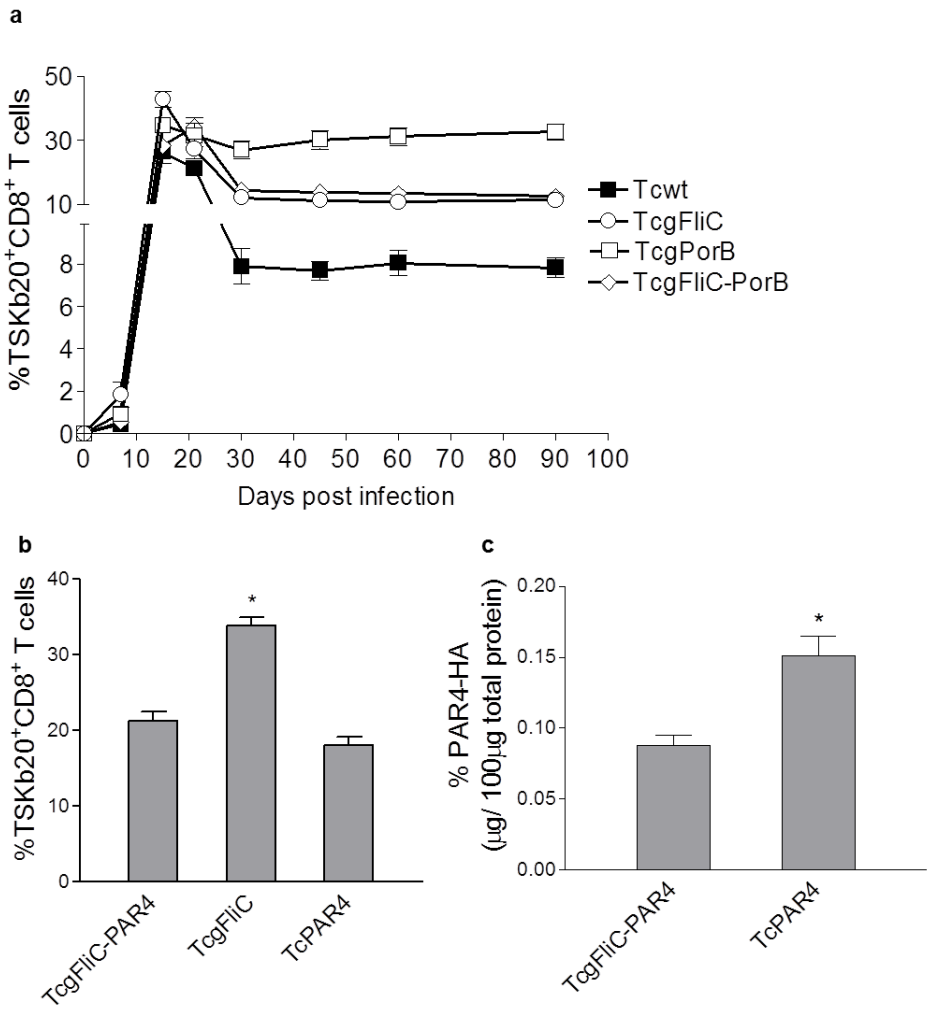
Figure 2.6. FliC transgenesis enhances control of *T. cruzi* infection in mice**.** (a) Frequency of $CD8^+$ T cells in circulation having Tcm like phenotype ($CD127^{hi}$ $TSKb20^+$ $CD8^+$ (left) and $KLRG1^{lo}$ $CD44^+$ $CD8^+$ (right)), in Tcwt or TcgFliC infected C57BL/6 mice, 296 dpi. Data represents 3 separate experiments with 3-6 mice/ group. (b) *T. cruzi* DNA in skeletal muscle of C57BL/6 mice inoculated with Tcwt, Tc-GPI-FliC, Tc-GPI+FliC or TcgFliC as determined by quantitative real-time PCR, 400 dpi. Horizontal bars represent the mean. Naïve mice served as control. The dotted line represents the threshold of detection for the assay. Data are representative of 3 separate experiments initiated with at least 5 mice/ group. * or ** indicates $p \leq 0.01$ comparing the indicated groups to naïve or Tcwt infected mice respectively, as determined by student t-test.



Supplementary figure 2.1. PAMP transgenic *T. cruzi* secretes the PAMPs and presents it in all life cycle stages. (a) *S. typhimurium* flagellin (FliC) or *N. meningitidis* porin (PorB) cloned in pTREX expression vector with gp72 signal sequence at their 5` end to make transgenic *T. cruzi* secreting FliC (TcgFliC) or PorB (TcgPorB). (b) TcgFliC or Tcwt trypomastigote stage lysate and culture supernatant immuno-blotted with antibodies against FliC or α-tubulin. (c) FliC or PorB expression in the epimastigote, trypomastigote and amastigote stages of TcgFliC or TcgPorB respectively determined by indirect immuno-fluorescence/DIC microscopy modifying the protocol described elsewhere(Agrawal et al., 2009). Anti-FliC (Biolegend) or anti-HA (Roche) antibodies were used to mark the target proteins. Images were acquired with an Applied Precision Delta Vision microscope, were deconvolved and adjusted for contrast using its Softworx software (Applied Precision). Scale bar represents 15µ.

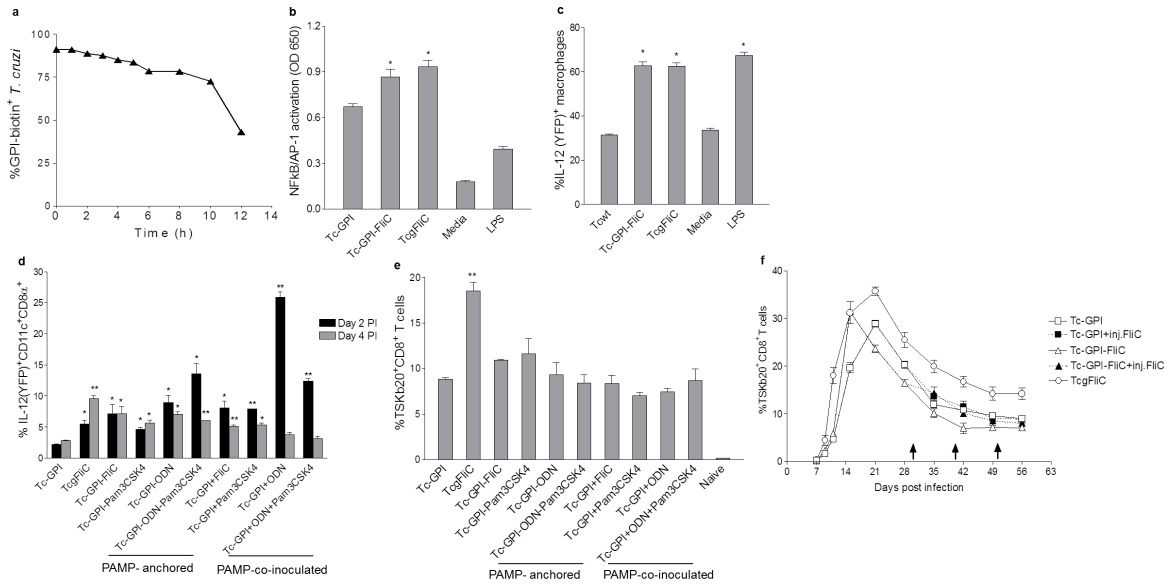


Supplementary figure 2.2. PAMP transgenesis in *T. cruzi* enhances the innate immune responses NFkB/ AP-1 activation in reporter cells incubated with epimastigote stage lysates of Tcwt, TcgFliC or TcgPorB for 18 hrs. Media or *E. coli* derived LPS served as controls. Data represented as mean $\pm$ s.e.m from one of 3 separate experiments. * or ** indicates $p \leq 0.05$ or ≤ 0.01 respectively, as determined by student t-test comparing the indicated groups to Tcwt.



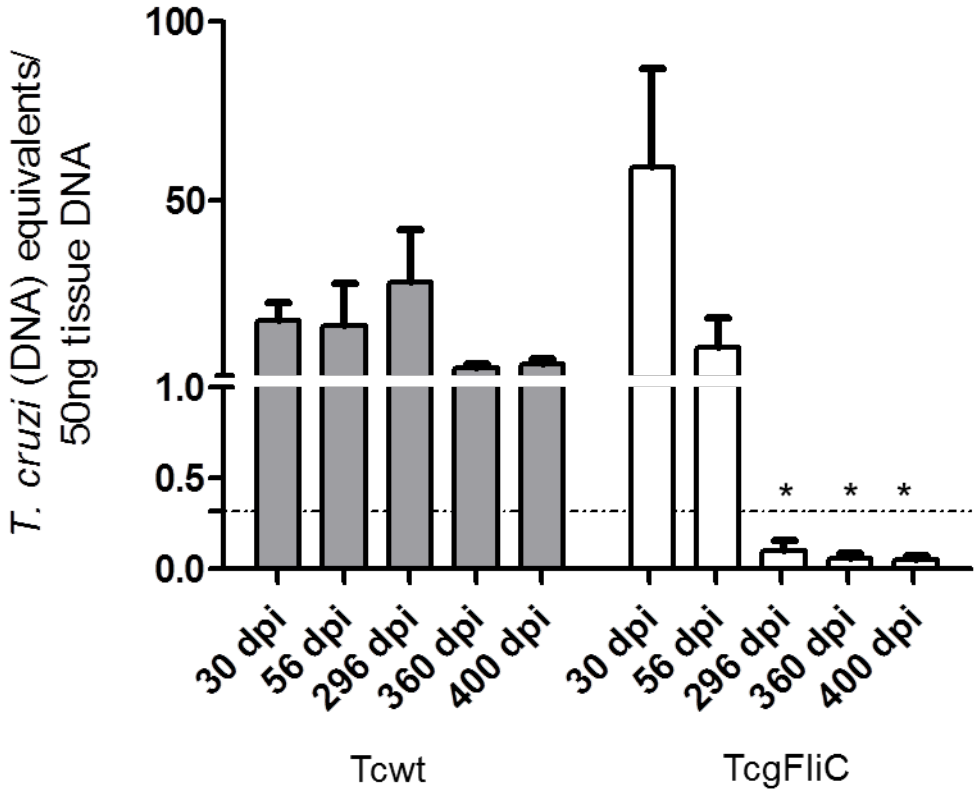
Supplementary figure 2.3. Over-expression of multiple protein PAMPs in *T. cruzi* does not improve the *T. cruzi* specific $CD8^+$ T cell response generated. (a) The kinetics of $TSKb20^+CD8^+$ T cell frequency in circulation on Tcwt, TcgFliC, TcgPorB or TcgFliC-PorB infection of C57BL/6 mice. Data represented as mean ± s.e.m from one of 3 separate experiments, with 3 mice/ group. (b) Frequency of $TSKb20^+$ $CD8^+$ T cells in circulation of C57BL/6 mice infected with TcgFliC-PAR4, TcgFliC or TcPAR4, 21 dpi. Data presented as mean ± s.e.m and is representative of 2 separate experiments with at least 3 mice/ group. (c) The quantity of (HA-tagged) PAR4 expressed by TcgFliC-

PAR4 or TcPAR4 represented as percentage of the total cellular protein in the epimastigote stage, as determined by ELISA. Data presented as mean $\pm$ s.e.m and is representative of 2 separate experiments. * or ** indicates $p \leq 0.05$ or ≤ 0.01 respectively, as determined by student t-test comparing the indicated groups to TcgFliC-PAR4.

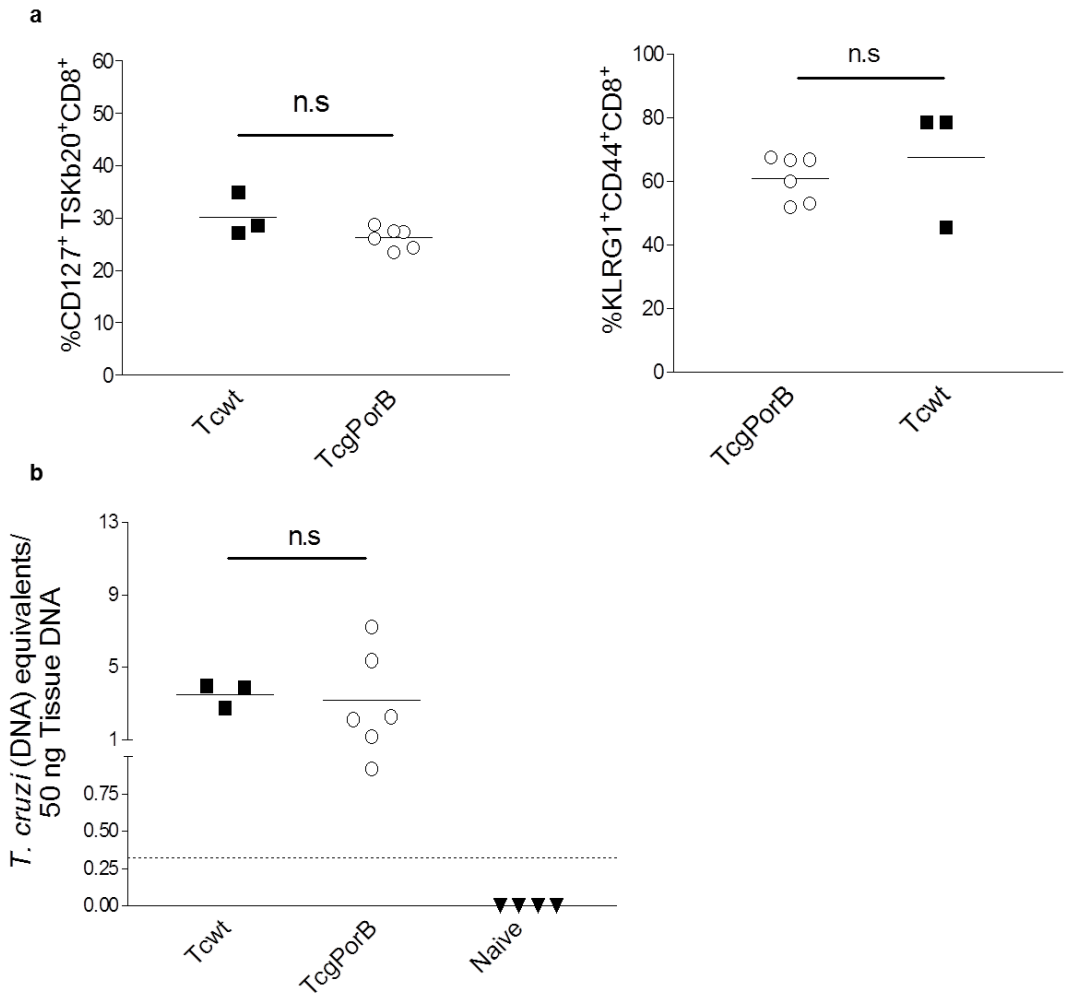


Supplementary figure 2.4. Continuous expression of FliC is required to sustain the enhanced adaptive immunity. (a) Percentage of *T. cruzi* retaining GPI-biotin at various time-points post surface-anchoring, maintained at 37^{o}C as determined by flow cytometry. Data represents two trials. (b and c) NFkB/ AP-1 activation in reporter cells (b) and the proportion of peritoneal exudate macrophages with induced IL-12 production (c) on incubation with Tc-GPI, Tc-GPI-FliC or TcgFliC trypomastigotes for 12 or 18 hrs respectively. Media or *E. coli* LPS served as controls. Data represented as mean$\pm$ s.e.m from two trials. * indicates $p \leq 0.05$ as determined by student t-test comparing the indicated groups to Tc-GPI or Tcwt. (d) The percentage IL-12 producing cDCs recruited into the draining lymph nodes observed on various days post infection with *T. cruzi* having the indicated PAMPs temporarily anchored on, co-inoculated with or constitutively expressed (TcgFliC), compared to the background strain (Tc-GPI), in IL-12*yet40* reporter mice. Data represented as mean $\pm$ s.e.m from one of 2 separate

experiments, with at least 3 mice/group/time point. (e) TSKb20^{+}CD8^{+} T cell frequency in circulation on infection with *T. cruzi* having the indicated PAMPs temporarily anchored on, co-inoculated with or heterologously over-expressed (TcgFliC), compared to the background strain (Tc-GPI) in C57BL/6 mice, 240 days post infection. Data represented as mean $\pm$ s.e.m from one of 3 separate experiments, with 3 mice/ group. (f) TSKb20^{+}CD8^{+} T cell frequency in circulation on Tc-GPI, Tc-GPI-FliC, Tc-GPI+FliC or TcgFliC infection of C57BL/6 mice. The arrows indicate the time points when FliC was inoculated in mice infected with Tc-GPI (Tc-GPI+inj.FliC) or Tc-GPI-FliC (Tc-GPI-FliC+inj.FliC). Data represented as mean $\pm$ s.e.m from 2 separate experiments, with 3 mice/ group. In (d) and (e), * or ** indicates $p \leq 0.05$ or ≤ 0.01 respectively, determined by student t-test comparing the indicated groups to Tc-GPI, at the corresponding time points.

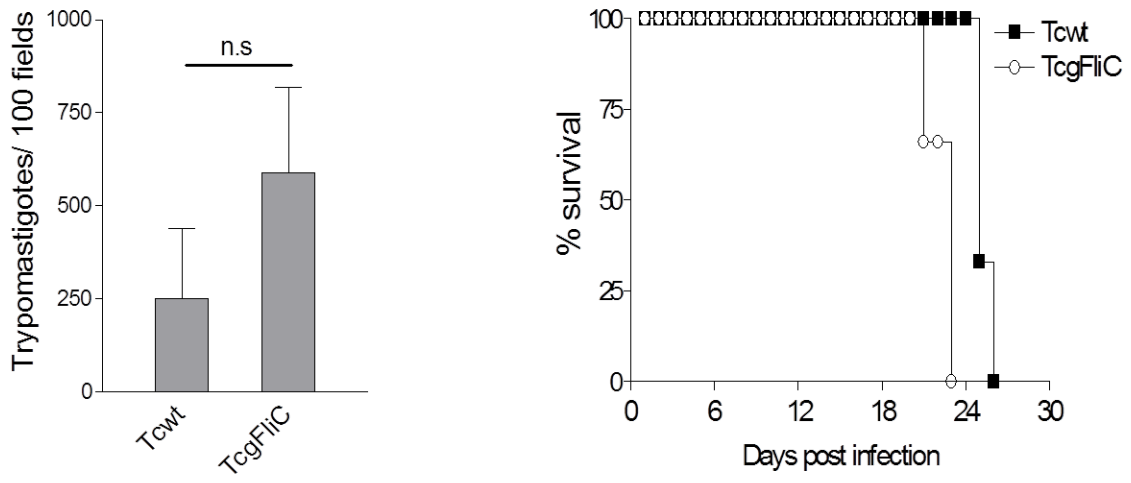


Supplementary figure 2.5. Better control of TcgFliC infection compared to Tcwt in the chronic, but not initial phase of infection in C57BL/6 mice. *T. cruzi* DNA in skeletal muscle of C57BL/6 mice inoculated with Tcwt or TcgFliC as determined by quantitative real-time PCR at various time-points post infection. Horizontal bars represent the mean. Naïve mice served as controls. The dotted line represents the threshold of detection for the assay. Data combined from separate trials initiated with at least 3-6 mice/ group. * indicates $p \leq 0.01$ comparing TcgFliC to Tcwt at the corresponding time points as determined by student t-test.

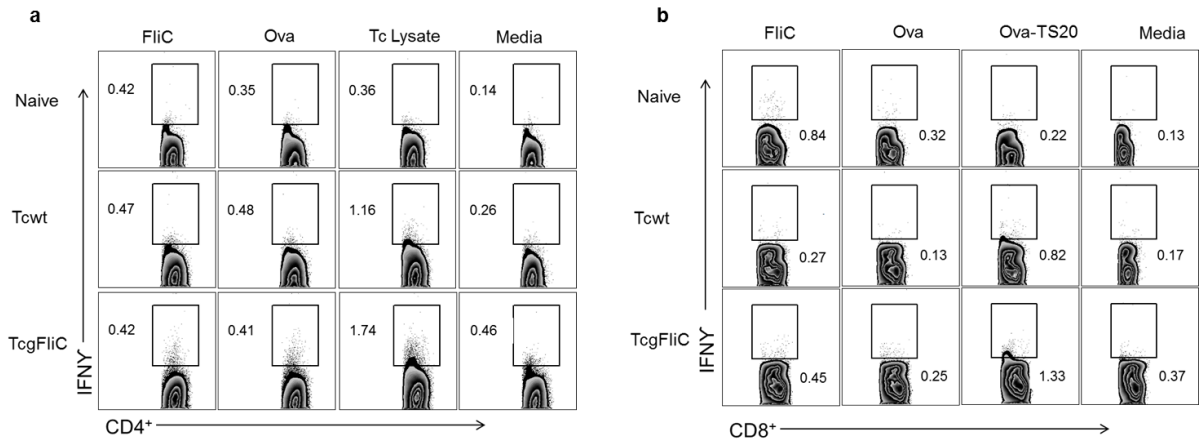


Supplementary figure 2.6 PorB transgenesis does not enhance the control of *T. cruzi* infection in mice**.** (a) Frequency of $CD8^+$ T cells in circulation having Tcm like phenotype ($CD127^{hi}$ $TSKb20^+$ $CD8^+$ (left) and $KLRG1^{lo}$ $CD44^+$ $CD8^+$ (right)), in Tcwt or TcgPorB infected C57BL/6 mice, 360 dpi. Data represents 2 separate experiments with 3-6 mice/ group. (b) *T. cruzi* DNA in skeletal muscle of C57BL/6 mice inoculated with Tcwt or TcgPorB as determined by quantitative real-time PCR, 369 dpi. Horizontal bars represent the mean. Naïve mice served as controls. The dotted line represents the threshold of detection for the assay. Data are representative of 2 separate experiments

initiated with at least 6 mice/ group, n.s indicates p≥ 0.05 as determined by student t-test.



Supplementary figure 2.7: PAMP transgenesis does not influence the virulence of *T. cruzi.* Left panel shows *T. cruzi* trypomastigotes in circulation observed in Tcwt or TcgFliC infected IFNγ$^{-/-}$ mice, 21 dpi. Data are represented as mean ± s.e.m from one of 2 separate experiments, with at least 3 mice/ group. The panel on right shows the mortality observed in Tcwt or TcgFliC infected IFNγ$^{-/-}$ mice. Data representative of at least 2 separate experiments with 3 mice/ group. n.s indicates $p > 0.05$, as determined by student t-test.



Supplementary figure 2.8. TcgFliC infection do not induce FliC-specific T cell responses $CD4^+$ and $CD8^+$ T cells producing IFNγ in response to re-stimulation of splenocytes with the native FliC, 120 dpi. Ova or *T. cruzi* lysate/ ova-TS20 served as controls. The numbers inset indicates the percentage of IFNγ producing $CD4^+$ ($CD44^+$) and $CD8^+$ ($CD44^+$) T cells. Data representative of 3 similar experiments, with 3-6 mice/ group.

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CHAPTER 3

OVER-EXPRESSION OF *TRYPANOSOMA CRUZI* FLAGELLAR PROTEIN ELICITS PROTECTIVE T CELL RESPONSES

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3.1 Abstract

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi,* affects millions of people in different parts of the world. The $CD8^+$ T cells crucial to the control of *T. cruzi* infection are often directed at members of large gene families, including trans-sialidase-like proteins that are highly diverse and vary widely among *T. cruzi* strains. We hypothesized that $CD8^+$ T cell responses directed against sub-dominant, invariant, non gene-family proteins may induce better cross-strain protection from *T. cruzi* infection and thus have sought to identify such targets. The observation that *T. cruzi* sacrifices its flagellum during intracellular amastigogenesis prompted the investigation of $CD8^+$ T cell targets among the abundant flagellar proteins, with the focus on Paraflagellar Rod Protein (PAR)4. Transgenic *T. cruzi* over-expressing PAR4 generated a stronger and more effective PAR4-specific $CD8^+$ T cell response that also conferred better protection to subsequent *T. cruzi* challenges. Transgenic over-expression of appropriate endogenous proteins of pathogens may be a viable strategy to improve live attenuated vaccines.

3.2 Introduction

Chagas disease, caused by the hemoflagellate protozoan parasite, *Trypanosoma cruzi* has a large and growing impact. It is the leading cause of endocarditis in humans, with millions infected, primarily in endemic areas of the Americas. Although *T. cruzi* infection induces robust humoral and cellular immune responses that normally result in the control of the acute infection, the infection is rarely cleared. The deficits in the immune response that prevents parasitological cure are of significant interest since it is the persistence of *T. cruzi* that ultimately leads to the clinical disease decades after the initial infection.

$CD8^+$ T cells are known to be key players in the control of *T. cruzi* infection (Tarleton et al., 1992; Tarleton et al., 1994), and a significant proportion of the anti-*T. cruzi* $CD8^+$ T cells are specific for epitopes derived from parasite trans-sialidase family proteins (Martin et al., 2006). Trans-sialidases are encoded by a family of >3000 highly variable genes that differ between isolates and are also capable of intra-isolate recombination. The trans-sialidase specific $CD8^+$ T cell response, despite being surprisingly large numerically, is dispensable for normal control of the infection (Rosenberg et al., 2010). The potential epitope variability in the trans-sialidase family has created interest in the identification of other, invariable, non-large gene-family proteins of *T. cruzi* that could elicit $CD8^+$ T cell responses to epitopes that are less capable of variation.
Among the multiple factors that govern the strength of $CD8^+$ T cell response to a particular epitope, the access of the protein to the host cell cytoplasm (Garg et al., 1997) and the amount of that protein that reaches the antigen processing machinery

(Wherry et al., 2002) are especially critical. In addition to the strength, in frequency, of the T cells response, the ability to detect and destroy pathogen-infected cells early in the infection cycle, thus restricting parasite replication potential, contributes to success in infection control (Yates et al., 2011). The intracellular life cycle of *T. cruzi* involves conversion of the infecting flagellated trypomastigote to the morphologically distinct amastigotes. The remodeling required by this conversion is presumed to result in the release and subsequent degradation of parasite proteins which could represent the earliest such products to gain access to class I MHC presentation pathways. Increasing the expression of any of these native, non-variant structural proteins in *T. cruzi* that normally serve as sub-dominant $CD8^+$T cell epitopes would be expected to enhance immunity to *T. cruzi*.

In this study, we observed that *T. cruzi* sacrifices its flagellum during amastigogenesis in the host cell shortly after invasion, making the released flagellar proteins among the earliest parasite proteins potentially presented as possible $CD8^+$ T cell targets. We identified $CD8^+$ T cell responses specific to the *T. cruzi* flagellar protein, paraflagellar rod protein (PAR) 4 with possible $CD8^+$ T cell epitopes in its amino terminus. A *T. cruzi* strain transgenically overexpressing PAR4 (TcPAR4) induced enhanced PAR4-specific $CD8^+$ T cell responses compared to wild-type (WT) *T. cruzi* (Tcwt) and yielded better protection from an acute challenge infection, thus validating a strategy for improving live attenuated vaccines.

3.3 Results

3.3.1 *T. cruzi* sacrifices its flagella during intracellular amastigogenesis

The flagellum of *T. cruzi* was visualized in trypomastigote stages by expression of tagged version of PAR4 in *T. cruzi*, PAR4-tdTomato. PAR4 appeared to localize only to the flagellar compartment and the basal body of the extracellular trypomastigotes (Fig 3.1a) unless the carboxy- terminal flagellar localization signal was removed (tr.PAR4) (Supplementary figs 3.1 and 3.2). The distribution of the orthologous PAR4 in *T. brucei* is similar (Bastin et al., 1999). Intracellular *T. cruzi* lost their flagella approximately 6-9 hrs after invading the host cell during intracellular amastigogenesis, giving rise to amastigotes and a separated flagellum (Fig 3.1b) in host cell cytoplasm. The detached *T. cruzi* flagellum also appeared to be associated with DNA (Fig 3.1b-e). This process resembled that in *T. brucei* procyclic forms (Sharma et al., 2008), which prior to mitosis undergo asymmetric division producing two daughter cells- one with a short flagellum (resembling *T. cruzi* amastigotes (Andrews et al., 1987)) and the other with an unusually long flagellum associated with nuclear DNA, which do not progress further in the lifecycle. The intact association of a basal body with the *T. cruzi* flagellum in trypomastigote stages (fig 3.1c), that would continue beyond the sacrifice of the flagellum (fig 3.1d, e), supports the presence of a similar asymmetric division in *T. cruzi* amastigogenesis. Only the amastigotes progress further in their intra-cellular lifecycle, for the next 4-5 days, to eventually convert into flagellated trypomastigotes and rupture out of the host cells (Supplementary fig 3.3). These observations also suggested that the entire repertoire of proteins in *T. cruzi* flagella may be available to the infected host

cell immuno-proteasome machinery for antigen processing and presentation early in its infection cycle.

3.3.2 *T. cruzi* flagellar component PAR4 contains CD8$^+$ T cell epitopes, at the amino terminal

One critical factor in the generation of CD8$^+$ T cell responses against *T. cruzi* proteins is the access of these proteins to the host cell cytoplasm (Garg et al., 1997). Given the possibility that CD8$^+$ T cells that target epitopes presented early in an infection cycle may have an advantage in controlling the infection, the observed loss of *T. cruzi* flagella during amastigogenesis prompted us to search for possible CD8$^+$ T cell targets in *T. cruzi* flagella. The integral flagellar component, PAR4 is one of the more abundant proteins in *T. cruzi* trypomastigotes (Atwood et al., 2005) and thus a potential target of CD8$^+$ T cell response. A distinct CD8$^+$T cell response to PAR4 was observed in *T. cruzi* infected mice (Fig 3.2a and b). The MHC-I restricted epitope in PAR4 appeared to be at its amino terminal region (PAR4(N)) (Fig 3.2c, supplementary fig 3.2), with PAR4(N) specific, functional CD8$^+$ T cells being generated in *T. cruzi* infected mice (Fig 3.2d). These results illustrate that flagellar sacrifice associated with *T. cruzi* amastiogogenesis may prompt a CD8$^+$ T cell response directed at one of the major flagellar component, PAR4, with possible epitopes at its amino terminus.

3.3.3 Over-expression of PAR4 in *T. cruzi* enhances PAR4-specific $CD8^+$ T cell responses during an infection.

Although PAR4 is relatively abundant in *T. cruzi* trypomastigotes, we hypothesized that an increased expression would further promote the relative immunodominance of this protein. *T. cruzi* over-expressing its native PAR4 (TcPAR4) protein were generated by single gene transgenesis, and were selected under strong drug pressure to achieve a ~2-fold increase PAR4 expression (fig 3.3a) Flagellar localization was critical to the enhanced PAR4-specific responses as Tctr.PAR4 lacking the flagellar localization signal induced reduced PAR4-specific $CD8^+$ T cell response as compared to TcPAR4 (Supplementary fig 3.4). Further, TcPAR4 infection also elicited higher in vitro (Fig 3.3c) and in vivo (Fig 3.3d) target cell lysis compared to infection with Tcwt. These results indicated that infection with *T. cruzi* overexpressing PAR4 can induce a greater and highly functional PAR4-specific $CD8^+$ T cell response.

3.3.4 Augmented epitope specific $CD8^+$ T cell responses in TcPAR4 infection

Based on the SYFPEITHI algorithm (Rammensee et al., 1999), we identified several potential $CD8^+$ T cell epitopes from PAR4(N) in C57BL/6 mice- of which, eight 8, 9 or 10-mer peptides were synthesized to identify the PAR4 H-2b epitopes. Two PAR4 derived peptides: DSLLNEVSL (PAR4(3)) and KALSDEMEEM (PAR4(5)) were recognized by $CD8^+$ T cells from *T. cruzi*-infected C57BL/6 mice (Fig 3.4A). In agreement with our earlier observation, TcPAR4 infection induced higher PAR4(3) and PAR4(5) specific $CD8^+$ T cell responses (Fig 3.4b) with increased(though only marginally with PAR4(3)) lysis of peptide-sensitized target cells (Fig 3.4c) compared to

that in Tcwt infection, further supporting the hypothesis that PAR4 overexpression would enhance protective *T. cruzi*-specific T cells responses during infection.

3.3.5 PAR4 transgenesis induces better control of *T. cruzi* infection

Given the enhanced PAR4-specific $CD8^+$ T cell response observed in infections with TcPAR4, we hypothesized that immunization with TcPAR4 would provide better, cross-strain protection against wild-type *T. cruzi* challenge than Tcwt. To test this hypothesis, we infected mice with TcPAR4 or Tcwt parasites and then drug-cured the infection using benznidazole (Bustamante et al., 2008). Infected and cured mice were then challenged in each footpad with tdTomato fluorescent protein-expressing *T. cruzi* (CL strain) (Canavaci et al., 2010), and parasite load at the site of infection was monitored from 2 to 11 days post-challenge by in vivo imaging (Collins et al., 2011). TcPAR4 immunized mice displayed a significant, early drop in the fluorescence signal compared to Tcwt vaccinated, or the control unvaccinated (primi-infection) mice (Fig 3.5 a,b). TcPAR4 vaccinated mice also exhibited significantly reduced parasite loads in skeletal muscle tissue at day 30 post-challenge, as compared to Tcwt vaccinated mice (Fig 3.5c). These results indicate that immunization with PAR4-over-expressing *T. cruzi* stimulates more effective adaptive immunity that better protects mice from challenge with WT *T. cruzi*.

3.4 Discussion

Though the life history of *T. cruzi* has been systematically elucidated over more than one hundred years (Brener, 1973; de Souza, 1984; Tyler and Engman, 2001), some

aspects of it still remain a mystery. One such intriguing feature is the cell biology of intracellular amastogogenesis, where the elongated trypomastigotes remodel into oval, aflagellate amastigotes. The *T. cruzi* ubiquitin-proteasome system has been shown to be key in this morphogenesis, apparently responsible for recycling of now unneeded trypomastigote structures (Gonzalez et al., 1996). However some key structural proteins have remained unaccounted for. The absence of the exclusive flagellar component of *T. cruzi*: paraflagellar rod protein, among the parasite proteasome-targeted proteins during amastigogenesis (de Diego et al., 2001) suggests the possibility of an alternate mechanism of dealing with the flagella during remodeling. This, along with the intriguing observation of $CD8^+$ T cells specific for certain flagellar structural proteins in *T. cruzi* infection (Michailowsky et al., 2003; Wrightsman et al., 2002) led us to hypothesize that the *T. cruzi* flagellum is 'lost' into the host cell cytosol during amastigogenesis. In this study, we make the novel observation of a detached *T. cruzi* flagellum in the cystol of the host cell during amastigogenesis. The released flagellum remains associated with the basal body and DNA and is thus reminiscent of the asymmetric division in *T. brucei* procyclic forms in tsetse flies (Sharma et al., 2008). In *T. brucei* procyclic stage, asymmetric division gives rise to an extensively flagellated daughter cell that do not progress further in their life cycle (Sharma et al., 2008) and is possibly degraded. Degradation also appears to be the fate of the flagellum in *T. cruzi* infection , as we observed HA-tagged (PAR4) 'particles' in TcPAR4 infected host cell cytosol at time points immediately following amastigogenesis (data not shown). It was also observed that as *T. cruzi* progressed through its amastigote stage, the expressed PAR4 localized sub-cellularly in the parasite cytoplasm, basal body or the endoflagellum

at various time points, until the transformation of amastigotes into the trypomastigote forms. These observations, in addition to providing critical insights into the biology of *T. cruzi*, also shed light on the hitherto unclear mechanism by which $CD8^+$ T cells could be generated against certain structural components in *T. cruzi* flagella that are absent from the pathogen's intracellular stage (Michailowsky et al., 2003; Portman and Gull, 2010). $CD8^+$ T cells are critical to the adaptive immune control of most intracellular pathogens owing to their ability to produce a variety of cytokines, and to directly target infected host cells for destruction. In its cytoplasmic niche, *T. cruzi* releases various proteins that are processed and presented on class I MHC molecules (Garg et al., 1997), and may be detected by specific $CD8^+$ T cells possessing cognate T cell receptors. However, unlike the genetically simpler viral or bacterial model pathogens, protozoans, including *T. cruzi,* appear to generate $CD8^+$ T cell responses against relatively higher numbers of epitopes, spanning multiple proteins (Doolan et al., 2003; Frickel et al., 2008; Martin et al., 2006). These responses also appear in a focused, reproducible hierarchy, with epitope-specificity in which certain clones are represented in higher numbers (dominant) than others (subdominant) in a phenomenon termed immunodominance (Yewdell and Bennink, 1999). Given the complexity of the *T. cruzi* genome, with hugely expanded sets of variant gene families encoding various surface-expressed and secreted proteins (El-Sayed et al., 2005), we observe multiple clones of $CD8^+$ T cells forming an immunodominance hierarchy, led by those specific to the trans-sialidase family of proteins (Martin et al., 2006; Rosenberg et al., 2010). Trans-siladisases are part of an enormous gene- family in *T. cruzi*, encoded by >3000 genes (Weatherly et al., unpublished), and are also the targets of the majority of *T. cruzi* specific $CD8^+$ T cells in

mice, with the responses to other proteins appearing significantly subdominant. Though *T. cruzi* trans-sialidases have been used as vaccine candidates in the past (Costa et al., 1998; Fontanella et al., 2008), their strain-variant nature (Martin et al., 2006) is likely to make them unreliable targets for immunization against *T. cruzi*. For this reason, our focus has shifted to the identification of other sub-dominant, strain-invariant epitopes from non-large gene-family proteins as potential vaccine candidates.

The diverse protein expression profiles of various life cycle stages in *T. cruzi* presents the opportunity to target stage-specific epitopes for the generation of immune responses directed at the respective stages. Thus, in lines of conventional T cell based vaccines, vaccine studies in *T. cruzi* have mostly focused on antigens expressed predominantly in its major intracellular stage- the amastigotes (Dumonteil, 2009; Sanchez-Burgos et al., 2007). However, a number of characteristics make proteins in the *T. cruzi* flagellum particularly attractive as immunological targets. They are naturally abundant, single gene-encoded and are non-variant among *T. cruzi* strains. Furthermore, the flagellar proteins appear to be among the first proteins released by *T. cruzi* upon infection of the host cell, making them ideal early indicators of *T. cruzi* infection. On the negative side, the flagellar proteins are only present in the host cell ephemerally – apparently for only the first ~12 hours of the ~96 hr intracellular infection cycle. So, if PAR4-specific T cells do not engage parasite-infected cells soon after their infection, these T cells will be ineffective.

There have been multiple attempts to develop vaccines against *T. cruzi*, that would generate cell mediated and/or humoral immune responses against various epitopes, with variable success rates (Cazorla et al., 2009; Vazquez-Chagoyan et al., 2011). The

studies aimed at improving the T cell responses have mostly favored the targeting of various immunodominant epitopes present in highly abundant proteins. However, studies in viral models have demonstrated a lack of correlation between the general epitope span in its proteins, or the overall strength of T cell responses and pathogen loads (Addo et al., 2003; Betts et al., 2001). In contrast, the stage specific expression of epitopes in a pathogen, which determines the timing and efficiency of T cell responses generated, appears to correlate best with protection (Adnan et al., 2006; Yates et al., 2011). Thus, it seemed logical that, even though strong, immuno-dominant $CD8^+$ T cell responses may be generated, an un-timely expression of its target epitopes by the intracellular stage of *T. cruzi*, may still offer the parasite an opportunity to establish an infection during a challenge. *T. cruzi* infects and persists chronically in spite of the strong, $CD8^+$ T cell responses directed at various trans-sialidase epitopes. Flagellar epitopes have the advantage of being presented on host cell surface very early (possibly within the first few hours) in *T. cruzi* infection cycles, likely letting PAR4 specific $CD8^+$ T cells to relatively rapidly identify infected cells in a challenge. TcPAR4 immunized mice, with higher proportions of PAR4 specific $CD8^+$ T cells showed an earlier and progressively better control of challenge infection, strengthening this proposition.

In this study, we make the novel observation that *T. cruzi* sacrifices its flagellum during morphogenesis into the amastigote stage in the host cell cytosol. This flagellum appeared to source a $CD8^+$ T cell response directed at its integral component-PAR4, that could further be amplified by transgenically over-expressing PAR4 in *T. cruzi.* The now enhanced, functional PAR4 specific $CD8^+$ T cell response imparted better immunity

to the host from challenge infections, making *T. cruzi* PAR4 a promising candidate to direct vaccine efforts at. This is a novel approach where, the prominent proteins expressed in the intracellular stage of a pathogen are not the chosen primary T cell targets for immunization, but are those proteins degraded early during the morphogenesis into the intracellular stage. We believe, the approach of over-expressing native, immunogenic proteins in vaccine strains of pathogens can be used to improve the immunogenicity of the various live-attenuated vaccines being generated or currently in use.

3.5 Methods

3.4.1 Mice, parasites, and infections

C57BL/6 mice used throughout in this study were purchased from The Jackson Laboratory or bred and maintained in our animal facility under specific pathogen-free conditions. *T. cruzi* epimastigotes (Brazil strain) were transfected as described previously (Garg et al., 1997) with pTREX plasmid (Lorenzi et al., 2003) containing the coding sequence of full length (1743 bp) or truncated (1693 bp) *T.cruzi* paraflagellar rod protein 4 (PAR4) gene (NCBI accession no: XM_800732), with or without fusion to an upstream td-Tomato gene or influenza haemagglutinin (HA)-tag, to generate transgenic *T. cruzi*. All infections were initiated by inoculating vero cell culture passaged trypomastigote stage *T. cruzi,* intra-peritoneally (i.p) (10^4 parasites) or subcutaneously in the foot pad (f.p) (10^4 parasites). All animal protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

3.5.2 Microscopy

Immunofluorescence microscopy was performed to determine presence of the PAR4 or tr.PAR4 in epimastigote, trypomastigote and amastigote stages of *T. cruzi*, modifying the protocol described before (Agrawal et al., 2009). Anti-PAR4 polyclonal serum from in mice, td-Tomato or anti-HA (Roche) antibodies were used to track PAR4 or tr.PAR4. Filipin bound to plasma membrane cholesterol marked the parasite cell surface and Propidium iodide or DAPI indicated DNA. Mouse Anti-centrin (*Chlamydomonas reinhardtii*) (millipore) was used to mark *T. cruzi* basal body. Images were acquired with an Applied Precision Delta Vision microscope, were deconvolved and adjusted for contrast using its Softworx software.

3.5.3 ELISPOT assay for IFNϒ

T cells specific to rPAR4, rPAR4(N), PAR4(3), PAR4(5), TSKB20, SIINFEKL or rOVA were determined in TcPAR4 or Tcwt infected, or naïve control mice by ELISPOT assay as described previously (Eickhoff et al., 2011). In short, splenocytes from the mice (1×10^6 per well) were incubated for 18 hrs with the corresponding antigen at 1ug/ well for the proteins or 5uM for the peptides, in nitrocellulose bottom ELISPOT plates (Millipore) coated with anti-mouse IFNϒ (BD Biosciences). After overnight stimulation and washing off the splenocytes, wells were subsequently incubated with biotinylated-anti-mouse IFN-γ (BD Biosciences), streptavidin-HRP (BD Biosciences) and AEC substrate (Sigma). Images of developed ELISPOT plates were captured using a CTL Analyzer and spots counted using Immunospot Software v3.2 (CTL). PMA/ionomycin

(iono) combination served as control. Data are represented as the number of IFN-γ spot forming cells per million total spleen cells.

3.5.4 Intracellular cytokine staining for IFNϒ

To determine intracellular IFNγ, $1.5x10^6$ spleen cells from TcPAR4, Tctr.PAR4 or Tcwt infected, or naïve mice were restimulated with recombinant proteins (at 1µg/ well): *T. cruzi* PAR4 (rPAR4), ovalbumin (rOVA), ovalbumin with its SIINFEKL epitope replaced by the immune-dominant ANYKFTLV (Martin et al., 2006) from *T. cruzi* (rOVA-TSKb20) , or peptides (at 5µM): PAR4 (3), PAR4(5), TSKb20 (5 µM) or SIINFEKL; or transgenic MC57G murine (C57BL/6) fibroblasts expressing: PAR4 protein's amino terminal (MC-PAR4(N)), middle portion (MC-PAR4(M)) or carboxy terminal (MC-PAR4(C)) (Supplementary fig 3.2), ovalbumin (MC-Ova) or ovalbumin with its SIINFEKL epitope replaced by the immune-dominant ANYKFTLV (Martin et al., 2006) from *T. cruzi* (MC-OVA-TSKb20), and processed for intracellular cytokine staining (ICS). The splenocytes were washed in PAB (2% BSA, 0.02% azide in PBS) and stained for surface expression of CD4, CD44 and CD8 using anti-CD4 PE, CD44 FITC and anti-CD8 eFluor450 (BD Pharmingen). All cells for ICS were fixed and permeabilized using Cytofix/Cytoperm (BD Pharmingen) on ice for 15 min and washed in PermWash (BD Pharmingen). The cells were then stained with anti-IFNγ APC (BD Pharmingen) for 30 min on ice. Cells were washed and fixed in 2% formaldehyde for 20 min at 4°C, then washed and resuspended in PAB for flow cytometric analysis using a CyAn flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

3.5.5 ELISA

To determine the relative concentrations of PAR4 in the trypomastigote stage from TcPAR or Tcwt, *T. cruzi* whole cell lysates (50µg/ well) were probed with serial dilutions of polyclonal anti-PAR4 sera raised in mice.

3.5.6 In vivo cytotoxicity assay

Equal numbers of MC-PAR4(N) or MC-Ova were labeled with different concentrations of CellTrace Violet (CTV) (Invitrogen) to produce CTV ^{hi} MC-PAR4(N) and CTV ^{lo} MC-Ova. Equal numbers of either were then transferred i.p into recipients, and after 18 h, reisolated by peritoneal lavage to detect CTV stained cells by flow cytometry. Similarly, Spleen cells from naive mice incubated for 1 h at 37°C with 10 µM PAR4 (3), PAR4(5), or SIINFEKL peptides were labeled with different concentrations of CFSE (Molecular Probes) as described before (Martin et al., 2006) to produce CFSE high and low populations. Equal numbers of CFSE-labeled cells were transferred i.v into recipients, and after 18 h, splenocytes were isolated and CFSE-labeled cells were detected by flow cytometry. Percentage of specific lysis was determined using the equation: 1 − [(% CTV or $CFSE^{lo}$ naive/% CTV or $CFSE^{hi}$ naive)/(% CTV or $CFSE^{lo}$ infected/CTV or $CFSE^{hi}$ infected)] × 100%.

3.5.7 In vitro cytotoxicity assay

In vitro cytotoxicity of splenocyte effector cells derived from TcPAR4 or Tcwt infected mice on target MC-PAR4(N) or Mc-Ova were as described before (Cao et al., 2010). Briefly, effector cells were harvested, counted, washed, and resuspended to 1 ×

10^6 cells/ml in media. Effectors and CFSE-labeled target cells were mixed at a range of E:T, in sterile 96-well flat bottom plates at 1×10^5 targets/well. Cultures were incubated for 5 h at 37°C under 5% CO_2. 7-AAD was then added to samples, incubated for 30 min in the dark, washed and cytolysis assessed by flow cytometry. Maximum cytolysis control was achieved by treating target cells with Cytofix/Cytoperm (BD Pharmingen) and minimum cytolysis control represented untreated target cells. Percentage of specific lysis was determined using the equation: [(% cytolysis $_{sample}$ - %cytolysis $_{min}$)/ (%cytolysis $_{max}$ - %cytolysis $_{min}$)] × 100%., where %cytolysis= 1-% live cells.

3.5.8 Assessing protection

The acute control of *T. cruzi* challenge was determined as described before (Collins et al., 2011). $2x10^5$ *T. cruzi* (CL) expressing the tdTomato protein (Canavaci et al., 2010) was subcutaneously inoculated into the footpads of naïve mice, or mice drug-cured from TcPAR4 or Tcwt infections. Mice feet were imaged every day using the Maestro 2 *in vivo* imaging system (Caliper Life sciences) with the green set of filters (acquisition settings: 560 to 750 in 10-nm steps; exposure time of 88.18 ms and 2 × 2 binning). The total fluorescent signal was quantified and the values represented as photons/cm^2/second.

3.5.9 Real-time PCR

The skeletal muscle tissue from challenged mice were analyzed at 30 dpi, by real-time PCR for the presence of *T. cruzi* (DNA) as described before (Cummings and Tarleton, 2003)

3.5.10 Statistical analysis

Data are presented as the mean plus/ minus the standard error of mean (s.e.m). Statistical analyses compared the groups with a two-tailed student t-test. Only p values of less than 0.05 were considered statistically significant.

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Figures

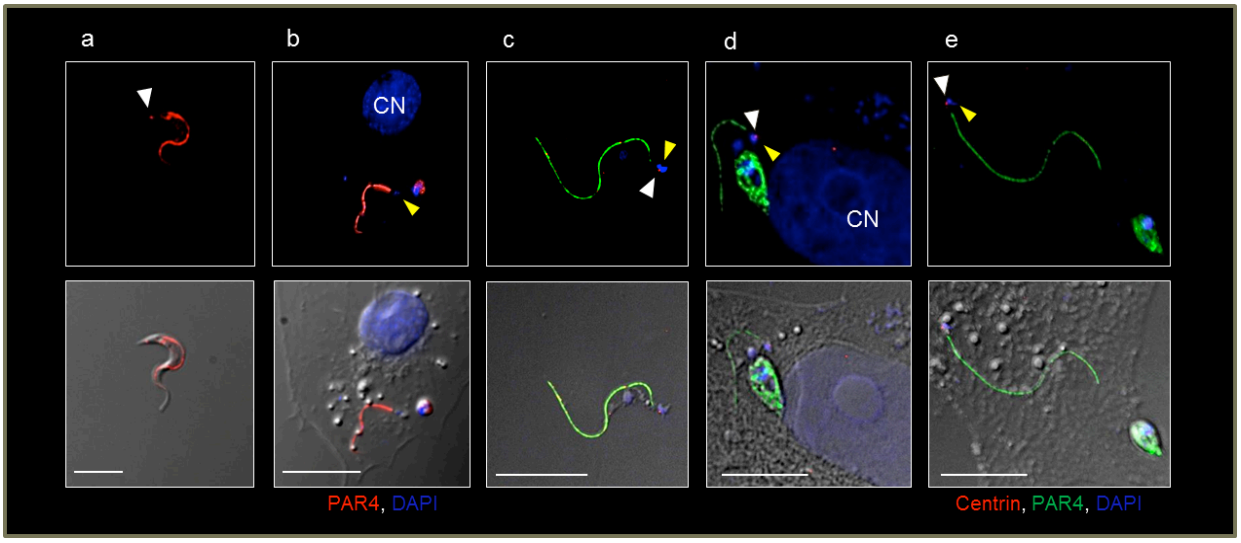
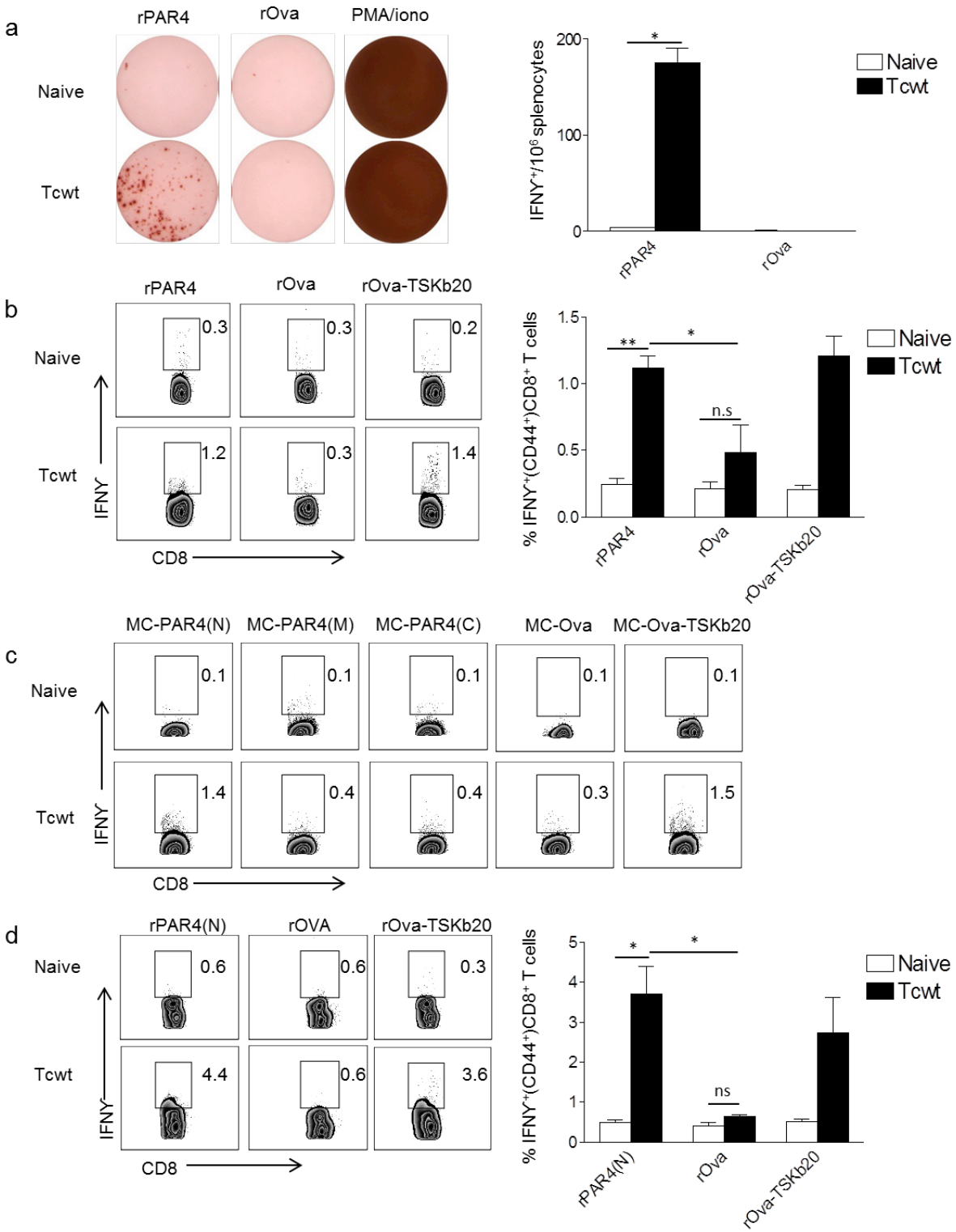


Figure 3.1 *T. cruzi* sacrifices its flagella during intracellular amastigogenesis. (a) Immunofluorescence microscopy (top panel) and DIC overlay (bottom panel) showing an intact *T. cruzi* flagellum containing PAR4-tdTomato fusion protein in the extracellular trypomastigote stage and (b) separated from the newly formed amastigote (6-9 hrs post infection) in the host cell cytoplasm. The white and yellow arrows indicate the basal body and the DNA associated with the detached flagellum, respectively. (c) The basal body (anti- centrin antibody, white arrow) and DNA (DAPI, yellow arrow) associated with the flagellum of extracellular trypomastigotes (anti-PAR4) but is sacrificed along with the flagellum (d) during amastigogenesis in the host cell. (e) The newly formed intracellular amastigote, adjacent to the free flagella (6-9 hrs post infection). CN indicates host cell nucleus. Scale bar= 10u


a
rPAR4
rOva
PMA/iono
Naive
Tcwt
IFNY+/10^6 splenocytes
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100
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*
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Tcwt
rPAR4
rOva
b
rPAR4
rOva
rOva-TSKb20
Naive
Tcwt
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CD8
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1.2
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% IFNY+(CD44+)CD8+ T cells
1.5
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rPAR4
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MC-PAR4(C)
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rPAR4(N)
rOva
rOva-TSKb20

Figure 3.2 *T. cruzi* PAR4 may contain $CD8^+$ T cell epitopes, in the amino terminal. (a) Representative wells of IFN-γ ELISPOT assay, where splenocytes from naïve or Tcwt infected (30 dpi) mice were re-stimulated with rPAR4, rOVA or PMA/ionomycin for 18 hrs. Bar graph indicates the average number of spots per well for either group from an experiment with 3 mice/group. (b) Intracellular IFN-γ staining of splenocytes derived from naïve or Tcwt infected (180 dpi) mice, re-stimulated with rPAR4, rOva or rOva-TSKb20 for 16hrs. The bar graph summarizes data from an experiment with 3 mice/ group. (c) Intracellular IFN-γ staining of splenocytes derived from naïve or Tcwt infected (48 dpi) mice, co-cultured for 16 hrs with MC-PAR4(N), MC-PAR4(M), MC-PAR4(C), MC-Ova or MC-Ova-TSKb20 cells. (d) Intracellular IFN-γ staining of splenocytes derived from naïve or Tcwt infected (90 dpi) mice, re-stimulated with rPAR4(N), rOva or rOva-TSKb20. The bar graph summarizes data from an experiment with 3 mice/ group. All histograms are gated on $CD8^+$ $CD44^+$ lymphocytes, with the inset numbers indicating the percentage of IFN-γ producing $CD8^+$ T cells. All data representative of 2-4 similar experiments, with the bar graphs presenting data as mean $\pm$ s.e.m. **, * or n.s indicate $p \leq 0.01$, $p \leq 0.05$ or $p > 0.05$ respectively, as determined by student t-test.

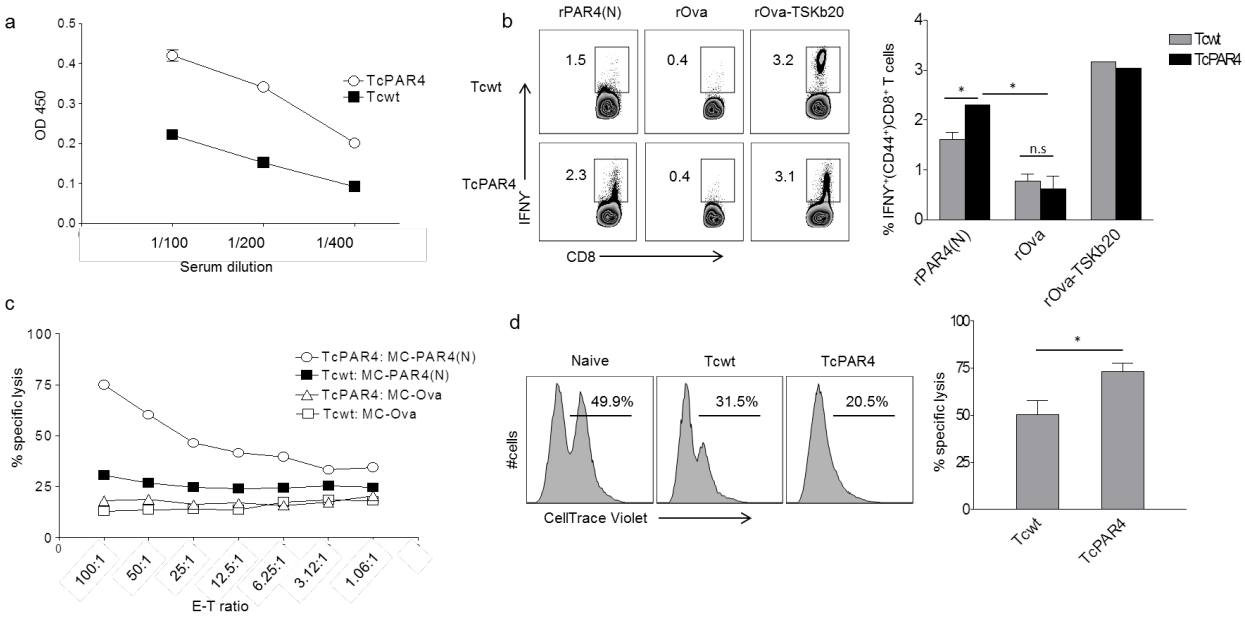


Figure 3.3 Over-expression of PAR4 in *T. cruzi* enhances PAR4-specific $CD8^+$ T cell responses in an infection (a) Relative expression levels of PAR4 in Tcwt and TcPAR4 trypomsatigote stage (lysate) as determined by ELISA using serial dilutions of anti-PAR4 hyper-immune sera. (b) Intracellular IFN-γ staining of splenocytes derived from Tcwt or TcPAR4 infected (120 dpi) mice, restimulated with rPAR4(N), rOva or rOva-TSKb20 for 16hrs. Histograms are gated on $CD8^+$ $CD44^+$ lymphocytes, with the inset numbers indicating the percentage of IFN-γ producing $CD8^+$ T cells. The bar graph summarizes data from an experiment with 3 mice/ group. (c) Cytolytic activity of splenocytes from TcPAR4 or Tcwt infected mice (96 dpi) against MC-PAR4(N) or MC-Ova target cells at different effector-target ratios. (d) Representative histograms comparing specific killing of CellTrace Violet (CTV) stained MC-PAR4(N) (CTV^{hi})/ MC-OVA (CTV^{lo}) target cells inoculated into and recovered 18h later from the peritoneal cavity of naïve, Tcwt or TcPAR4 infected (300 dpi) mice. The inset numbers represent the relative proportions of recovered MC-PAR4(N) compared to MC-OVA . The bar

graph shows the specific lysis of MC-PAR4(N) in Tcwt or TcPAR4 infections. Data represents an experiment with 3 mice/ group. All data representative of at least three similar experiments with the bar graphs presenting data as mean $\pm$ s.e.m. * or n.s indicate $p \leq 0.05$ or $p > 0.05$ respectively, as determined by student t-test.

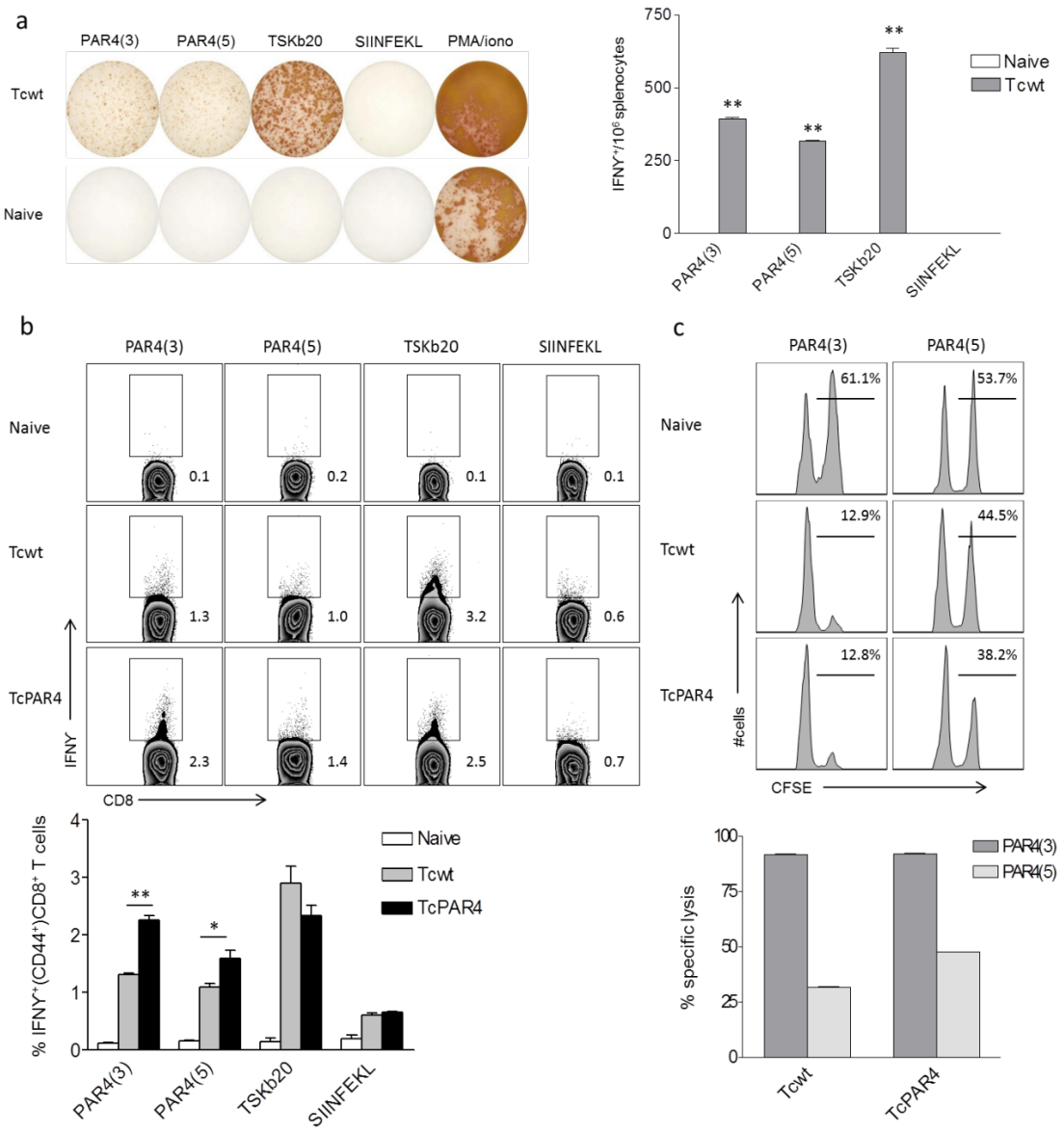


Figure 3.4 Augmented epitope specific $CD8^+$ T cell responses in TcPAR4 infection (a) Representative wells of IFN-γ ELISPOT assay, where splenocytes from naïve or Tcwt infected (30 dpi) mice were restimulated with the PAR4(3), PAR4(5), TSKb20 or SIINFEKL peptides, or PMA/ionomycin for 18 hrs. Bar graph indicates the average numbers (±s.e.m) of spots per well for either group from a representative of 3 trials with 3 mice/ group. ** indicates $p \leq 0.01$ comparing the indicated groups to SIINFEKL control

by student t-test. (b) Representative intracellular IFN-γ staining of splenocytes derived from Naïve, Tcwt or TcPAR4 infected (30 dpi) mice, restimulated with PAR4(3), PAR4(5), TSKb20 or SIINFEKL for 5 hrs. Histograms are gated on $CD8^+$ $CD44^+$ lymphocytes, with the inset numbers indicating the percentage of IFN-γ producing $CD8^+$ T cells. The bar graph summarizes data from an experiment with 3 mice/ group. (c) Representative histograms comparing specific killing of PAR4(3) or PAR4(5) ($CFSE^{hi}$)/ SIINFEKL ($CFSE^{lo}$) peptide pulsed target splenocytes inoculated into and recovered 18h later from naïve, Tcwt or TcPAR4 infected (300 dpi) mice. The inset numbers represent the relative proportion of PAR4(3) or PAR4(5) pulsed to SIINFEKL pulsed splenocytes recovered. The bar graph shows the specific lysis of PAR4(3) or PAR4(5) pulsed splenocytes in Tcwt or TcPAR4 infections. Data represent an experiment with 3 mice/ group. ** or * indicates $p \leq 0.01$ or ≤ 0.05 respectively by student t-test. All data representative of at least 3 separate trials.

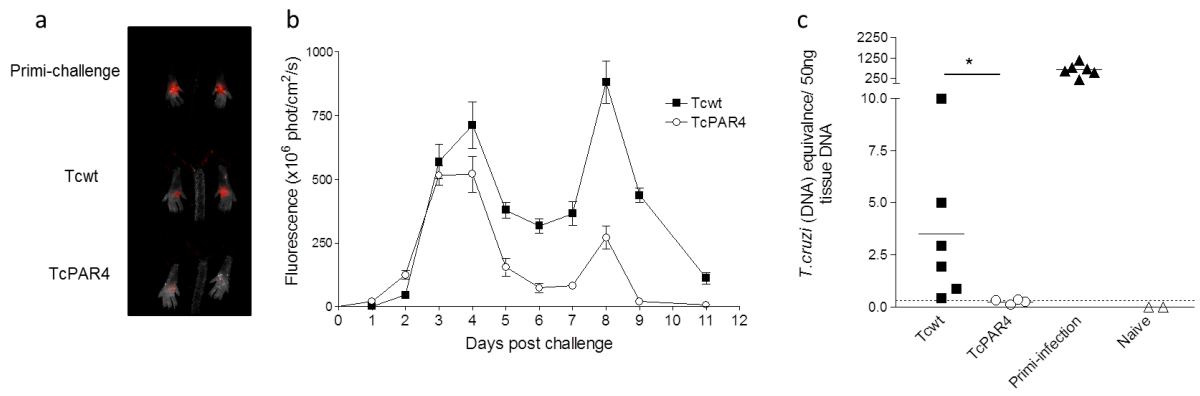
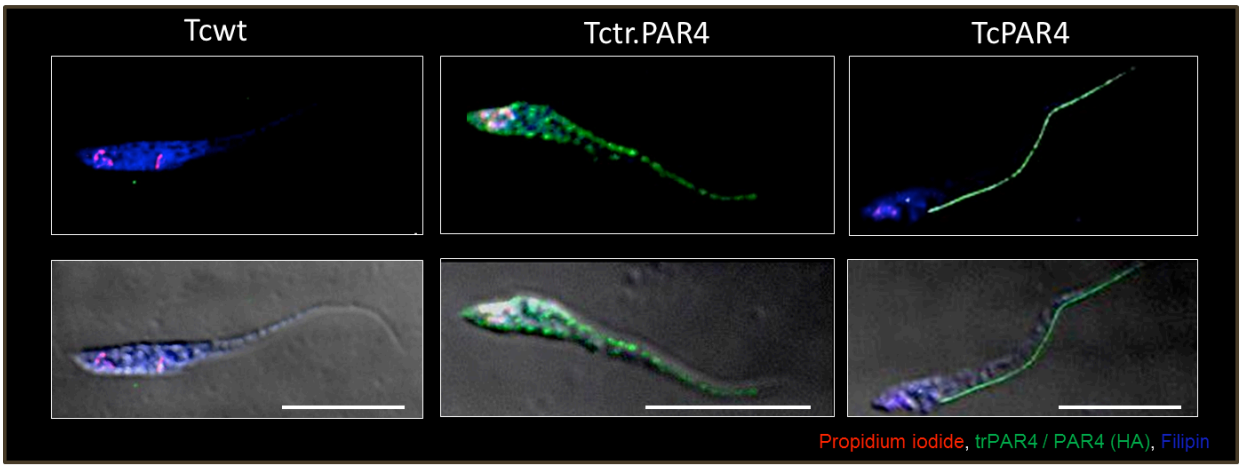


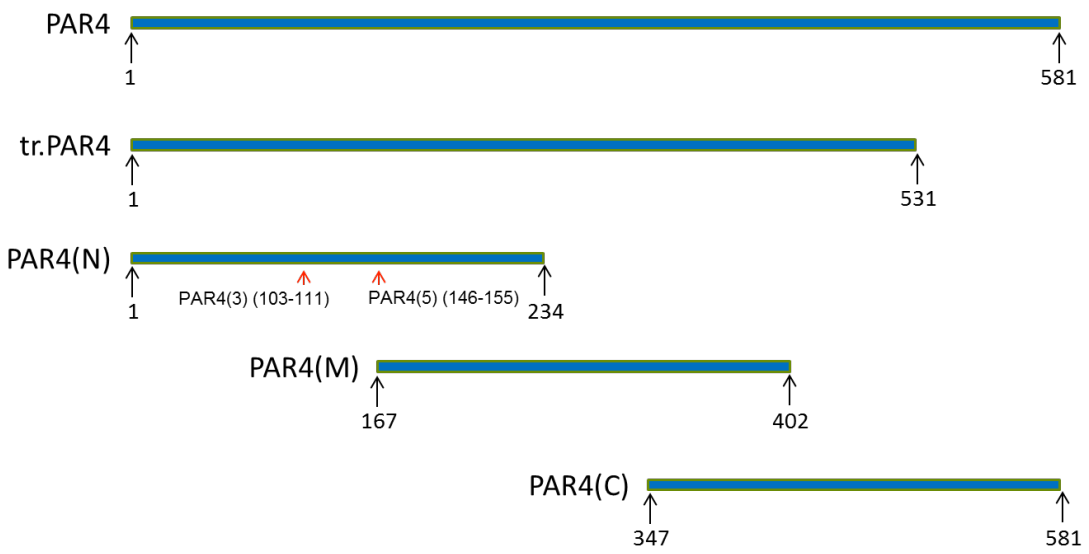
Figure 3.5 TcPAR4 immunization may protect mice from *T. cruzi* challenge.

(a) Representative picture from day 4 post challenge showing the acute control of challenge (2.5×10^5 fluorescent *T. cruzi)* at the site of infection (superficial subcutaneous tissue of each footpad) in naive (primi-challenge), Tcwt or TcPAR4 immunized mice. The parasite load in foot pad, represented by the fluorescent signal is determined by *in vivo* imaging. (b) Protection observed through the first 11 days of *T. cruzi* challenge, with the graph showing the mean fluorescent signal of all feet in Tcwt or TcPAR4 immunized mice at indicated time points. Data from an experiment with 3 mice/ group, that is representative of 3 separate trials. (c) *T. cruzi* DNA in skeletal muscle of 2.5×10^5 fluorescent *T. cruzi* challenged C57BL/6 mice, immunized with Tcwt or TcPAR4 as determined by quantitative real-time PCR, 30 days post challenge. Horizontal bars represent the mean. Unimmunized (primi-infection) or naïve mice served as controls. The dotted line represents the threshold of detection for the assay. Data representative of 2 separate experiments with 3-6 mice/ group. * indicates $p \leq 0.05$ by student t-test



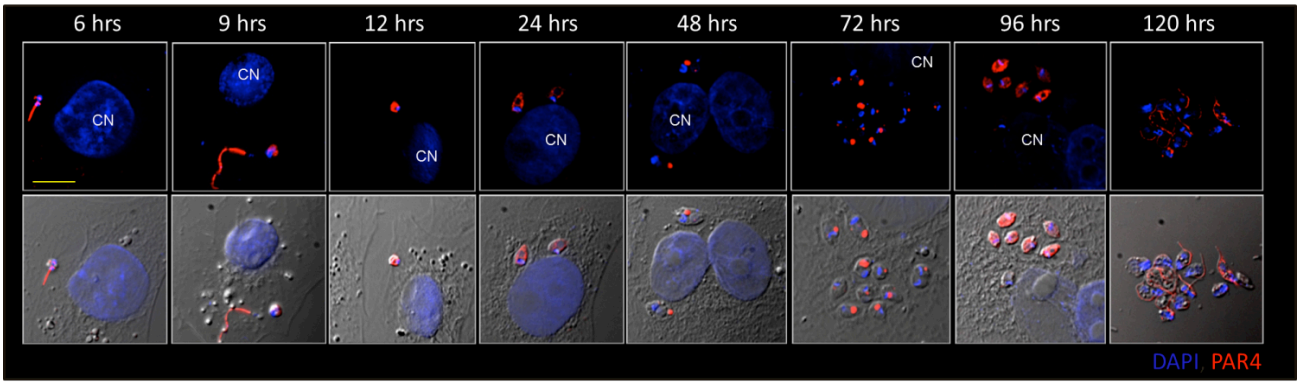
Supplementary figure 3.1 Deleting carboxy terminal flagellar localization signal retains PAR4 in *T. cruzi* cytoplasm.

Immunofluorescence microscopy (top panel) and DIC overlay (bottom panel) showing localization of transgenic tr.PAR4 (anti-HA) or PAR4 (anti-HA) in the epimastigote stages of Tcwt, Tctr.PAR4 or TcPAR4 strains respectively. Scale bar= 10u



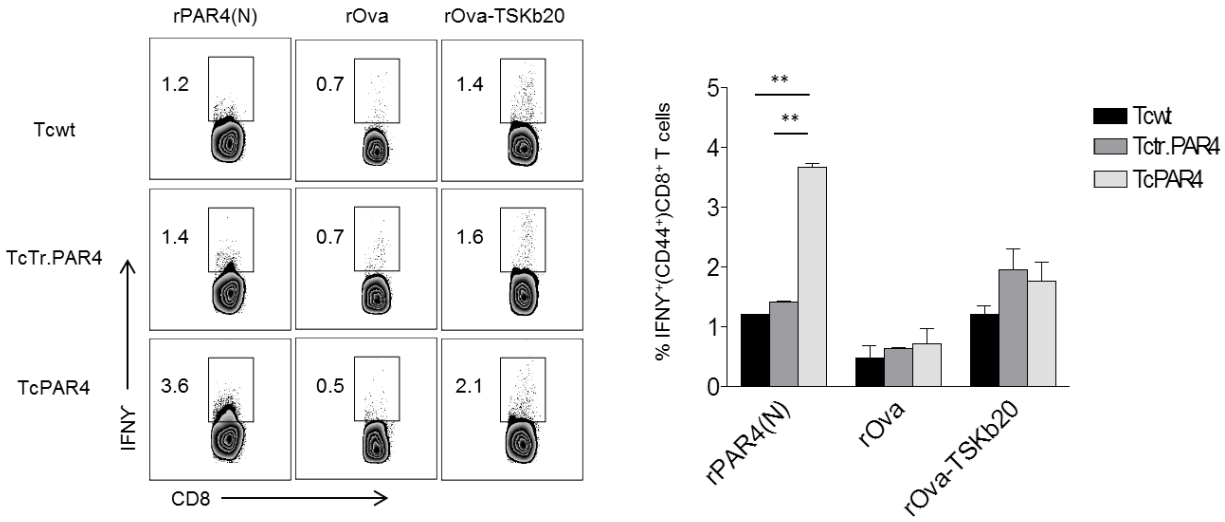
Supplementary figure 3.2 Schematic representation of the relative sizes and positions of transgenically expressed proteins.

Relative lengths and positions of the various full or truncated gene products expressed in transgenic *T. cruzi*, bacteria or mammalian cell lines. The numbers beneath the arrows indicate the amino acid positions of the fragments or epitopes.



Supplementary figure 3.3 Localization of PAR4 in intracellular life cycle stages of TcPAR4

Representative immunofluorescence microscopy (top panel) and DIC overlay (bottom panel) indicating the localization of PAR4 (PAR4-tdTomato) in TcPAR4 tracked through the specified time points post infection. CN indicates host cell nucleus. Scale bar= 10u



Supplementary figure 3.4 PAR4 localization to flagella critical to enhanced PAR4 specific response in TcPAR4

Intracellular IFN-γ staining of splenocytes derived from Tcwt, Tctr.PAR4 or TcPAR4 infected (180 dpi) mice, restimulated with rPAR4(N), rOva or rOva-TSKb20 for 16hrs. Histograms are gated on $CD8^+$ $CD44^+$ lymphocytes, with the inset numbers indicating the percentage of IFN-γ producing $CD8^+$ T cells. The bar graph presents mean $\pm$ s.e.m from one of three separate experiments with 3 mice/ group. ** indicates $p \leq 0.01$ by student t-test.

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CHAPTER 4

CONCLUSIONS

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi* is one of the high impact human parasitic disease in the Americas, affecting more than 300,000 people in the United States and a total of 8-10 million people in Latin America. Even with its huge public health and economic impact, safe drugs or reliable vaccines against this infection are unavailable, making it a priority in the field to direct efforts at achieving these. The quest for a vaccine against Chagas disease has made understanding the immunology of *T. cruzi* infection critical.

In the first part of this project, we attempted to understand the role of innate immunity against *T. cruzi*, in instructing the adaptive immune responses, which are seminal to controlling *T. cruzi* infection. Though multiple PAMPs have been predicted in *T. cruzi* (Almeida and Gazzinelli, 2001; Bafica et al., 2006; Campos et al., 2001; Golgher and Gazzinelli, 2004; Ouaissi et al., 2002), the relatively 'silent' nature of its host cell invasion (Costales et al., 2009), the irrelevance of these endogenous PAMPs in influencing the strength of adaptive immune responses (Oliveira et al., 2010), that are also unusually delayed (Padilla et al., 2009a; Padilla et al., 2009b), led us to predict a deficiency of host PRR accessible PAMPs in intact, infective *T. cruzi*. Heterologous expression of bacterial PAMPs- *S. typhimurium* FliC and *N. meningitides* PorB in *T. cruzi* led to a more rapid and significantly stronger innate and adaptive immune responses to *T. cruzi*. Co-inoculating or temporarily surface-anchoring PAMPs on *T.*

cruzi also elicited similar initial responses, but unless PAMPs were continuously expressed by the parasite, these responses returned to nomal levels as the infection progressed. PAMP transgenesis in *T. cruzi* also led to a better parasite control, and even complete clearance in some cases. This study provides significant new insights into the *T. cruzi*-host interface and identifies some of the contributing factors in the ability of *T. cruzi* to persist indefinitely in most hosts, despite the generation of potent immune responses. Our findings also advance the idea that innate immune responses may have an extended, instructive role on the adaptive immunity, thus playing an even more significant part in the effective control of pathogens, than was previously appreciated. Our study suggests that expression of heterologous PAMPs could aid in enhancing the effectiveness of live vaccines and periodic or continuous exposure to PAMPs might have therapeutic benefit in persistent infections, where endogenous PAMPs may be inadequate.

In the second part of this project, we took a closer look at the $CD8^+$ T cell responses generated against *T. cruzi*, from the aspect of its immuno-dominance hierarchy and the ways to modify the dominance pattern in order to improve immune control. The majority of the $CD8^+$ T cell responses generated against *T. cruzi* are directed against epitopes in the strain-variant, large gene family encoded trans-sialidase family of proteins (Martin et al., 2006). We hypothesized that $CD8^+$ T cell responses directed against sub-dominant, invariant, non gene-family proteins may induce better cross-strain protection from *T. cruzi* infection and thus, attempted to identify such targets. Based on the observation that *T. cruzi* sacrifices its flagellum relatively early during its intracellular amastigogenesis, possibly leading to the presentation of its constituent epitopes on the

infected host cells relatively early also, we investigated for $CD8^+$ T cell targets among the abundant flagellar proteins. We transgenically over-expressed the $CD8^+$ T cell epitope-containing flagellar component, PAR4 in *T. cruzi*. Infection of mice with these PAR4-overexpressors resulted in a significantly stronger and functional PAR4-specific $CD8^+$ T cell response, that also imparted better protection to subsequent *T. cruzi* challenges. These findings demonstrated that overexpression of suitable, native proteins in pathogens may enhance protective T cell responses - supporting the feasibility of this approach in designing better live-attenuated vaccines.

This work attempts to shed light on some critical aspects of the immunology of *T. cruzi* infection. However, many important questions still remain unanswered. Despite generating an improved adaptive immune response that led to a better control of *T. cruzi*, the delay in the appearance of *T. cruzi* specific $CD8^+$ T cell responses was only marginally affected by PAMP transgenesis. Until *T. cruzi* completed its first round of replication, adaptive anti-*T. cruzi* immune responses against it remained undetected. This suggested that host damage associated molecular patterns (DAMPs) that are released from infected host cells, along with *T. cruzi* at the end of the latter's intracellular life cycle may be playing a significant role in *T. cruzi* immunology. Though overexpression of PAR4 improved the dominance of PAR4 specific immune responses, it only forms a part of the *T. cruzi* flagellar protein repertoire. We need to look for other protein targets in *T. cruzi* that can be overexpressed in *T. cruzi* either alone, or in tandem, to improve their immunodominance- that may impart better protection against the pathogen.

This work demonstrates how we may be able to manipulate the immune responses generated against *T. cruzi*, to the advantage of the host, to possibly design better vaccines to control the infection. A prominent direction, in which research is heading in the field of Chagas disease, is towards the development of transmission blocking live-attenuated vaccines for companion animals. The strategies described in this work may be utilized in improving the immunogenicity of these vaccine strains. In general, PAMP transgenesis in live-vaccines may work as 'live adjuvants' that would improve the immunogenicity of these attenuated pathogens.

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